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# 6S RNA-Dependent Susceptibility to RNA Polymerase Inhibitors

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**ABSTRACT** Bacterial small RNAs (sRNAs) contribute to a variety of regulatory mechanisms that modulate a wide range of pathways, including metabolism, virulence, and antibiotic resistance. We investigated the involvement of sRNAs in rifampicin resistance in the opportunistic pathogen *Staphylococcus aureus*. Using a competition assay with an sRNA mutant library, we identified 6S RNA as being required for protection against low concentrations of rifampicin, an RNA polymerase (RNAP) inhibitor. This effect applied to rifabutin and fidaxomicin, two other RNAP-targeting antibiotics. 6S RNA is highly conserved in bacteria, and its absence in two other major pathogens, *Salmonella enterica* and *Clostridioides difficile*, also impaired susceptibility to RNAP inhibitors. In *S. aureus*, 6S RNA is produced from an autonomous gene and accumulates in stationary phase. In contrast to what was reported for *Escherichia coli*, *S. aureus* 6S RNA does not appear to play a critical role in the transition from exponential to stationary phase but affects  $\sigma^B$ -regulated expression in prolonged stationary phase. Nevertheless, its protective effect against rifampicin is independent of alternative sigma factor  $\sigma^B$  activity. Our results suggest that 6S RNA helps maintain RNAP- $\sigma^A$  integrity in *S. aureus*, which could in turn help bacteria withstand low concentrations of RNAP inhibitors.

**KEYWORDS** 6S RNA, rifampicin, fidaxomicin, *Staphylococcus aureus*, *Salmonella enterica*, *Clostridioides difficile*, regulatory RNA, antibiotic resistance, sigma factors, RNA polymerase

*Staphylococcus aureus* is a commensal Gram-positive bacterium but also an opportunistic pathogen responsible for diseases ranging from benign (mostly cutaneous forms) to life-threatening (visceral or osteoarticular forms) infections (reviewed in references 1 and 2). Due to the emergence of resistant strains, mainly methicillin-resistant *S. aureus* (MRSA) and vancomycin-intermediate *S. aureus* (VISA), *S. aureus* has become a high-priority target for the discovery of new antibiotics (3).

In standard antibiotic treatment regimens, if antistaphylococcal penicillins (i.e., penicillinase-resistant penicillins) and glycopeptides give unsatisfactory results, combination therapy with rifampicin may be considered, particularly in complicated prosthetic device-associated infections (4). Rifampicin, a rifamycin derivative, is an inhibitor of bacterial RNA polymerase (RNAP) (5–7). The molecule binds to the RNAP  $\beta$ -subunit in the DNA/RNA channel to prevent transcription by steric hindrance. This effect occurs during a narrow window, just after the synthesis of the first ribonucleotides; rifampicin is ineffective on transcripts once they are elongated (7).

Highly conserved among bacteria, the core RNA polymerase contains four essential subunits (two  $\alpha$ ,  $\beta$ , and  $\beta'$ ) and one accessory subunit ( $\omega$ ) (8–10). Among Gram-positive bacteria, RNA polymerase includes two other accessory subunits,  $\delta$  and  $\epsilon$  ( $\epsilon$  is specific to *Firmicutes*). These accessory subunits may enhance transcriptional specificity and recycling of RNAP. A sigma factor subunit completes the core enzyme: when present, the complex is called RNAP holoenzyme.  $\sigma$  factors recognize bacterial promoters and

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This work is dedicated to the memory of our colleague Brice Felden, who passed away unexpectedly on 5 March 2021.

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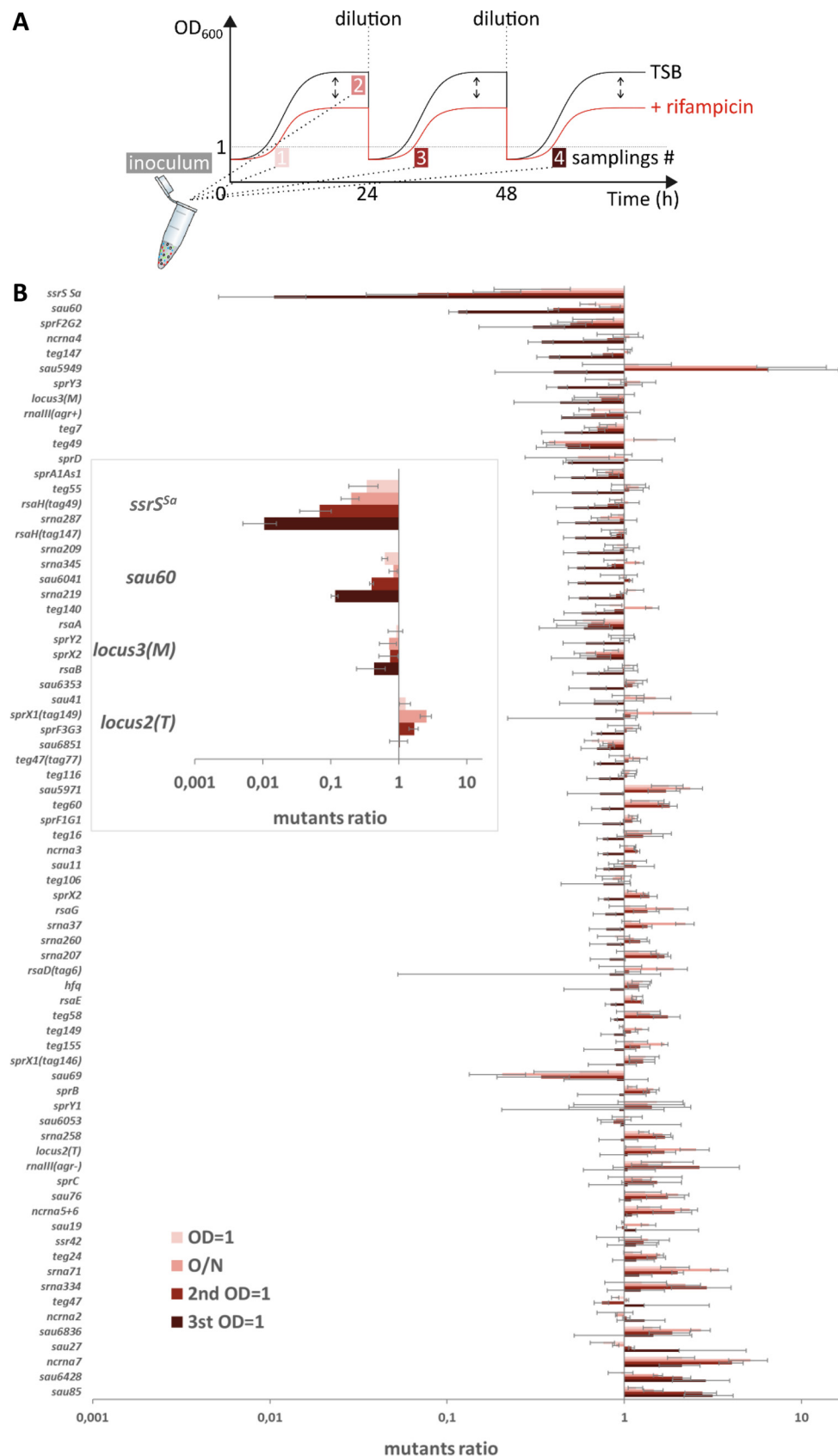
participate in adaptation to changing growth conditions (11). The  $\sigma$  factors are associated with specific transcriptional programs whose function and features may differ among species (11). The number of  $\sigma$  factors varies between species. For example, seven  $\sigma$  factors have been identified in *Escherichia coli* and *Salmonella enterica* (12) and 22 in the spore-forming bacterium *Clostridioides difficile* (13). In contrast, *S. aureus* possesses only four  $\sigma$  factors,  $\sigma^A$ ,  $\sigma^B$ ,  $\sigma^H$ , and  $\sigma^S$ .  $\sigma^A$  is the vegetative factor responsible for transcription of housekeeping genes (14),  $\sigma^B$  is the main alternative sigma factor contributing to stress adaptation (15–17). The last two factors are expressed only in response to specific conditions:  $\sigma^H$  is involved in the regulation of competence (18, 19) and  $\sigma^S$  in the response to miscellaneous environmental stresses (20). A number of transcriptional factors participate together with  $\sigma$  factors in modulating bacterial transcription (21).

Small RNAs (sRNAs) are recognized as ubiquitous elements that fine-tune gene expression at transcriptional and posttranscriptional levels (22, 23). sRNAs are well studied in Gram-negative bacteria. However, in Gram-positive bacteria, including *S. aureus*, their roles in virulence, metabolism, and antibiotic resistance are less well understood, although there is no doubt about their involvement in these processes (24–26). The majority of characterized sRNAs interact with mRNAs. However, some sRNAs interact directly with protein complexes. This is the case for 6S RNA, one of the first-described sRNAs, identified in *Escherichia coli* in 1967 (27) and sequenced in 1970 (28). In *E. coli*, 6S RNA binds preferentially to RNAP associated with the  $\sigma^{70}$  factor. 6S RNA accumulates during exponential growth and reaches its maximum levels in stationary phase (29). The 6S RNA/RNAP interaction leads to inhibition of numerous *E. coli*  $\sigma^{70}$ -dependent promoters and consequently reorients transcription dependent on alternative sigma factors, allowing adaptation to many environmental conditions (reviewed in references 30 and 31). Although 6S RNA is conserved among bacteria (32), its role(s) and function(s) in many of them remain unknown.

We recently developed a platform to assess *S. aureus* sRNAs required for fitness based on an sRNA mutant library (33). Using this platform, we identified a rifampicin susceptibility phenotype associated with the lack of 6S RNA, pointing to a possible new mechanism of resistance against low rifampicin concentrations. We showed that this phenotype is restricted neither to rifampicin nor to *S. aureus* but extends to other RNAP inhibitors and bacterial species. Characterization of 6S RNA in *S. aureus* indicates its partial involvement in  $\sigma^B$ -dependent transcription regulation at late stationary phase, rather than during transition from exponential to stationary phase. Additional experimental evidence suggests that *S. aureus* 6S RNA has a role in RNAP holoenzyme cohesion.

## RESULTS

**Absence of 6S RNA confers increased susceptibility to rifampicin in *S. aureus* and *S. enterica*.** At the beginning of this study, we examined the possible involvement of sRNAs in processes underlying *S. aureus* susceptibility to antibiotics. To uncover sRNA-associated phenotypes, our laboratory previously developed a fitness assay based on competition between sRNA-tagged deletion mutants within a library that includes mutants of most *S. aureus* bona fide sRNAs, defined as those expressed by an autonomous gene without antisense transcription (33, 34). Deletions were designed to remove most sRNA gene sequences, leading to inactive sRNAs, in most cases keeping intact promoters and terminators. Briefly, the fitness of individual sRNA deletion mutants growing within a collection of mutants is tested by comparing their proportion in the presence or absence of different compounds. The accumulation or reduction of individual strains is identified by monitoring the tagged sequences. This method distinguishes strains showing even subtle growth differences. Three identical libraries containing 74 putative sRNA mutants and 3 control mutants were challenged with rifampicin at a sublethal concentration ( $6 \mu\text{g L}^{-1}$ ). After 3 days of growth, one mutant was underrepresented  $\sim 100$ -fold compared to the other mutants when normalized to the same libraries grown in the same medium, without rifampicin (Fig. 1). The mutant with reduced fitness due to the presence of rifampicin carried a deletion of the



**FIG 1** Fitness loss of the *ssrS<sup>Sa</sup>* mutant in the presence of a sublethal concentration of rifampicin. (A) Scheme of fitness experiment sampling. Three libraries were cultured for 3 days in tryptic soy broth (TSB) with or without (Continued on next page)

*ssrS* gene (referred to here as the *ssrS<sup>Sa</sup>* strain), which encodes 6S RNA, an sRNA known to interact with RNAP, the rifampicin target (5–7).

We asked whether *ssrS<sup>Sa</sup>* mutant susceptibility to rifampicin observed in the fitness experiment is detectable in monocultures. Serial dilutions of overnight cultures of the wild-type (WT) and *ssrS<sup>Sa</sup>* strains were spotted on solid medium containing low levels of rifampicin ( $5 \mu\text{g L}^{-1}$ ); under this condition, the *ssrS<sup>Sa</sup>* mutant was 100-fold more sensitive to rifampicin than the parental strain (Fig. 2A). This susceptibility was reversed by insertion of an *ssrS<sup>Sa</sup>* copy at an ectopic chromosomal locus ( $\Delta\textit{ssrS}^{\text{Sa}} \text{ ecto-ssrS}^{\text{Sa}}$ ) (see Fig. S1 in the supplemental material; Fig. 2A, left panel). A longer growth lag in rifampicin-containing liquid medium was also observed for the *ssrS<sup>Sa</sup>* mutant than for the wild-type or complemented strains (Fig. 2C). These two tests indicated that the rifampicin susceptibility phenotype was solely due the absence of 6S RNA. This phenotype is observed within a narrow window of rifampicin concentrations, below the MIC ( $12 \mu\text{g L}^{-1}$ ). We conclude that 6S RNA protects *S. aureus* cells against sublethal concentrations of rifampicin.

As 6S RNA is widely conserved in the bacterial kingdom, we examined its protective role against rifampicin in the enteric pathogen *Salmonella enterica*, a Gram-negative species. The *ssrS* gene of *S. enterica* (*ssrS<sup>LT2</sup>*) was deleted (Fig. S2). As in *S. aureus*, *ssrS<sup>LT2</sup>* deletion led to a rifampicin susceptibility phenotype compared to its parental strain (Fig. 2B, left panel). However, this phenotype was only partly complemented by insertion of the *ssrS<sup>LT2</sup>* wild-type gene at a chromosomal ectopic position (Fig. 2B, left panel).

**6S RNA protection against rifampicin is partially interchangeable between *S. aureus* and *S. enterica*.** Since *S. aureus* and *S. enterica* *ssrS* mutants show a similar rifampicin susceptibility phenotype, we investigated whether the 6S RNA genes would be functional in heterologous backgrounds. For this, gene swaps were performed, replacing (i) the native *S. enterica* LT2 *ssrS<sup>LT2</sup>* gene with the *S. aureus* *ssrS<sup>Sa</sup>* homolog (*S. enterica*  $\Delta\textit{ssrS}^{\text{LT2}}::\textit{ssrS}^{\text{Sa}}$ ) (Fig. S2) and (ii) the native *S. aureus* *ssrS* gene (*ssrS<sup>Sa</sup>*) with the *S. enterica* *ssrS<sup>LT2</sup>* homolog (*S. aureus*  $\Delta\textit{ssrS}^{\text{Sa}}::\textit{ssrS}^{\text{LT2}}$ ) (Fig. S1).

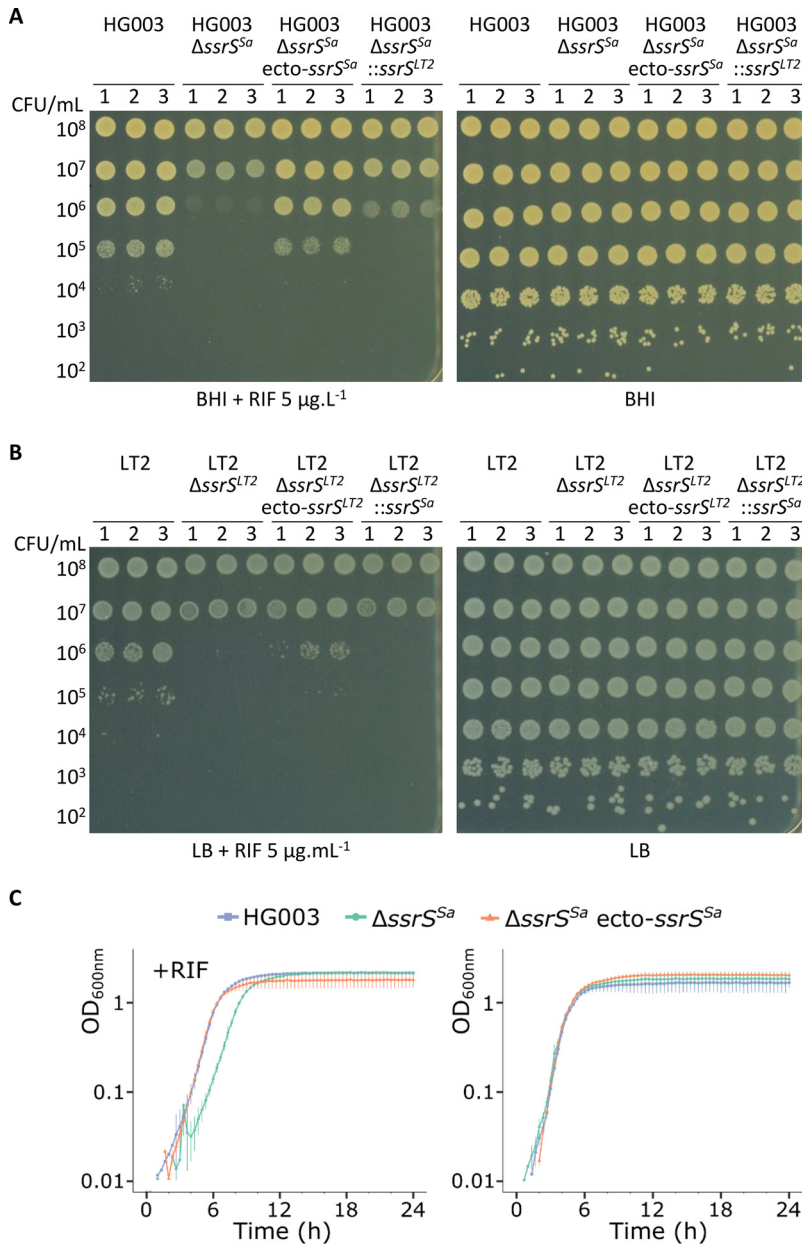
The *ssrS<sup>Sa</sup>* gene failed to compensate the *S. enterica*  $\Delta\textit{ssrS}^{\text{LT2}}$  strain rifampicin susceptibility (Fig. 2B, left panel). This is possibly due to reduced synthesis of staphylococcal 6S RNA in the *S. enterica* background, as suggested by the results of Northern blot analysis (Fig. S3A). Interestingly, however, the reverse swap in *S. aureus*  $\Delta\textit{ssrS}^{\text{Sa}}$  partially restored growth in rifampicin (Fig. 2A, left panel). Complementation of *ssrS<sup>Sa</sup>* by *ssrS* from an evolutionarily distant species suggests that different 6S RNAs shield against rifampicin using similar mechanisms.

**6S RNA protects RNAP against different RNAP inhibitors.** The family of RNAP inhibitors comprises molecules with different mechanisms of action. We chose two RNAP inhibitors, rifabutin and fidaxomicin, and a putative RNAP inhibitor, aureothricin, to test the impact of *ssrS<sup>Sa</sup>* on drug susceptibility.

Rifabutin, a spiropiperidyl rifamycin, is a rifampicin analog (35, 36). Fidaxomicin (also known as lipiarmycin [37, 38] and tiacumicin B [39]) is a narrow-spectrum antibiotic (40) that inhibits transcription initiation by locking RNAP through an open-clamp state that prevents an efficient interaction with the promoter (41–44). Aureothricin is a member of the dithiopyrrolone group and has broad-spectrum activity (45). However, the mechanism of action of this molecule remains unclear. For each drug, the appropriate sublethal concentrations to use were first established using *S. aureus* strain HG003. The *ssrS<sup>Sa</sup>* mutant showed an  $\sim 4$ -log-fold-greater susceptibility to rifabutin than HG003, almost

#### FIG 1 Legend (Continued)

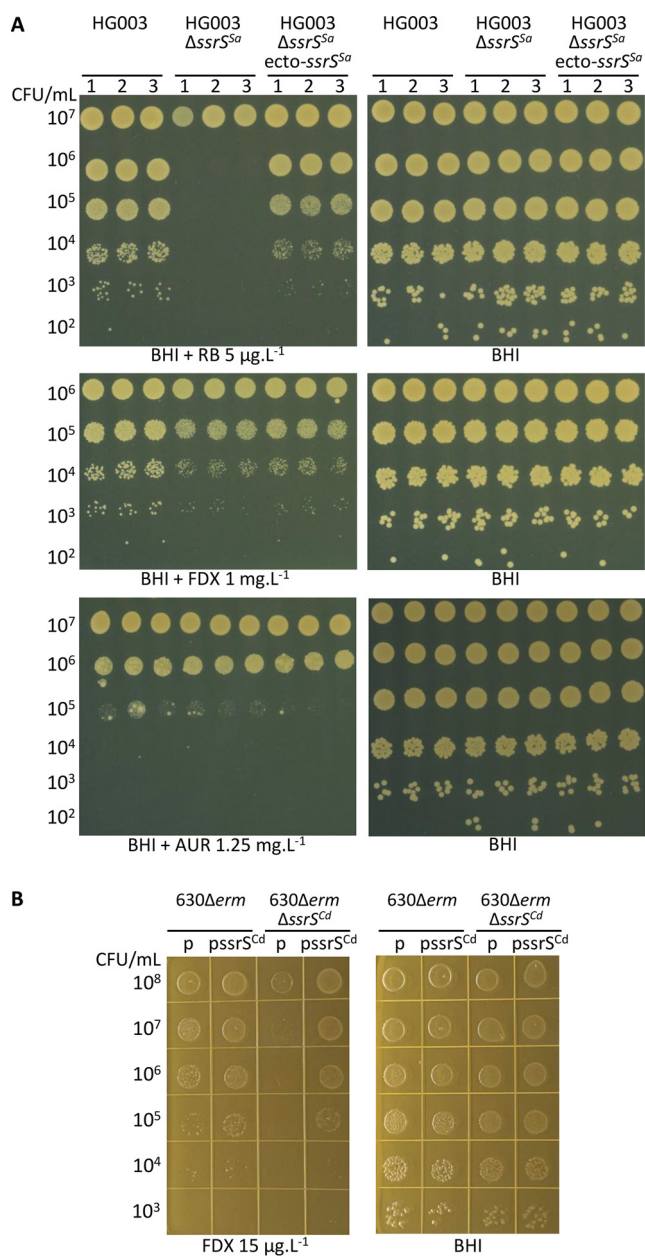
rifampicin. Cultures were diluted 1:1,000 at 24 and 48 h. After each dilution step (0, 24, and 48 h), samples were withdrawn for tag counting under both growth conditions, when the  $\text{OD}_{600}$  reached 1 (samplings 1, 3, and 4) and after the first overnight growth (sampling 2), as indicated. (B) Results of the competition assay between *S. aureus* sRNA mutants in the presence of  $6 \mu\text{g L}^{-1}$  rifampicin. Mutant strain names are on the y axis; the x axis shows the proportion of each mutant within the population grown in the presence of rifampicin normalized to the inoculum and to the corresponding sample grown in the absence of rifampicin. For each mutant, four histograms are shown; the color code corresponds to samplings indicated in panel A. Locus 2 and 3 mutants have tag insertions in loci likely not transcribed and not expected to alter the strain fitness. Error bars represent the experimental standard deviations between the three libraries. (Inset) Enlargement of bars for four relevant sRNA mutants: *ssrS<sup>Sa</sup>*, *sau60*, and the control strain loci 2 and 3.



**FIG 2** *ssrS* deletions confer a conserved rifampicin susceptibility phenotype from *S. aureus* to *S. enterica*. Three independent clones were grown overnight for each indicated strain. (A) Serial dilutions of overnight *S. aureus* (HG003 strain and its derivatives) cultures were spotted on BHI agar with or without 5 μg L<sup>-1</sup> rifampicin (RIF). (B) Serial dilutions of overnight *S. enterica* (LT2 strain and its derivatives) cultures were spotted on LB agar with or without 5 μg mL<sup>-1</sup> rifampicin. (C) Growth kinetics of *S. aureus* strains (HG003 and its derivatives) in BHI with or without 5 μg L<sup>-1</sup> rifampicin. OD<sub>600</sub> is an arbitrary value due to plate reader conditions, not representative of absorbance measurements of *S. aureus* in flasks. Error bars represent standard deviations from three experiments.

entirely complemented by *ssrS<sup>Sa</sup>* ectopic expression (Fig. 3A). The *ssrS<sup>Sa</sup>* mutant was moderately negatively affected by fidaxomicin compared to the parental strain, with visibly smaller colonies (Fig. 3A). The increased fidaxomicin susceptibility phenotype was not fully complemented by ectopic expression of *ssrS<sup>Sa</sup>*. Unlike with rifabutin and fidaxomicin, no aureothricin hypersusceptibility was associated with the absence of 6S RNA (Fig. 3A).

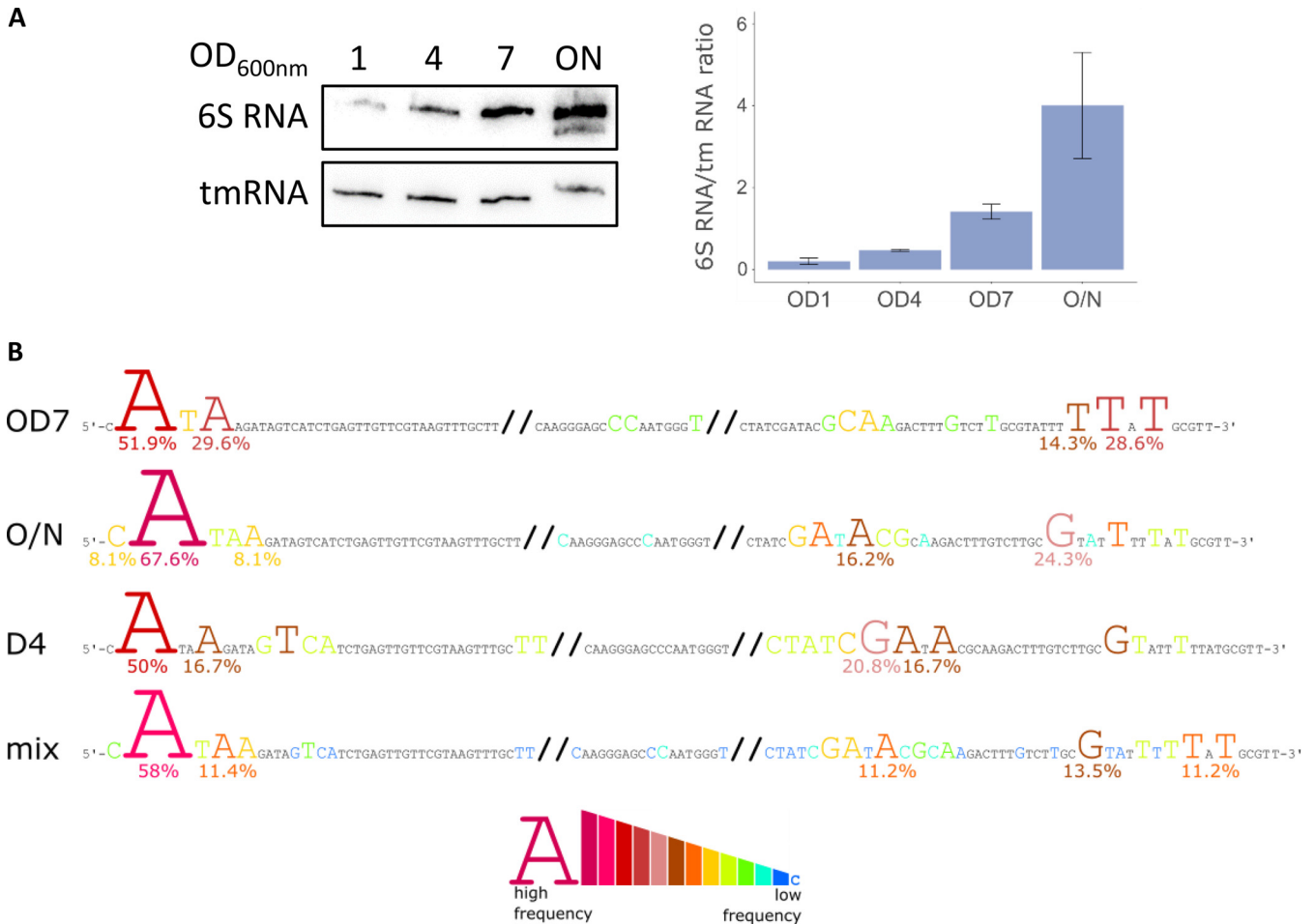
Fidaxomicin is mainly active against *C. difficile*, a major human intestinal pathogen (40). We decided to test whether *ssrS* deletion also impacts RNAP inhibitor susceptibility



**FIG 3** Susceptibility to RNAP inhibitors. (A) Serial dilution of *S. aureus* overnight cultures plated on solid medium containing rifabutin (RB), fidaxomicin (FDX), aureothricin (AUR), or no antibiotic. The numbers 1, 2, and 3 indicate independent clones. The antibiotic concentrations used were below the MIC. (B) Serial dilutions of *C. difficile* overnight cultures plated on solid medium containing FDX or no antibiotic. Pictures are representative of four replicates. Thiamphenicol was added in all plates (15  $\mu$ g mL<sup>-1</sup>) to maintain the plasmid. p, empty vector pMTL84121; psrS<sup>Cd</sup>, pMTL84121-ssrS<sup>Cd</sup>.

in this species. For this, we constructed a  $\Delta$ ssrS derivative ( $\Delta$ ssrS<sup>Cd</sup>) of *C. difficile* 630 $\Delta$ erm. *C. difficile*  $\Delta$ ssrS<sup>Cd</sup> was 1,000-fold more susceptible to fidaxomicin than its parental strain (Fig. 3B, left panel). A plasmid carrying the ssrS<sup>Cd</sup> gene introduced in the  $\Delta$ ssrS<sup>Cd</sup> strain complemented the phenotype by restoring wild-type level of susceptibility to fidaxomicin (Fig. 3B, left panel).

We conclude that ssrS-related susceptibility to antibiotics is a common feature of different RNAP inhibitors in evolutionarily distant bacterial species. The mechanism associated with this susceptibility phenotype is likely the same for different RNAP-targeted antibiotics and different species.



**FIG 4** *ssrS* gene expression and 6S RNA sequence in *S. aureus*. (A) *ssrS*<sup>50</sup> expression. Cultures of HG003 grown in BHI were sampled at OD<sub>600</sub> of 1, 4, and 7 and ON (20 to 24 h incubation). A Northern blot probing for 6S RNA and transfer-messenger RNA (tmRNA) (for normalization) was performed. A quantification of 6S RNA normalized to tmRNA is presented. The standard deviation is based on biological triplicates. (B) Identification of 6S RNA ends by 5'-3' RACE mapping. Sequences were analyzed separately at different time points (OD of 7, ON [20 to 24 h incubation], and day 4 [D4]) and compiled (mix). Colored letters represent extremities found in analyzed sequences. A color scale indicates the frequency at each 5' or 3' end. The highest frequencies are indicated below the corresponding nucleotides.

**Growth phase-dependent expression of *ssrS* in *S. aureus*.** The expression profile of 6S RNA differs according to species (30). We performed Northern blotting experiments to evaluate 6S RNA expression in *S. aureus* (Fig. 4A). 6S RNA was strongly expressed and accumulated to 20-fold-higher levels in stationary phase, as determined in *S. aureus* HG003. The expression profile of *S. aureus* 6S RNA was similar to that reported in *Salmonella* (46), *E. coli* (29), and *Bacillus subtilis* 6S-1 RNA, which carries a second 6S RNA (47–50).

In overnight (ON) samples where 6S RNA is the most abundant, a second, faster-migrating band was also observed (Fig. 4A and Fig. S3A). A second band was reported in *C. difficile* even during exponential phase (51). To determine a potential alternative 6S RNA form in *S. aureus*, also previously suggested (52), we performed 5'-3' RACE (rapid amplification of cDNA ends) on samples collected at different time points during growth in rich medium: at an optical density at 600 nm (OD<sub>600</sub>) of 7 (corresponding to entry into stationary phase), ON (i.e., after the first overnight growth), and on day 4. At all sampling points, the major transcription start site (TSS) is the same (Fig. 4B) and in agreement with the site determined by global TSS mapping (53). Concerning the 3' end, the longest form ending with a T is the most abundant in OD 7 samples, representing 28.6% of analyzed sequences. These data confirm that the size of the longest, most abundant form is 231 nucleotides (nt) (predicted at 230 nt [52]). Samples from



**TABLE 1** Transcriptomic analysis of  $\Delta$ ssrS<sup>Sa</sup> versus HG003 in late exponential phase<sup>a</sup>

Locus tag/gene name	FC	P <sub>adj</sub>	Function or relevant data <sup>b</sup>	Classification	Regulation	TU <sup>c</sup>
SAOUHSC_02911	0.48	2.2E-28	<i>Epoxyqueuosine reductase</i>	tRNA modification	$\sigma^A$	
SAOUHSC_02640/hrtA	0.48	4.2E-10	Hemin efflux ATP-binding protein HrtA	Transport and binding	$\sigma^A$ , HssR	a
SAOUHSC_01817	0.49	5.9E-10	<i>Integral component of membrane</i>		$\sigma^A$	b
SAOUHSC_01736	0.52	1.0E-08	<i>Unknown; downstream ssrS (34)</i>		$\sigma^A$	c
SAOUHSC_02641/hrtB	0.53	3.7E-08	Hemin efflux system permease protein HrtB	Transport and binding	$\sigma^A$ , HssR	a
SAOUHSC_01818/ald2	0.55	8.0E-07	Alanine dehydrogenase	Energy metabolism	$\sigma^A$ , CcpA	b
SAOUHSC_00874	0.56	3.5E-10	<i>Thioredoxin-like protein</i>	Hypothetical protein	$\sigma^A$	
SAOUHSC_02297	0.57	3.7E-14	S1 RNA-binding domain-containing protein	Protein synthesis	$\sigma^A$	d
SAOUHSC_02590	0.60	3.7E-09	<i>Amino acid permease</i>	Transport and binding	$\sigma^A$ , CcpA, CodY	
SAOUHSC_02296	0.62	1.7E-08	<i>SprT-like protein</i>		$\sigma^A$	d
SAOUHSC_00561/vraX	0.64	4.2E-10	<i>VraX</i>		$\sigma^A$	
SAOUHSC_00704	0.64	2.0E-08	<i>ABC-2 transporter</i>		$\sigma^A$	
SAOUHSC_03028/bstA	0.64	1.2E-03	<i>DinB-like protein</i>		$\sigma^B$	
SAOUHSC_01735/tcdA	0.65	1.9E-06	<i>ThiF domain-containing protein</i>	Cofactor biosynthesis	$\sigma^A$	c
SAOUHSC_02656	0.65	4.0E-10	<i>Cytochrome c oxidase-like protein</i>		$\sigma^A$	
SAOUHSC_00157/murQ	1.51	1.6E-03	<i>N-Acetylmuramic acid-6-phosphate etherase</i>	Cell envelope	$\sigma^A$ , MurR, CcpA	e
SAOUHSC_00156/mupG	1.52	1.2E-03	<i>6-Phospho-N-acetylmuramidase</i>	Cell envelope	$\sigma^A$ , MurR, CcpA	e
SAOUHSC_01121/hla	1.56	1.6E-05	Alpha-hemolysin	Virulence/toxin	$\sigma^A$ , SaeR, CcpA, RNA III	
SAOUHSC_02169/chp	1.60	1.8E-04	CHIPS	Virulence	$\sigma^A$	
SAOUHSC_00961/comK1	1.85	4.3E-08	<i>Competence protein</i>		$\sigma^A$ , CodY	

<sup>a</sup>Fold change (FC) represents the gene expression ratio between  $\Delta$ ssrS<sup>Sa</sup> and its parental strain at an OD<sub>600</sub> of 7. The top portion of the table contains genes with FC of <0.66, and the bottom shows genes with FC of >1.5. FC and adjusted P value (P<sub>adj</sub>) were determined using the DESeq2 method.

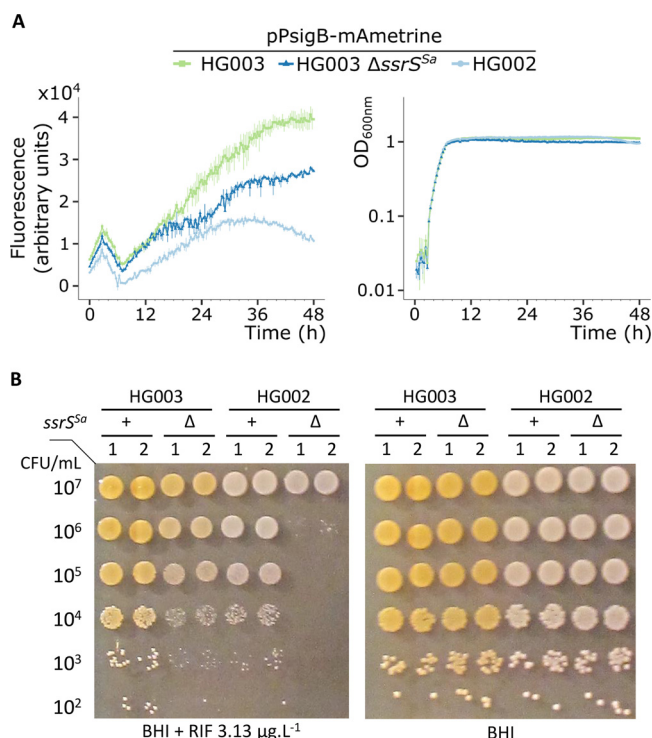
<sup>b</sup>Hypothetical proteins are in italics. CHIPS, chemotaxis inhibitory protein of *S. aureus*.

<sup>c</sup>TU, transcription unit.

overnight or day 4 cultures exhibited shorter forms, which may result from processing or degradation by 3' exonucleases.

**Moderate impact of 6S RNA on the global *S. aureus* transcription profile.** The role of 6S RNA in transcriptional regulation was suggested early (29) and then validated by transcriptomic analysis in *E. coli* (54–56); in transcriptome sequencing (RNA-seq) data, 35 genes were at least 2-fold differentially expressed in a 6S RNA-deficient strain compared to the parental strain at the onset of stationary phase. To determine whether *S. aureus* 6S RNA could play a similar role, the transcriptional profile of the  $\Delta$ ssrS<sup>Sa</sup> mutant was compared with that of its parental strain by RNA-seq on samples collected at an OD<sub>600</sub> of 7, which corresponds to the entry into stationary phase of *S. aureus* (Table 1). Transcriptome analyses were performed on biological triplicates, and features with a P value of <0.05 were retained for interpretation. Surprisingly, the transcriptional profiles of parental and  $\Delta$ ssrS<sup>Sa</sup> strains were highly similar. Only three genes were >2-fold downregulated in  $\Delta$ ssrS<sup>Sa</sup> (fold change [FC] < 0.5) (Table 1). They encode a hypothetical epoxyqueuosine reductase (QueH/SAOUHSC\_02911), a hemin transporter (HrtA/SAOUHSC\_02640; its cofunctional partner HrtB/SAOUHSC\_02641 is also downregulated), and a 30-amino-acid peptide (SAOUHSC\_01817) of unknown function. Other genes related to transporters, cell wall metabolism, and redox state were also significantly reduced but with a lower fold change. All of these genes are regulated by  $\sigma^A$  except *bstA*, a  $\sigma^B$  DNA-damage-induced gene encoding a putative DinB superfamily protein. Taken together, these results suggest that 6S RNA does not redirect transcription during the stationary-phase transition in a sigma-dependent manner.

Transcriptome results, obtained early in the stationary phase, did not provide evidence linking  $\sigma^B$  transcriptional activity to the presence of 6S RNA. We used a reporter fusion strategy to pursue this question: the gene encoding the fluorescent protein mAmetrine was placed under the transcriptional control of the  $\sigma^B$ -regulated SAOUHSC\_00624 promoter (57) (pPsigB-mAmetrine) (Fig. 5A). The HG002 strain is an HG003-isogenic strain containing an 11-bp deletion in the *rsbU* gene, which encodes a  $\sigma^B$  activator (58), used here as a negative control. No significant difference in fluorescence was observed between *ssrS*<sup>Sa</sup> and parental strains when fluorescence was measured for the first 18 h of growth in rich liquid medium. Thus, in keeping with transcriptomic findings, we conclude that 6S

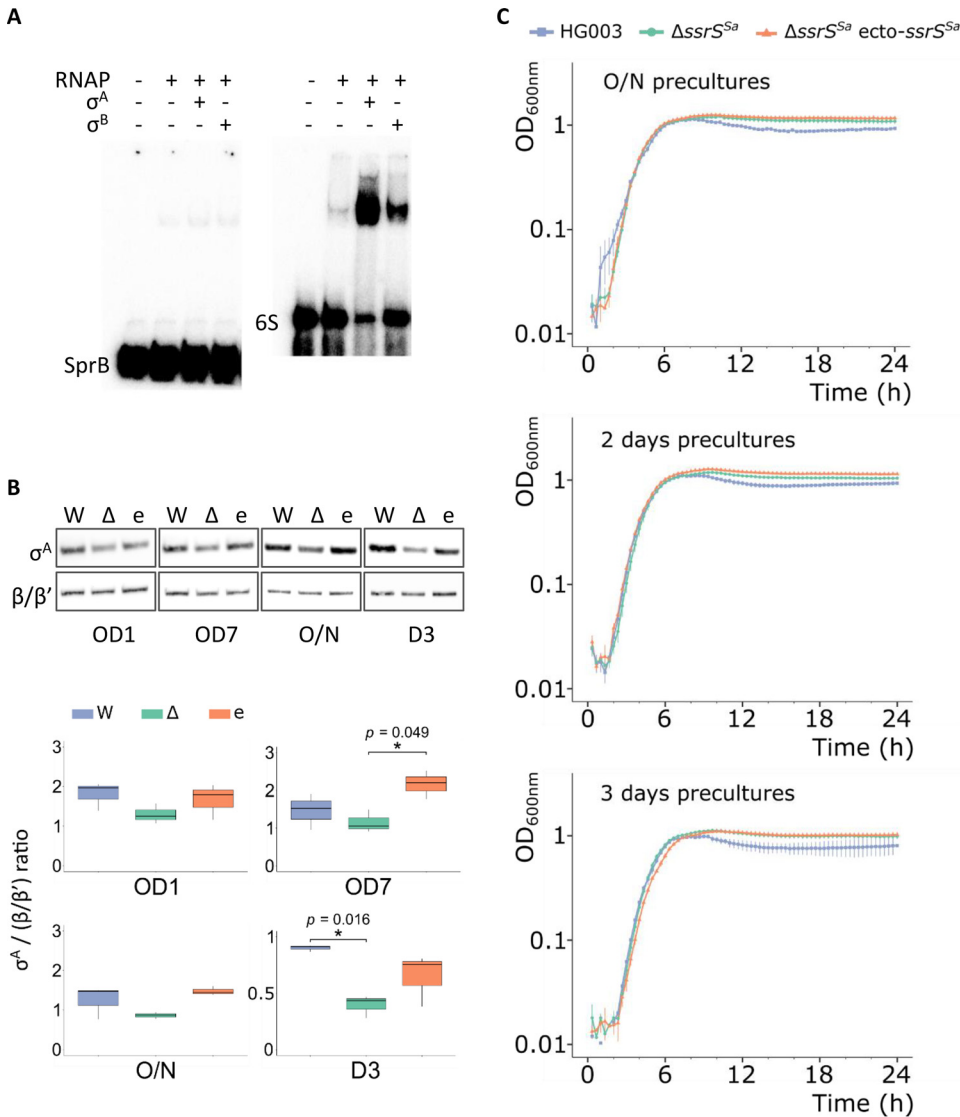


**FIG 5** 6S RNA and  $\sigma^B$  interplay in late stationary phase and in rifampicin response in *S. aureus*. (A) Fluorescence and OD<sub>600</sub> were monitored simultaneously in three strains (HG003 [parental], HG003  $\Delta$ ssrS<sup>5a</sup>, and HG002) expressing a fluorescent protein (mAmetrine) under the control of the  $\sigma^B$  promoter of SAOUHSC\_00624 from a plasmid (pPsigB-mAmetrine). HG002 (*rsbU* strain equivalent to the  $\sigma^B$  strain) is a negative control. Error bars represent standard deviations for biological triplicates. (B) Spot test comparing HG003 and HG002 (parental and  $\Delta$ ssrS<sup>5a</sup> strains, respectively) with a sublethal concentration of rifampicin (3.13  $\mu$ g L<sup>-1</sup>). Arbitrary values are shown as OD<sub>600</sub>. Experiment was done with independent duplicates.

RNA does not appear to redirect transcription during transition from exponential to stationary phase in *S. aureus*, which differs from what was reported for *E. coli* (56, 59). Interestingly, however, after 18 h of culture, mAmetrine expression continued to increase in the parental strain HG003, compared to markedly lower expression in the  $\Delta$ ssrS<sup>5a</sup> strain. This result, suggesting that 6S RNA could be important for efficient  $\sigma^B$ -dependent gene expression during starvation, remains to be investigated.

Since the absence of 6S RNA in *S. aureus* leads to an increased susceptibility to rifampicin, we questioned if this phenotype was related to  $\sigma^B$  regulation. HG002 is deficient in  $\sigma^B$  activity, illustrated by the absence of yellow pigmentation (60). We first noticed a greater susceptibility to rifampicin in HG002 than HG003 (Fig. 5). This observation indicates that lower  $\sigma^B$  activity *per se* confers increased rifampicin susceptibility, as described for a *C. difficile sigB* mutant (61). This effect is probably due to a stress adaptation deficiency related to the absence of  $\sigma^B$  regulation. To determine the effect of 6S RNA in this genetic context, the *ssrS<sup>5a</sup>* deletion was introduced into HG002. The resulting strain (HG002  $\Delta$ ssrS<sup>5a</sup>) was considerably more susceptible to rifampicin than the parental strain HG002 (Fig. 5B). This observation indicates that the absence of 6S RNA leads to increased rifampicin susceptibility through a pathway that is independent of  $\sigma^B$  activity.

**6S RNA plays a role in RNAP stability in *S. aureus*.** 6S RNA binds to RNAP- $\sigma^{70}$  in *E. coli* (29, 47). As RNAP holoenzyme is a protein complex with accessory subunits (especially  $\sigma$  factors), an element binding to this complex could directly influence its stability or composition. We first performed an electrophoretic mobility shift assay (EMSA) in *S. aureus* with radiolabeled 6S RNA (6S [<sup>32</sup>P]RNA), purified sigma factors ( $\sigma^A$ -His and  $\sigma^B$ -His), and RNAP (with His-tagged RpoC) to assess the interaction with 6S RNA (Fig. 6A). A <sup>32</sup>P-labeled unrelated sRNA, SprB (62), was used as a control. No interactions with



**FIG 6** 6S RNA and RNAP holoenzyme interactions in *S. aureus*. (A) EMSA with 6S [<sup>32</sup>P]RNA (6S RNA), RNAP,  $\sigma^A$ , and  $\sigma^B$ . All the proteins were His tagged and purified. [<sup>32</sup>P]SprB (SprB) is a control RNA. (B) Immunodetection of  $\sigma^A$  performed by Western blotting of samplings at OD<sub>600</sub> of 1 and 7, ON, and at day 3 (D3). Quantification of  $\sigma^A$  is relative to the amount of RNAP  $\beta/\beta'$  subunits. Experiments were carried out in biological triplicates and analyzed by one-way ANOVA and Tukey's HSD test [ $F_{OD1}(2,6) = 1.54, P = 0.288; F_{OD7}(2,6) = 5.21, P = 0.049, T_{OD7}(\text{adjusted } P \text{ value } [P_{adj}] = 0.045, 95\% \text{ confidence interval (CI) } = 0.026 \text{ to } 2.00; F_{ON}(2,6) = 4.50, P = 0.064; F_{D3}(2,6) = 8.91, P = 0.016, T_{D3}(P_{adj}) = 0.013, 95\% \text{ CI} = -0.871 \text{ to } -0.138$ ]. Significant differences of  $\sigma^A/(\beta/\beta')$  means between strains ( $P < 0.05$ ) are indicated by a star. W, wild type (HG003);  $\Delta$ ,  $ssrS^{Sa}$  mutant; e,  $\Delta ssrS^{Sa}$  ecto- $ssrS^{Sa}$ . (C) Growth curves of HG003,  $ssrS^{Sa}$  mutant ( $\Delta ssrS^{Sa}$ ) and complemented ( $\Delta ssrS^{Sa}$  ecto- $ssrS^{Sa}$ ) strains in BHI. Strains were cultured in independent triplicates from ON, 2-day, or 3-day precultures. Error bars represent standard deviations.

SprB were detected. In contrast, our results showed interaction between 6S RNA and RNAP coupled to the vegetative sigma factor,  $\sigma^A$ , and to a lesser extent between 6S RNA and RNAP- $\sigma^B$ .

We questioned if the absence of 6S RNA could alter RNAP holoenzyme composition. The amounts of  $\sigma^A$  and  $\beta/\beta'$  subunits were evaluated in  $ssrS^{Sa}$  and parental strain cultures at different time points. Western blots were performed with antibodies raised against  $\sigma^A$  and RNAP (Fig. 6B). Interestingly,  $\sigma^A$  pools were lower in the  $ssrS^{Sa}$  mutant than the parental strain and complemented strains under all tested conditions. At day 3,  $\sigma^A$  pools were significantly decreased by nearly 2-fold in the  $ssrS^{Sa}$  mutant compared to the parental strain. These results suggest that 6S RNA plays a role in RNAP

holoenzyme stability and could act as a protective belt for RNAP- $\sigma^A$ . We hypothesized that a reduced amount of  $\sigma^A$  could modify strain outgrowth. Levels of growth of HG003 (parental strain), HG003  $\Delta ssrS^{S\alpha}$  and its complemented mutant HG003  $\Delta ssrS^{S\alpha}$  ecto- $ssrS^{S\alpha}$  from precultures that had grown ON, for 2 days, and for 3 days were compared (Fig. 6C). Surprisingly, no growth difference was observed between the three strains in BHI, regardless of the preculture age. Despite the significant effect on  $\sigma^A$  levels, 6S RNA is not an essential factor for *S. aureus* growth in rich medium.

## DISCUSSION

Here, we demonstrated that the absence of 6S RNA in *S. aureus* leads to a fitness loss in the presence of low rifampicin concentrations. This marked phenotype was associated with only one sRNA gene ( $ssrS^{S\alpha}$ ) of 77 tested mutants in a competition experiment. This phenotype is conserved from Gram-positive to Gram-negative bacteria, suggesting a common protective effect.

In *S. aureus*, the rifampicin susceptibility phenotype was fully restored by ectopic gene complementation, indicating that it was solely due to the absence of 6S RNA. In *S. enterica*, however, similarly done complementation of the  $ssrS^{LT2}$  deletion was only partial, while the native and ectopic copies had similar expression levels (Fig. S3A). In *E. coli*, the  $ssrS$  and  $ygfA$  genes are in an operon (48, 63) and mature 6S RNA results from processing of 5' and 3' transcript ends (64, 65); similar organization and regulation are expected in *S. enterica*. Two hypotheses may explain the incomplete complementation of the  $\Delta ssrS^{LT2}$  rifampicin susceptibility phenotype: (i) the ectopic  $ssrS^{LT2}$  copy could be subjected to a slightly different processing pathway (not detected in the gel in Fig. S3A) and (ii)  $\Delta ssrS^{LT2}$  could affect  $ygfA$  expression (however, no growth defect has been observed for the mutant so far).

Despite weak similarity between *S. aureus*  $ssrS^{S\alpha}$  and the cognate *S. enterica*  $ssrS^{LT2}$  gene, we observed partial complementation of the rifampicin susceptibility phenotype in *S. aureus*  $\Delta ssrS^{S\alpha}$  by the  $\Delta ssrS^{LT2}$  allele. The lack of the reverse complementation ( $ssrS^{S\alpha}$  into *S. enterica*  $\Delta ssrS^{LT2}$ ) might be ascribable to the lower expression of  $ssrS^{S\alpha}$  in *S. enterica* (Fig. S3A) and/or to any of the hypotheses raised above for the *S. enterica*  $\Delta ssrS^{LT2}$  ecto- $ssrS^{LT2}$  phenotype.

Susceptibility of the  $ssrS$  mutants was not observed for all the compounds tested. Differences in the mechanisms of action, binding sites, and drug entry efficiencies could explain this observation (42, 66). The  $\Delta ssrS^{S\alpha}$  mutant showed increased susceptibility of *S. aureus* to rifampicin, rifabutin, and fidaxomicin. Similarly, cognate  $\Delta ssrS$  *S. enterica* and *C. difficile* mutants showed marked sensitivities to rifampicin and fidaxomicin, respectively. These drugs bind RNAP close to sites interacting with DNA, suggesting that 6S RNA interaction with the enzyme may, at least partially, prevent antibiotic access to their sites. Based on this reasoning, our results suggest that aureothricin, for which toxicity was unaffected by  $ssrS^{S\alpha}$  deletion, does not bind RNAP at the interface with DNA.

Our findings suggest differences in the regulatory roles of 6S RNA in *S. aureus* compared to those reported for *E. coli* (54). In the latter species, 6S RNA interaction with the RNAP holoenzyme is proposed to coordinate transcriptional regulation with growth (29). Accordingly, numerous transcriptome analyses performed under different conditions indicated that in *E. coli*, many 6S RNA-regulated genes were related to translational/transcriptional machinery or amino acid metabolism (29, 54–56, 67–70). In contrast, our *S. aureus* transcriptomic analysis and promoter assay revealed no obvious 6S RNA-related differences in expression during the transition to stationary phase. Two major features of *S. aureus* could explain this phenomenon. The first is lower diversity of sigma factors in *S. aureus*, which has only four  $\sigma$  factors, among which  $\sigma^A$  and  $\sigma^B$  control the majority of transcribed genes. Given that the main alternative sigma factor  $\sigma^B$  is involved in stress response and not only in stationary-phase adaptation,  $\sigma^B$  promoters could be less sensitive to 6S RNA during the transition phase. The second feature is the compensatory effect of a coregulator. The levels of the alarmone ppGpp

increase in *E. coli* *ssrS* mutants and might compensate for the lack of 6S RNA (55, 56, 59). This possibility provides an attractive explanation for the phenomenon in *S. aureus*, as ppGpp is synthesized in response to nutrient starvation and drives growth adaptation (71). Further experiments are needed to explore this pathway in *S. aureus*.

Our promoter assay (Fig. 5A) suggests a 6S RNA-dependent expression of  $\sigma^B$ -promoters in late stationary phase, after 18 h of culture. Among its known roles in *E. coli*, 6S RNA also influences transcription during long-term starvation (54, 67). Whether *S. aureus* 6S RNA interacts with  $\sigma^B$  for alternative promoter expression during late stationary phase remains unclear. In particular, in the absence of a functional  $\sigma^B$ , the absence of 6S RNA still generates rifampicin inhibition, indicating that this phenotype was not due to a lack of reprogramming transcription from  $\sigma^A$  to  $\sigma^B$ . The relationship between 6S RNA and  $\sigma^B$  remains to be characterized.

We showed that *S. aureus* 6S RNA interacts directly with RNAP- $\sigma^A$ , raising the question of whether this could directly affect the holoenzyme stability. Of note, the  $\Delta$ *sau60* mutant exhibits a moderate reduction in fitness in the presence of rifampicin (Fig. 1B).  $\Delta$ *sau60* corresponds to a deletion within the intergenic sequence upstream of *rpoB* encoding the  $\beta$  subunit of RNA polymerase; this deletion may alter the ratio of RNAP subunits and possibly the RNAP stability, leading to a rifampicin susceptibility phenotype. However, this attenuated phenotype was not detected by a spot test.

In *E. coli*, the majority of 6S RNA is coupled to RNAP- $\sigma^{70}$  (29). In *Streptococcus pneumoniae*, 6S RNA bound to RNAP was recently proposed to be a stockpile for inactive RNAP (72). In *S. aureus*, the absence of 6S RNA leads to a reduced amount of  $\sigma^A$  in prolonged stationary-phase cultures. A similar effect was observed with  $\sigma^{70}$  in *E. coli* (29) and in the soluble sigma fraction of *Synechocystis* sp. (73). In *S. aureus*,  $\sigma^A$  is unstable (74); our results indicate that it is probably stabilized by RNAP core enzyme and 6S RNA. Knowing that  $\sigma^A$  is the vegetative sigma factor in *S. aureus*, decreased levels in the  $\Delta$ *ssrS<sup>5a</sup>* strain could have a negative impact on growth, and particularly on outgrowth recovery. In comparison, in *B. subtilis*, which expresses two different 6S RNAs, outgrowth is delayed in cells lacking 6S-1 RNA (75), whereas no extended lag phase was noticed in *E. coli* 6S RNA-deficient cells (29). Similar to *E. coli* (29), no lag linked to *ssrS* was observed during *S. aureus* outgrowth from stationary phase, suggesting that reduced  $\sigma^A$  pools in the *ssrS<sup>5a</sup>* mutant are enough to manage growth restart and that 6S RNA is not essential for growth in rich medium.

RNAP inhibitors remain in use in combination therapies against difficult-to-treat infections (4). Antibiotic concentrations below the MIC are encountered by bacteria under many environmental conditions, including hosts undergoing antimicrobial treatments (76). We demonstrated that 6S RNA provides protection against low concentrations of RNAP inhibitors. 6S RNA is highly conserved, and the effects of *ssrS* deletion on RNAP inhibitor susceptibility were observed in unrelated pathogens. 6S RNA may significantly enhanced fitness to RNAP inhibitors under these conditions. Our studies indicate the importance of 6S RNA in stabilizing RNAP interactions with  $\sigma^A$  and suggest that it plays its main roles in prolonged stationary phase. Our findings give insight into the mode of action of 6S RNA in an important pathogen and suggest the need to develop strategies that prevent low-level rifampicin from persisting in the antibiotic-treated host.

This protective effect is possibly due to steric hindrance, as the presence of 6S RNA would reduce the accessibility of the RNAP to its inhibitors. A second nonexclusive proposal is that the destabilization of  $\sigma^A$  associated with the absence of 6S RNA affects the transcriptional program to adapt to low concentrations of RNAP inhibitors. Note that in *S. aureus*, this shielding effect is not associated with the sigma stress factor  $\sigma^B$ .

## MATERIALS AND METHODS

**Bacterial strains and culture.** All strains used in this study and their genotypes are listed in Table S1 in the supplemental material. Strains were cultured at 37°C, with 180-rpm agitation for liquid cultures except for *C. difficile*. The latter was cultured under anaerobic conditions (5% H<sub>2</sub>, 5% CO<sub>2</sub>, 90% N<sub>2</sub>) with 7.5  $\mu$ g mL<sup>-1</sup> (precultures) or 15  $\mu$ g mL<sup>-1</sup> (plates) thiamphenicol for plasmid selection. *E. coli* and

*S. enterica* (serovar Typhimurium) strains were cultured in lysogeny broth (LB), *S. aureus* strains in brain heart infusion (BHI) or tryptic soy broth (TSB), and *C. difficile* in BHI. When necessary, media were supplemented with antibiotics.

*S. aureus* mutants were constructed in the HG003 or HG002 background (58) by allelic exchange using pIMAY (77) derivatives (except strains for RNAP purification), as described elsewhere (33). Plasmids used in this study are described in Table S2. Most plasmids constructed for this study were obtained by Gibson assembly (78), using primers listed in Table S3, and cloned in *E. coli* IM08B (79). An ectopically complemented *S. aureus* mutant was obtained from the *ssrS<sup>S<sub>5</sub>a</sup>* mutant by a 2-step crossover recombination at locus 2 using a pIMAY derivative as described above. Genetic features of  $\Delta$ *ssrS<sup>S<sub>5</sub>a</sup>*,  $\Delta$ *ssrS<sup>S<sub>5</sub>a</sup>* ecto-*ssrS<sup>S<sub>5</sub>a</sup>* and  $\Delta$ *ssrS<sup>S<sub>5</sub>a</sup>::ssrS<sup>LT2</sup>* *S. aureus* mutants used in this study are described in Fig. S1.

To purify *S. aureus*  $\sigma$  factors, *sigA* and *sigB* from HG003 were PCR amplified using primers F-SigA/R-SigA-His and F-SigB/R-SigB-His, respectively, and cloned into the NdeI/XhoI restriction sites of the pET-21C vector. The resulting plasmids pET-21C-*sigA* and pET-21C-*sigB* were transformed into *E. coli* strain BL21 (DE3) pLysS, leading to strains producing  $\sigma^A$  and  $\sigma^B$  that were His<sub>6</sub> tagged in their C-terminal portions upon IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) induction.

A HG003 strain expressing a chromosomally encoded His-tagged RpoC for the purification of the RNAP core enzyme was constructed as followed. The recombinational transfer of the histidine sequence into *rpoC* gene was achieved by two-step PCR. A sequence encoding 10 histidines was added upstream from the termination codon, in frame with RpoC ( $\beta'$  subunit of RNA polymerase). The *rpoC-his* fragment was generated by long-flanking homology PCR using the primers listed in Table S3 and cloned between the BamHI and PstI restriction sites of temperature-sensitive pBT2 vector (80) to obtain pBT2-*rpoC-His* plasmid. The resulting plasmid was electroporated into *S. aureus* RN4220 and then transferred to HG003 strain. The gene encoding His-tagged RpoC protein was integrated into the *S. aureus* HG003 chromosome by double-crossover recombination as described elsewhere (81) to obtain the HG003 *rpoC-his* strain.

*S. enterica* serovar Typhimurium mutants were constructed in the background of strain LT2-derived MA7455 (82) using  $\lambda$ Red recombineering (83). The  $\Delta$ *ssrS<sup>LT2</sup>* ecto-*ssrS<sup>LT2</sup>* strain (ectopic complementation of the  $\Delta$ *ssrS<sup>LT2</sup>* mutation) was constructed by inserting an *ssrS<sup>LT2</sup>* copy fused to a chloramphenicol resistance cassette at the neutral *chiPQ* locus in *ssrS<sup>LT2</sup>* mutant as described above. See Fig. S2 for construction.

*C. difficile* mutants were constructed in the 630 $\Delta$ *erm* background (84). The knockout mutant was obtained using an allelic chromosomal exchange following the published method (85) with the primers CM57/CM58 and CM59/CM60 and pMSR vector pDIA7052. To complement the *ssrS<sup>cd</sup>* deletion mutant, the *ssrS<sup>cd</sup>* sequence and its promoter region were PCR amplified using the primer pair CM77/CM78 and cloned into pMTL84121 to produce pDIA7065. Two *E. coli* strains were used as intermediates: NEB 10-beta for plasmid construction (Table S2) and HB101 RP4 for conjugation.

**MIC determination.** Antimicrobial susceptibility testing by broth microdilution for rifampicin, rifabutin, fidaxomicin, and aureothricin MIC determination in *S. aureus* was performed as described elsewhere (86). The *S. enterica* serovar Typhimurium rifampicin MIC was determined as described elsewhere (87).

**Fitness experiment.** The fitness experiment was performed as described elsewhere (33), with three independent sRNA-tagged mutant libraries grown simultaneously in TSB with or without 6  $\mu$ g L<sup>-1</sup> rifampicin and sampled at four points: at an OD<sub>600</sub> of 1, ON, at an OD<sub>600</sub> of 1 after the first dilution, and at an OD<sub>600</sub> of 1 after the second dilution. All the mutants were tag sequenced with an adapted Illumina protocol. The amount of each mutant was normalized to the total amount of bacteria with and without rifampicin and to the inoculum (Fig. 1A). In total, three mutants [*locus1*, *rsaD(tag26)*, and *teg146* mutants] were discarded from the analysis because they were under-represented in the assembled library. Concerning *sprD* and *sau5949*, only two values were taken into account in the third dilution sampling.

**Spot test.** Overnight cultures were 10-fold serially diluted in NaCl 0.9% (*S. aureus* and *S. enterica*) or BHI (*C. difficile*) until a dilution of 10<sup>-8</sup> was reached and spotted on agar plates containing different sublethal antibiotic concentrations, namely, less than the MIC (rifampicin, <12  $\mu$ g L<sup>-1</sup> for *S. aureus* and <12  $\mu$ g mL<sup>-1</sup> for *S. enterica*; rifabutin, <15.6  $\mu$ g L<sup>-1</sup>; aureothricin, <6.25  $\mu$ g mL<sup>-1</sup>; fidaxomicin, <4 mg L<sup>-1</sup> for *S. aureus* and <30  $\mu$ g L<sup>-1</sup> for *C. difficile*). Pictures were taken after ON growth or 24 h for *C. difficile*.

**Growth curves.** *S. aureus* strains were cultured in microplates from ON triplicate cultures diluted 1/1,000 in BHI with or without 5  $\mu$ g L<sup>-1</sup> rifampicin. Two- and three-day cultures were also used as precultures for Fig. 6C. Absorbance at 600 nm (OD<sub>600</sub>) was measured over time with a plate reader (Clariostar).

**Fluorescence measurement.** mAmetrine expression (excitation wavelength [ $\lambda_{ex}$ ] = 425  $\pm$  15 nm; emission wavelength [ $\lambda_{em}$ ] = 525  $\pm$  15 nm) was monitored over time in microplates by a plate reader (Clariostar), simultaneously with absorbance measurement of overnight triplicates cultures diluted 1:1,000 in TSB to limit autofluorescence.

**RNA extraction.** Strains were cultured until the desired OD<sub>600</sub> was reached. After centrifugation, pellets were frozen in dry ice-ethanol. RNAs were then extracted by phenol-chloroform treatment as described elsewhere (88). When necessary, RNAs were incubated with Turbo DNase treatment (Thermo Fisher Scientific) prior to a second phenol-chloroform extraction.

**Northern blotting.** Ten micrograms of total RNAs per well was separated on 1.3% agarose or 10% Tris-borate-EDTA (TBE)-urea polyacrylamide gels (Criterion Precast gels) as described elsewhere (89). For polyacrylamide gels, electrophoresis in 1 $\times$  TBE was followed by transfer to Hybond-N<sup>+</sup> membranes in 0.5 $\times$  TBE using a TE70 ECL semidry transfer unit (Amersham Pharmacia Biotech). Probes (Table S3) were [ $\alpha$ -<sup>32</sup>P]dCTP labeled.

**RNA-seq and transcriptomic analysis.** RNAs (DNA free) extracted from triplicate cultures sampled at an  $OD_{600}$  of 7 were sequenced using a NextSeq 500/550 high-output kit v2 (75 cycles). Sequences were aligned to the reference genome (CP000253, NCTC8325) with the Bowtie2 tool and quantified with the Feature Counts program. Differential gene expression analysis was performed using the DESeq2 algorithm (90).

**5'-3' RACE.** 5' and 3' ends were determined using the circularization method described in reference 51. Ten micrograms of total RNA of the wild-type strain (HG003) was extracted from samplings at an  $OD_{600}$  of 7, ON, and on day 4. Primers used to amplify the 5'-3' junction with Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific) are listed in Table S3. A CloneJET PCR cloning kit (Thermo Fisher Scientific) was used to clone the final PCR products.

**Purification of  $\sigma^A$ ,  $\sigma^B$ , and RNAP core enzyme.** For  $\sigma$  purification, *E. coli* strains BL21(DE3) pET-21C-*sigA* and BL21(DE3) pET-21C-*sigB* were grown in 1 L of LB broth at 37°C to an  $OD_{600}$  of 0.5. After induction with 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside for 3 h, bacteria were collected by centrifugation, resuspended in 10 mL of buffer A (10 mM HEPES [pH 7.5], 200 mM NaCl, 1 mM  $MgCl_2$ , 20 mM imidazole, 5% glycerol) with 0.25 mg  $mL^{-1}$  lysozyme per gram of pellet and frozen and thawed twice. Lysates were treated with DNase I (100 U  $mL^{-1}$ ) for 20 min at 30°C, and supernatants containing  $\sigma^A$ -His or  $\sigma^B$ -His proteins were obtained by centrifugation at  $8,000 \times g$  for 15 min. For RNA polymerase purification, a fresh overnight culture of *S. aureus* HG003 *rhoC-his* was used to inoculate 500 mL of BHI at an  $OD_{600}$  of 0.1 and grown for 5 h at 37°C. The culture was harvested by centrifugation at  $4,000 \times g$  for 15 min. The cell pellet was resuspended in 10 mL of buffer A with 1 mg of lysostaphin and DNase I (100 U  $mL^{-1}$ ). After incubation for 20 min at 37°C, the lysate was clarified by centrifuging for 30 min at  $40,000 \times g$ . For affinity purification of  $\sigma^A$ -His,  $\sigma^B$ -His, and His<sub>10</sub>-tagged RNAP, a HiTrap Talon column (5 mL; GE Healthcare) was connected to an AKTA Prime chromatography system (GE Healthcare) equilibrated with buffer B (10 mM HEPES [pH 7.5], 1 M NaCl, 20 mM imidazole, 5% glycerol). After loading the lysate containing either  $\sigma^A$ -His,  $\sigma^B$ -His, or His<sub>10</sub>-tagged RNAP, the column was washed with 100 mL of buffer B. His-tagged proteins were then eluted using an imidazole gradient, dialyzed in buffer C (10 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM  $MgCl_2$ , 5% glycerol), and then subjected to a second step of purification on heparin column. Proteins were loaded on a HiTrap heparin HP column (1 mL; GE Healthcare) equilibrated with buffer C. After a wash with 20 mL of buffer C, proteins were eluted using a NaCl gradient, dialyzed in buffer C, and then concentrated in a centrifugal concentrator with a 10-kDa-molecular-weight-cutoff membrane (Merck Millipore).

**EMSA.** *ssrS<sup>5a</sup>* and *sprB* were *in vitro* transcribed from PCR product templates containing a T7 promoter (primers listed in Table S3) with a MEGAscript T7 transcription kit (Thermo Fisher Scientific). RNAs were separated by 8% polyacrylamide-7 M urea gel electrophoresis and eluted overnight in G50 elution buffer (20 mM Tris-HCl [pH 7.5], 2 mM EDTA and 0.25% SDS). RNAs were precipitated in cold ethanol and 0.3 M sodium acetate and dephosphorylated using calf intestinal alkaline phosphatase (New England Biolabs), according to manufacturer protocol. The RNAs obtained were 5' radiolabeled with T4 polynucleotide kinase (New England Biolabs) and [ $\gamma$ -<sup>32</sup>P]ATP (ATP) and purified with MicroSpin G-50 columns (Amersham Pharmacia Biotech). RNAP (140 nM) alone or preincubated 10 min at 37°C with 420 nM sigma factors was mixed with 4 nM radiolabeled 6S RNA or SprB in buffer D (15 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM  $MgCl_2$ , 5% glycerol, 100  $\mu$ g  $mL^{-1}$  BSA, 200  $\mu$ g  $mL^{-1}$  *E. coli* tRNA). Complex formation was performed at 37°C for 10 min, and samples were loaded on 5% polyacrylamide-5% glycerol gels under nondenaturing conditions. Gels were dried and visualized using a Typhoon phosphorimager (Molecular Dynamics).

**$\sigma^A$  and  $\beta/\beta'$  subunit quantification.** *S. aureus* strains were cultured in triplicate until the desired  $OD_{600}$  was reached. Frozen pellets were lysed in 50 mM Tris-HCl buffer (pH 7.5) with glass beads. The total protein amount in the supernatant was determined by Bradford protein assay. Western blot electrophoresis was performed with 3  $\mu$ g proteins per well, using 8% bis-Tris Plus polyacrylamide gels (Bolt; Invitrogen). Transfer and hybridization followed iBlot and iBind manufacturer instructions (Invitrogen), respectively. Membranes were prehybridized at 4°C ON with human serum (1:10,000 dilution) to saturate unspecific binding. Rabbit primary antibodies were used for immunodetection of  $\sigma^A$  (anti- $\sigma^A$ ; 1:5,000 dilution) and  $\beta/\beta'$  subunits (anti-RNAP; 1:10,000 dilution). A horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Advansta; 1:4,000 dilution) was chosen as the secondary antibody. Pictures were taken with a charge-coupled device (CCD) camera. The statistical analysis for the comparison of  $\sigma^A$  normalized to  $\beta/\beta'$  quantity between strains was done by a one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test to identify pairs with significantly different amounts of  $\sigma^A$ .

**Data availability.** The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB50160 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB50160>).

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 1.5 MB.

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## REFERENCES

- Lowy FD. 1998. *Staphylococcus aureus* infections. *N Engl J Med* 339: 520–532. <https://doi.org/10.1056/NEJM199808203390806>.
- Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev* 28:603–661. <https://doi.org/10.1128/CMR.00134-14>.
- Mancuso G, Midiri A, Gerace E, Biondo C. 2021. Bacterial antibiotic resistance: the most critical pathogens. *Pathogens* 10 10:1310. <https://doi.org/10.3390/pathogens10101310>.
- Zimmerli W, Sendi P. 2019. Role of rifampin against staphylococcal biofilm infections in vitro, in animal models, and in orthopedic-device-related infections. *Antimicrob Agents Chemother* 63:e01746-18. <https://doi.org/10.1128/AAC.01746-18>.
- Sippel A, Hartmann G. 1968. Mode of action of rifamycin on the RNA polymerase reaction. *Biochim Biophys Acta* 157:218–219. [https://doi.org/10.1016/0005-2787\(68\)90286-4](https://doi.org/10.1016/0005-2787(68)90286-4).
- Hartmann G, Honikel KO, Knusel F, Nuesch J. 1967. The specific inhibition of the DNA-directed RNA synthesis by rifamycin. *Biochim Biophys Acta* 145:843–844. [https://doi.org/10.1016/0005-2787\(67\)90147-5](https://doi.org/10.1016/0005-2787(67)90147-5).
- Campbell EA, Korzhveva N, Mustaev A, Murakami K, Nair S, Goldfarb A, Darst SA. 2001. Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell* 104:901–912. [https://doi.org/10.1016/s0092-8674\(01\)00286-0](https://doi.org/10.1016/s0092-8674(01)00286-0).
- Darst SA. 2001. Bacterial RNA polymerase. *Curr Opin Struct Biol* 11:155–162. [https://doi.org/10.1016/s0959-440x\(00\)00185-8](https://doi.org/10.1016/s0959-440x(00)00185-8).
- Murakami KS. 2015. Structural biology of bacterial RNA polymerase. *Biomolecules* 5:848–864. <https://doi.org/10.3390/biom5020848>.
- Murakami KS, Darst SA. 2003. Bacterial RNA polymerases: the whole story. *Curr Opin Struct Biol* 13:31–39. [https://doi.org/10.1016/s0959-440x\(02\)00005-2](https://doi.org/10.1016/s0959-440x(02)00005-2).
- Feklistov A, Sharon BD, Darst SA, Gross CA. 2014. Bacterial sigma factors: a historical, structural, and genomic perspective. *Annu Rev Microbiol* 68: 357–376. <https://doi.org/10.1146/annurev-micro-092412-155737>.
- Gruber TM, Gross CA. 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu Rev Microbiol* 57:441–466. <https://doi.org/10.1146/annurev.micro.57.030502.090913>.
- Soutourina O, Dubois T, Monot M, Shelyakin PV, Saujet L, Boudry P, Gelfand MS, Dupuy B, Martin-Verstraete I. 2020. Genome-wide transcription start site mapping and promoter assignments to a sigma factor in the human enteropathogen *Clostridioides difficile*. *Front Microbiol* 11: 1939. <https://doi.org/10.3389/fmicb.2020.01939>.
- Deora R, Misra TK. 1996. Characterization of the primary sigma factor of *Staphylococcus aureus*. *J Biol Chem* 271:21828–21834. <https://doi.org/10.1074/jbc.271.36.21828>.
- Deora R, Tseng T, Misra TK. 1997. Alternative transcription factor sigmaSB of *Staphylococcus aureus*: characterization and role in transcription of the global regulatory locus *σ*. *J Bacteriol* 179:6355–6359. <https://doi.org/10.1128/jb.179.20.6355-6359.1997>.
- Kullik I, Giachino P, Fuchs T. 1998. Deletion of the alternative sigma factor sigmaB in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. *J Bacteriol* 180:4814–4820. <https://doi.org/10.1128/JB.180.18.4814-4820.1998>.
- van Schaik W, Abee T. 2005. The role of sigmaB in the stress response of Gram-positive bacteria—targets for food preservation and safety. *Curr Opin Biotechnol* 16:218–224. <https://doi.org/10.1016/j.copbio.2005.01.008>.
- Morikawa K, Inose Y, Okamura H, Maruyama A, Hayashi H, Takeyasu K, Ohta T. 2003. A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. *Genes Cells* 8:699–712. <https://doi.org/10.1046/j.1365-2443.2003.00668.x>.
- Fagerlund A, Granum PE, Havarstein LS. 2014. *Staphylococcus aureus* competence genes: mapping of the SigH, ComK1 and ComK2 regulons by transcriptome sequencing. *Mol Microbiol* 94:557–579. <https://doi.org/10.1111/mmi.12767>.
- Shaw LN, Lindholm C, Prajsnar TK, Miller HK, Brown MC, Golonka E, Stewart GC, Tarkowski A, Potempa J. 2008. Identification and characterization of sigma, a novel component of the *Staphylococcus aureus* stress and virulence responses. *PLoS One* 3:e3844. <https://doi.org/10.1371/journal.pone.0003844>.
- Browning DF, Butala M, Busby SJW. 2019. Bacterial transcription factors: regulation by Pick “N” Mix. *J Mol Biol* 431:4067–4077. <https://doi.org/10.1016/j.jmb.2019.04.011>.
- Waters LS, Storz G. 2009. Regulatory RNAs in bacteria. *Cell* 136:615–628. <https://doi.org/10.1016/j.cell.2009.01.043>.
- Wagner EGH, Romby P. 2015. Small RNAs in bacteria and archaea: who they are, what they do, and how they do it. *Adv Genet* 90:133–208. <https://doi.org/10.1016/bs.adgen.2015.05.001>.
- Romby P, Charpentier E. 2010. An overview of RNAs with regulatory functions in Gram-positive bacteria. *Cell Mol Life Sci* 67:217–237. <https://doi.org/10.1007/s00018-009-0162-8>.
- Bouloc P, Felden B. 2011. Ribonucleic acids: regulators of *Staphylococcus aureus* and role in bacterial virulence. *Med Sci* 27:238–241.
- Bouloc P, Repoila F. 2016. Fresh layers of RNA-mediated regulation in Gram-positive bacteria. *Curr Opin Microbiol* 30:30–35. <https://doi.org/10.1016/j.mib.2015.12.008>.
- Hindley J. 1967. Fractionation of 32P-labelled ribonucleic acids on polyacrylamide gels and their characterization by fingerprinting. *J Mol Biol* 30: 125–136. [https://doi.org/10.1016/0022-2836\(67\)90248-3](https://doi.org/10.1016/0022-2836(67)90248-3).
- Brownlee GG. 1971. Sequence of 6S RNA of *E. coli*. *Nat New Biol* 229:147–149. <https://doi.org/10.1038/newbio229147a0>.
- Wassarman KM, Storz G. 2000. 6S RNA regulates *E. coli* RNA polymerase activity. *Cell* 101:613–623. [https://doi.org/10.1016/s0092-8674\(00\)80873-9](https://doi.org/10.1016/s0092-8674(00)80873-9).
- Wassarman KM. 2018. 6S RNA, a global regulator of transcription. *Microbiol Spectr* 6:RWR-0019. <https://doi.org/10.1128/microbiolspec.RWR-0019-2018>.
- Cavanagh AT, Wassarman KM. 2014. 6S RNA, a global regulator of transcription in *Escherichia coli*, *Bacillus subtilis*, and beyond. *Annu Rev Microbiol* 68:45–60. <https://doi.org/10.1146/annurev-micro-092611-150135>.
- Wehner S, Damm K, Hartmann RK, Marz M. 2014. Dissemination of 6S RNA among bacteria. *RNA Biol* 11:1467–1478. <https://doi.org/10.4161/rna.29894>.
- Le Lam TN, Morvan C, Liu W, Bohn C, Jaszczyszyn Y, Bouloc P. 2017. Finding sRNA-associated phenotypes by competition assays: an example with *Staphylococcus aureus*. *Methods* 117:21–27. <https://doi.org/10.1016/j.ymeth.2016.11.018>.
- Liu W, Rochat T, Toffano-Nioche C, Le Lam TN, Bouloc P, Morvan C. 2018. Assessment of bona fide sRNAs in *Staphylococcus aureus*. *Front Microbiol* 9:228. <https://doi.org/10.3389/fmicb.2018.00228>.
- Della Bruna C, Schioppacassi G, Ungheri D, Jabes D, Morvillo E, Sanfilippo A. 1983. LM 427, a new spiro-piperidylrifamycin: *in vitro* and *in vivo* studies. *J Antibiot (Tokyo)* 36:1502–1506. <https://doi.org/10.7164/antibiotics.36.1502>.
- Sanfilippo A, Della Bruna C, Marsili L, Morvillo E, Pasqualucci CR, Schioppacassi G, Ungheri D. 1980. Biological activity of a new class of rifamycins. Spiro-piperidyl-rifamycins. *J Antibiot (Tokyo)* 33:1193–1198. <https://doi.org/10.7164/antibiotics.33.1193>.
- Coronelli C, White RJ, Lancini GC, Parenti F. 1975. Lipiamycin, a new antibiotic from Actinoplanes. II. Isolation, chemical, biological and biochemical characterization. *J Antibiot* 28:253–259. <https://doi.org/10.7164/antibiotics.28.253>.



38. Parenti F, Pagani H, Beretta G. 1975. Lipiarmycin, a new antibiotic from *Actinoplanes*. I. Description of the producer strain and fermentation studies. *J Antibiot* 28:247–252. <https://doi.org/10.7164/antibiotics.28.247>.
39. Theriault RJ, Karwowski JP, Jackson M, Girolami RL, Sunga GN, Vojtko CM, Coen LJ. 1987. Tiacumicins, a novel complex of 18-membered macrolide antibiotics. I. Taxonomy, fermentation and antibacterial activity. *J Antibiot* 40:567–574. <https://doi.org/10.7164/antibiotics.40.567>.
40. Zhanel GG, Walkty AJ, Karlowsky JA. 2015. Fidaxomicin: a novel agent for the treatment of *Clostridium difficile* infection. *Can J Infect Dis Med Microbiol* 26:305–312. <https://doi.org/10.1155/2015/934594>.
41. Talpaert M, Campagnari F, Clerici L. 1975. Lipiarmycin: an antibiotic inhibiting nucleic acid polymerases. *Biochem Biophys Res Commun* 63:328–334. [https://doi.org/10.1016/s0006-291x\(75\)80047-7](https://doi.org/10.1016/s0006-291x(75)80047-7).
42. Lin W, Das K, Degen D, Mazumder A, Duchi D, Wang D, Ebricht YW, Ebricht RY, Sineva E, Gigliotti M, Srivastava A, Mandal S, Jiang Y, Liu Y, Yin R, Zhang Z, Eng ET, Thomas D, Donadio S, Zhang H, Zhang C, Kapanidis AN, Ebricht RH. 2018. Structural basis of transcription inhibition by fidaxomicin (lipiarmycin A3). *Mol Cell* 70:60–71.E15. <https://doi.org/10.1016/j.molcel.2018.02.026>.
43. Artsimovitch I, Seddon J, Sears P. 2012. Fidaxomicin is an inhibitor of the initiation of bacterial RNA synthesis. *Clin Infect Dis* 55(Suppl 2):S127–S131. <https://doi.org/10.1093/cid/cis358>.
44. Tupin A, Gualtieri M, Leonetti JP, Brodolin K. 2010. The transcription inhibitor lipiarmycin blocks DNA fitting into the RNA polymerase catalytic site. *EMBO J* 29:2527–2537. <https://doi.org/10.1038/emboj.2010.135>.
45. Qin Z, Huang S, Yu Y, Deng H. 2013. Dithiopyrrolone natural products: isolation, synthesis and biosynthesis. *Mar Drugs* 11:3970–3997. <https://doi.org/10.3390/md11103970>.
46. Kroger C, Colgan A, Srikumar S, Handler K, Sivasankaran SK, Hammarlof DL, Canals R, Grissom JE, Conway T, Hokamp K, Hinton JC. 2013. An infection-relevant transcriptomic compendium for *Salmonella enterica* serovar Typhimurium. *Cell Host Microbe* 14:683–695. <https://doi.org/10.1016/j.chom.2013.11.010>.
47. Trotochaud AE, Wassarman KM. 2005. A highly conserved 6S RNA structure is required for regulation of transcription. *Nat Struct Mol Biol* 12:313–319. <https://doi.org/10.1038/nsmb917>.
48. Barrick JE, Sudarsan N, Weinberg Z, Ruzzo WL, Breaker RR. 2005. 6S RNA is a widespread regulator of eubacterial RNA polymerase that resembles an open promoter. *RNA* 11:774–784. <https://doi.org/10.1261/rna.7286705>.
49. Ando Y, Asari S, Suzuma S, Yamane K, Nakamura K. 2002. Expression of a small RNA, BS203 RNA, from the *yocJ*-*yocJ* intergenic region of *Bacillus subtilis* genome. *FEMS Microbiol Lett* 207:29–33. <https://doi.org/10.1111/j.1574-6968.2002.tb11023.x>.
50. Thuring M, Ganapathy S, Schluter MAC, Lechner M, Hartmann RK. 2021. 6S-2 RNA deletion in the undomesticated *B. subtilis* strain NCIB 3610 causes a biofilm derepression phenotype. *RNA Biol* 18:79–92. <https://doi.org/10.1080/15476286.2020.1795408>.
51. Soutourina OA, Monot M, Boudry P, Saujet L, Pichon C, Sismeiro O, Semenova E, Severinov K, Le Bouguenec C, Coppee JY, Dupuy B, Martin-Verstraete I. 2013. Genome-wide identification of regulatory RNAs in the human pathogen *Clostridium difficile*. *PLoS Genet* 9:e1003493. <https://doi.org/10.1371/journal.pgen.1003493>.
52. Pichon C, Felden B. 2005. Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains. *Proc Natl Acad Sci U S A* 102:14249–14254. <https://doi.org/10.1073/pnas.0503838102>.
53. Prados J, Linder P, Redder P. 2016. TSS-EMOTE, a refined protocol for a more complete and less biased global mapping of transcription start sites in bacterial pathogens. *BMC Genomics* 17:849. <https://doi.org/10.1186/s12864-016-3211-3>.
54. Lal A, Krishna S, Seshasayee ASN. 2018. Regulation of global transcription in *Escherichia coli* by Rsd and 6S RNA. *G3 (Bethesda)* 8:2079–2089. <https://doi.org/10.1534/g3.118.200265>.
55. Geissen R, Steuten B, Polen T, Wagner R. 2010. *E. coli* 6S RNA: a universal transcriptional regulator within the centre of growth adaptation. *RNA Biol* 7:564–568. <https://doi.org/10.4161/rna.7.5.12969>.
56. Neusser T, Polen T, Geissen R, Wagner R. 2010. Depletion of the non-coding regulatory 6S RNA in *E. coli* causes a surprising reduction in the expression of the translation machinery. *BMC Genomics* 11:165. <https://doi.org/10.1186/1471-2164-11-165>.
57. Mader U, Nicolas P, Depke M, Pane-Farre J, Debarbouille M, van der Kooi-Pol MM, Guerin C, Derozier S, Hiron A, Jarmer H, Leduc A, Michalik S, Reilman E, Schaffer M, Schmidt F, Bessieres P, Noirot P, Hecker M, Msadek T, Volker U, van Dijl JM. 2016. *Staphylococcus aureus* transcriptome architecture: from laboratory to infection-mimicking conditions. *PLoS Genet* 12:e1005962. <https://doi.org/10.1371/journal.pgen.1005962>.
58. Herbert S, Ziebandt AK, Ohlsen K, Schafer T, Hecker M, Albrecht D, Novick R, Gotz F. 2010. Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. *Infect Immun* 78:2877–2889. <https://doi.org/10.1128/IAI.00088-10>.
59. Cavanagh AT, Chandransu P, Wassarman KM. 2010. 6S RNA regulation of relA alters ppGpp levels in early stationary phase. *Microbiology (Reading)* 156:3791–3800. <https://doi.org/10.1099/mic.0.043992-0>.
60. Liu W, Boudry P, Bohn C, Bouloc P. 2020. *Staphylococcus aureus* pigmentation is not controlled by Hfq. *BMC Res Notes* 13:63. <https://doi.org/10.1186/s13104-020-4934-4>.
61. Kint N, Janoir C, Monot M, Hoys S, Soutourina O, Dupuy B, Martin-Verstraete I. 2017. The alternative sigma factor sigma(B) plays a crucial role in adaptive strategies of *Clostridium difficile* during gut infection. *Environ Microbiol* 19:1933–1958. <https://doi.org/10.1111/1462-2920.13696>.
62. Bordeau V, Cady A, Revest M, Rostan O, Sassi M, Tattevin P, Donnio PY, Felden B. 2016. *Staphylococcus aureus* regulatory RNAs as potential biomarkers for bloodstream infections. *Emerg Infect Dis* 22:1570–1578. <https://doi.org/10.3201/eid2209.151801>.
63. Hsu LM, Zagorski J, Wang Z, Fournier MJ. 1985. *Escherichia coli* 6S RNA gene is part of a dual-function transcription unit. *J Bacteriol* 161:1162–1170. <https://doi.org/10.1128/jb.161.3.1162-1170.1985>.
64. Kim KS, Lee Y. 2004. Regulation of 6S RNA biogenesis by switching utilization of both sigma factors and endoribonucleases. *Nucleic Acids Res* 32:6057–6068. <https://doi.org/10.1093/nar/gkh939>.
65. Chae H, Han K, Kim KS, Park H, Lee J, Lee Y. 2011. Rho-dependent termination of *ssrS* (6S RNA) transcription in *Escherichia coli*: implication for 3' processing of 6S RNA and expression of downstream *ygfA* (putative 5-formyl-tetrahydrofolate cyclo-ligase). *J Biol Chem* 286:114–122. <https://doi.org/10.1074/jbc.M110.150201>.
66. Chen J, Boyaci H, Campbell EA. 2021. Diverse and unified mechanisms of transcription initiation in bacteria. *Nat Rev Microbiol* 19:95–109. <https://doi.org/10.1038/s41579-020-00450-2>.
67. Cavanagh AT, Klocko AD, Liu X, Wassarman KM. 2008. Promoter specificity for 6S RNA regulation of transcription is determined by core promoter sequences and competition for region 4.2 of sigma70. *Mol Microbiol* 67:1242–1256. <https://doi.org/10.1111/j.1365-2958.2008.06117.x>.
68. Gildehaus N, Neußer T, Wurm R, Wagner R. 2007. Studies on the function of the riboregulator 6S RNA from *E. coli*: RNA polymerase binding, inhibition of *in vitro* transcription and synthesis of RNA-directed *de novo* transcripts. *Nucleic Acids Res* 35:1885–1896. <https://doi.org/10.1093/nar/gkm085>.
69. Kim EY, Shin MS, Rhee JH, Choy HE. 2004. Factors influencing preferential utilization of RNA polymerase containing sigma-38 in stationary-phase gene expression in *Escherichia coli*. *J Microbiol* 42:103–110.
70. Trotochaud AE, Wassarman KM. 2004. 6S RNA function enhances long-term cell survival. *J Bacteriol* 186:4978–4985. <https://doi.org/10.1128/JB.186.15.4978-4985.2004>.
71. Haurlyuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K. 2015. Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nat Rev Microbiol* 13:298–309. <https://doi.org/10.1038/nrmicro3448>.
72. Hor J, Garriss G, Di Giorgio S, Hack LM, Vanselow JT, Forstner KU, Schlosser A, Henriques-Normark B, Vogel J. 2020. Grad-seq in a Gram-positive bacterium reveals exonucleolytic sRNA activation in competence control. *EMBO J* 39:e103852. <https://doi.org/10.15252/embj.2019103852>.
73. Heilmann B, Hakkila K, Georg J, Tyystjarvi T, Hess WR, Axmann IM, Dienst D. 2017. 6S RNA plays a role in recovery from nitrogen depletion in *Synechocystis* sp. PCC 6803. *BMC Microbiol* 17:229. <https://doi.org/10.1186/s12866-017-1137-9>.
74. Mondal R, Ganguly T, Chanda PK, Bandhu A, Jana B, Sau K, Lee CY, Sau S. 2010. Stabilization of the primary sigma factor of *Staphylococcus aureus* by core RNA polymerase. *BMB Rep* 43:176–181. <https://doi.org/10.5483/bmbrep.2010.43.3.176>.
75. Cavanagh AT, Sperger JM, Wassarman KM. 2012. Regulation of 6S RNA by pRNA synthesis is required for efficient recovery from stationary phase in *E. coli* and *B. subtilis*. *Nucleic Acids Res* 40:2234–2246. <https://doi.org/10.1093/nar/gkr1003>.
76. Andersson DI, Hughes D. 2014. Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol* 12:465–478. <https://doi.org/10.1038/nrmicro3270>.
77. Monk IR, Shah IM, Xu M, Tan MW, Foster TJ. 2012. Transforming the untransformable: application of direct transformation to manipulate

- genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. mBio 3:e00277-11. <https://doi.org/10.1128/mBio.00277-11>.
78. Gibson DG. 2011. Enzymatic assembly of overlapping DNA fragments. Methods Enzymol 498:349–361. <https://doi.org/10.1016/B978-0-12-385120-8.00015-2>.
79. Monk IR, Tree JJ, Howden BP, Stinear TP, Foster TJ. 2015. Complete bypass of restriction systems for major *Staphylococcus aureus* lineages. mBio 6:e00308-15. <https://doi.org/10.1128/mBio.00308-15>.
80. Bruckner R. 1997. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosus*. FEMS Microbiol Lett 151:1–8. [https://doi.org/10.1016/S0378-1097\(97\)00116-X](https://doi.org/10.1016/S0378-1097(97)00116-X).
81. Chabelskaya S, Gaillot O, Felden B. 2010. A *Staphylococcus aureus* small RNA is required for bacterial virulence and regulates the expression of an immune-evasion molecule. PLoS Pathog 6:e1000927. <https://doi.org/10.1371/journal.ppat.1000927>.
82. Figueroa-Bossi N, Coissac E, Netter P, Bossi L. 1997. Unsuspected prophage-like elements in *Salmonella typhimurium*. Mol Microbiol 25:161–173. <https://doi.org/10.1046/j.1365-2958.1997.4451807.x>.
83. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645. <https://doi.org/10.1073/pnas.120163297>.
84. Hussain HA, Roberts AP, Mullany P. 2005. Generation of an erythromycin-sensitive derivative of *Clostridium difficile* strain 630 (630 $\Delta$ erm) and demonstration that the conjugative transposon Tn916 $\Delta$ E enters the genome of this strain at multiple sites. J Med Microbiol 54:137–141. <https://doi.org/10.1099/jmm.0.45790-0>.
85. Peltier J, Hamiot A, Garneau JR, Boudry P, Maikova A, Hajnsdorf E, Fortier LC, Dupuy B, Soutourina O. 2020. Type I toxin-antitoxin systems contribute to the maintenance of mobile genetic elements in *Clostridioides difficile*. Commun Biol 3:718. <https://doi.org/10.1038/s42003-020-01448-5>.
86. Wiegand I, Hilpert K, Hancock RE. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163–175. <https://doi.org/10.1038/nprot.2007.521>.
87. Brandis G, Pietsch F, Alemayehu R, Hughes D. 2015. Comprehensive phenotypic characterization of rifampicin resistance mutations in *Salmonella* provides insight into the evolution of resistance in *Mycobacterium tuberculosis*. J Antimicrob Chemother 70:680–685. <https://doi.org/10.1093/jac/dku434>.
88. Oh ET, So JS. 2003. A rapid method for RNA preparation from Gram-positive bacteria. J Microbiol Methods 52:395–398. [https://doi.org/10.1016/S0167-7012\(02\)00218-x](https://doi.org/10.1016/S0167-7012(02)00218-x).
89. Luo X, Esberard M, Bouloc P, Jacq A. 2021. A small regulatory RNA generated from the malK 5' untranslated region targets gluconeogenesis in *Vibrio* species. mSphere 6:e00134-21. <https://doi.org/10.1128/mSphere.00134-21>.
90. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. <https://doi.org/10.1186/s13059-014-0550-8>.