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RESEARCH ARTICLE

Long Noncoding RNA SSR42 Controls *Staphylococcus aureus* Alpha-Toxin Transcription in Response to Environmental Stimuli

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ABSTRACT *Staphylococcus aureus* is a human pathogen causing a variety of diseases by versatile expression of a large set of virulence factors that most prominently features the cytotoxic and hemolytic pore-forming alpha-toxin. Expression of alpha-toxin is regulated by an intricate network of transcription factors. These include two-component systems sensing quorum and environmental signals as well as regulators reacting to the nutritional status of the pathogen. We previously identified the repressor of surface proteins (Rsp) as a virulence regulator. Acute cytotoxicity and hemolysis are strongly decreased in *rsp* mutants, which are characterized by decreased transcription of toxin genes as well as loss of transcription of a 1,232-nucleotide (nt)-long noncoding RNA (ncRNA), SSR42. Here, we show that SSR42 is the effector of Rsp in transcription regulation of the alpha-toxin gene, *hla*. SSR42 transcription is enhanced after exposure of *S. aureus* to subinhibitory concentrations of oxacillin which thus leads to an SSR42-dependent increase in hemolysis. Aside from Rsp, SSR42 transcription is under the control of additional global regulators, such as CodY, AgrA, CcpE, and σ^B, but is positioned upstream of the two-component system SaeRS in the regulatory cascade leading to alpha-toxin production. Thus, alpha-toxin expression depends on two long ncRNAs, SSR42 and RNAIII, which control production of the cytolytic toxin on the transcriptional and translational levels, respectively, with SSR42 as an important regulator of SaeRS-dependent *S. aureus* toxin production in response to environmental and metabolic signals.

IMPORTANCE *Staphylococcus aureus* is a major cause of life-threatening infections. The bacterium expresses alpha-toxin, a hemolysin and cytotoxin responsible for many of the pathologies of *S. aureus*. Alpha-toxin production is enhanced by subinhibitory concentrations of antibiotics. Here, we show that this process is dependent on the long noncoding RNA, SSR42. Further, SSR42 itself is regulated by several global regulators, thereby integrating environmental and nutritional signals that modulate hemolysis of the pathogen.

KEYWORDS *Staphylococcus aureus*, β-lactams, hemolysin gene regulation, noncoding RNA

Community-acquired and health care-associated methicillin-resistant *Staphylococcus aureus* (MRSA) strains are major causes for a variety of diseases (1, 2) ranging from superficial skin infections and skin and deep abscesses to severe conditions with high morbidity, such as osteomyelitis, endocarditis, and sepsis (3). *S. aureus* adapts to different host environments by coordinated expression of certain virulence factors (4–6). A major virulence factor of *S. aureus* is the pore-forming alpha-toxin (encoded by *hla*). In addition to the hemolytic, cytolytic, and dermonecrotic properties of alpha-toxin

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(7, 8), the generation of alpha-toxin pores within target cell membranes triggers release of cytokines and host cell apoptosis (9, 10). Alpha-toxin thereby acts on a multitude of different cell types, including macrophages, monocytes, lymphocytes, epithelial cells, and fibroblasts (7, 9, 11), thus having a major impact on the pathogenesis of infections such as pneumonia (12), corneal infections (13), and sepsis (14). Alpha-toxin production in *S. aureus* is tightly regulated in a time-dependent manner by two-component systems, such as the quorum sensing system accessory gene regulator (*agr*) (15, 16) and SaeRS (17), MarR-type DNA-binding proteins belonging to the staphylococcal accessory regulator (*sar*) family, as well as other regulators (reviewed in reference 18).

SaeRS is essential for *S. aureus* virulence, regulating the transcription of toxin-encoding genes, such as *hla*, and surface proteins as well as capsular biosynthesis (17, 19–21). Targets of the SaeRS two-component system are classified into two groups. Class I genes, e.g., *coa*, *fnbA*, *eap*, *efb*, *saeP*, *fib*, and *emp*, depend on the histidine kinase activity of SaeS, whereas for transcriptional activation of class II genes such as *hla* and *hlb*, a low basal phosphorylation level of the response regulator SaeR is sufficient (22; reviewed in references 17 and 23). *sae* is transcribed from two promoters, P1 and P3. Transcription from promoter P1 results in transcript T1 encoding a four-gene operon, *saePQRS*. *saeP* and *saeQ* are auxiliary genes (19, 24). Both resulting proteins, SaeP and SaeQ, were described to form a ternary complex activating phosphatase activity within SaeS while being dispensable for its kinase activity (22). The T1 transcript is unstable. It is processed by the endoribonuclease RNase Y to create the more stable transcript T2, consisting of *saeQRS* (25, 26). Transcript T3 is driven by the constitutively active promoter P3 and contains *saeRS* (25). A fourth transcript harboring only *saeP*, T4, is initiated at P1 and results either from processing of T1 or by premature termination (24, 27).

The *agr* quorum and diffusion sensing system constitutes another major virulence regulator in *S. aureus*. An auto-inducing peptide pheromone (AIP) derived from the precursor AgrD is secreted and modified by the membrane protein, AgrB. At high densities of bacteria, the AIP sensor histidine kinase AgrC phosphorylates the response regulator AgrA, which in turn leads to transcription of the main *agr* effector, RNAIII. Whereas some virulence factor genes are directly dependent on AgrA, a majority are regulated by the RNA (28) on either the transcriptional or translational level (29, 30). Noncoding RNAs (ncRNAs) have been shown to regulate gene expression acting in *cis* or *trans*, thereby modulating transcription, translation, and mRNA stability (31), and especially in bacteria ncRNAs play major roles in gene and virulence regulation (32). *S. aureus* produces an array of small ncRNAs whose full regulatory properties have not yet been completely deciphered (33–35). RNAIII regulates staphylococcal alpha-toxin on a translational level by base pairing to *hla* mRNA, thereby liberating a Shine-Dalgarno sequence which otherwise is sequestered within a secondary structure and inaccessible to the ribosomes (30).

In a transposon mutant screen we previously identified repressor of surface proteins(Rsp) as another virulence regulator. We demonstrated that deletion of *rsp* resulted in reduced hemolysis and cytotoxicity of *S. aureus* as well as a shift in pathotype (36). We identified the long ncRNA SSR42 as a major target of Rsp since SSR42 transcription was completely lost in insertional as well as deletion mutants of *rsp* (36). Prior to that study, SSR42 had been identified as an 891-nucleotide (nt) ncRNA that was stable during the stationary growth phase and thus was designated a small stable RNA (SSR) (37, 38). SSRs were implicated in bacterial adaption to detrimental conditions, thereby enhancing survival (38). SSR42 further regulates the transcription of 80 mRNA species in *S. aureus* strains UAMS-1 and LAC. Among the regulated genes were alpha-toxin, Panton-Valentine leukocidin (PVL), and protein A as well as genes for capsule biosynthesis (37). SSR42 has also been identified in other studies, where it has been designated Teg27 (39), sRNA363 (4), or srn_4470_RsaX28 (40), although with slightly different length estimates. However, transcriptome sequencing (RNA-seq) revealed a primary SSR42 transcript of 1,232 nt in the methicillin-resistant *S. aureus* (MRSA) strain JE2 (36). Another study demonstrated cleavage of SSR42 (there

termed RsaX28) by the endoribonuclease RNase Y in a so-called EMOTE (exact mapping of transcriptome ends) assay (40).

In the present study, we show that SSR42 is the effector of Rsp and that SSR42 is required for efficient transcription of *hla* message in an SaeRS-dependent manner. We further show that hemolysis is enhanced upon exposure of *S. aureus* to subinhibitory concentrations of antibiotics and that this process is dependent on SSR42. In order to elucidate the signal transduction cascade involved in this process, we demonstrated that SSR42 transcription is influenced by global regulators and thus serves as an integrator for various stimuli, which results in increased hemolysis.

RESULTS

SSR42 is the effector of Rsp-mediated hemolysis in *S. aureus*. We recently identified the AraC-type transcriptional regulator Rsp as a regulator of various virulence traits of *S. aureus* ranging from hemolysis to cytotoxicity. However, loss of *rsp* did not influence staphylococcal survival in whole blood or deep-abscess formation, suggesting that Rsp deficiency altered the pathotype of *S. aureus*. Rsp-dependently transcribed genes were determined by RNA-seq and included a set of virulence factors as well as the long noncoding RNA (ncRNA) SSR42. SSR42 is located directly upstream of *rsp* in an antiparallel orientation, and its transcription is strongly dependent on functional Rsp (36). Deletion of *rsp* strongly reduced hemolysis of *S. aureus* (36). Since SSR42 was previously described to influence hemolysis (37), we tested whether the hemolysis defect of an *rsp* mutant resulted from the loss of SSR42 and thus determined the potential role of SSR42 as a potential effector of Rsp. We generated a markerless SSR42 knockout mutant (ΔSSR42) by allelic replacement in the cytolytic strain *S. aureus* 6850, which encompassed the complete primary ncRNA transcript. Whereas deletion of SSR42 did not alter bacterial growth dynamics (see Fig. S1 in the supplemental material), hemolysis on sheep blood agar was strongly reduced (Fig. 1A). Similarly, hemolysis of erythrocyte suspensions in samples treated with culture supernatants of *S. aureus* ΔSSR42 were reduced to 30% of the wild-type level. Hemolysis was readily complemented in *trans* by expression of SSR42 from a plasmid, illustrating that the regulatory cascade of alpha-toxin expression was still intact in the deletion strain (Fig. 1B). We further excluded potential polar effects of the gene deletion by using various complementation plasmids (Fig. S1B and C).

Northern blotting using two different probes against SSR42 (Fig. S2A) demonstrated a transcript of an approximate length of 1,200 nt (Fig. S2A to C), confirming the RNA-seq data (36). Next to the predominant full-length SSR42 we identified a smaller RNA, which suggested processing of the primary transcript. Global mapping of potential RNase processing sites demonstrated an RNase Y cleavage site within SSR42 (Fig. S2H) (40). Indeed smaller transcript versions of SSR42 were not detectable in a Δ*rny* mutant of *S. aureus* (Fig. 2D). We thus analyzed SSR42 transcript stability in both wild-type and *rny*-negative backgrounds. However, these results did not reveal a role for RNase Y in destabilizing the SSR42 transcript (Fig. S2E and F).

Next, we generated a double-knockout *S. aureus* strain, ΔSSR42-*rsp*, in which we deleted the chromosomal region encoding both SSR42 and *rsp* (Fig. 1C and S1). We further transformed complementation plasmids into *S. aureus* ΔSSR42-*rsp* that reintroduced either SSR42, *rsp*, or both of the genes (pSSR42, p*rsp*, or pSSR42-*rsp*, respectively) (Fig. 1C to F) and tested the functional complementation of hemolysis (Fig. 1C). *S. aureus* ΔSSR42-*rsp* displayed strongly reduced hemolysis which was not complemented by either pSSR42 or p*rsp* (Fig. 1C). In contrast, reintroduction of the complete region encompassing SSR42 and *rsp* in *trans* (pSSR42-*rsp*) completely restored wild-type hemolysis levels.

We then isolated RNA from wild-type, mutant, and complemented mutant strains and analyzed transcript levels of SSR42, *rsp*, and *hla* message RNAs by quantitative PCR (qPCR). *hla* transcription was restored only in *S. aureus* ΔSSR42-*rsp* carrying pSSR42-*rsp* (Fig. 1D), corroborating that Rsp is required for SSR42 transcription. We therefore generated a plasmid which encoded either SSR42 (pAHT-SSR42) or *rsp* (pAHT-*rsp*) under

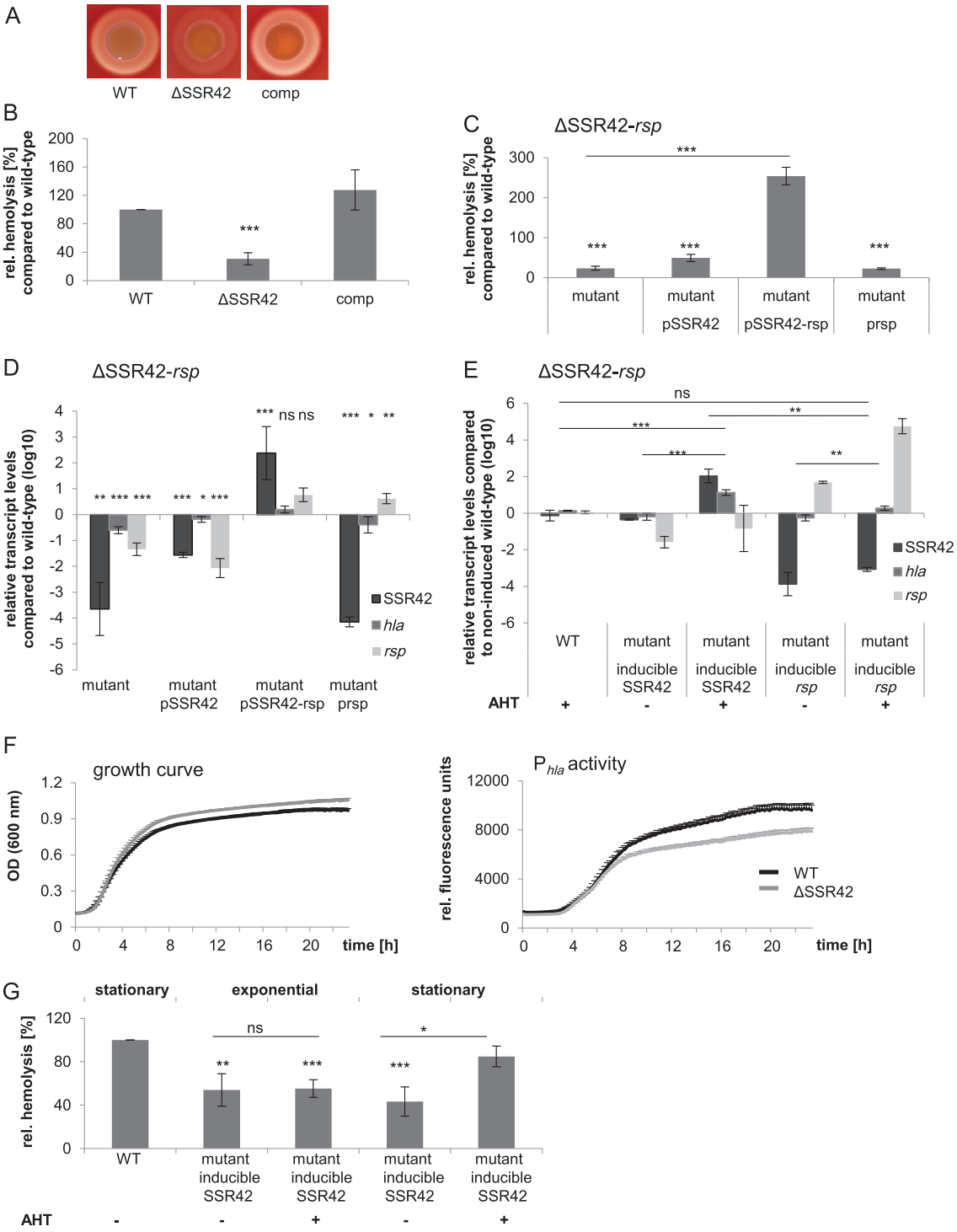

FIG 1 SSR42 is required for hemolysis in *S. aureus* and is Rsp dependent. *S. aureus* 6850 wild-type (WT) hemolysis is lost in a ΔSSR42 mutant but is complemented in *trans* (comp) on sheep blood agar (A) or upon exposure of sheep erythrocytes to stationary-growth-phase culture supernatants (B) rel, relative. (C) Episomal complementation with only SSR42 (pSSR42) or *rsp* (p*rsp*) is not sufficient to complement hemolysis in an SSR42-*rsp* double-deletion mutant, whereas reintroduction of the locus *rsp*-SSR42 in *trans* readily complements hemolysis (pSSR42-*rsp*). (D) Quantitative real-time PCR analysis of wild-type *S. aureus*, a ΔSSR42-*rsp* double-knockout mutant, and complemented mutants for comparison of transcript levels of SSR42, *hla*, and *rsp*. The transcription levels of SSR42, *hla*, and *rsp* were significantly reduced in the double-knockout mutant. SSR42 and *hla* transcription could be restored only when complementation plasmid pSSR42-*rsp* (harboring both SSR42 and *rsp*) was used. *hla* transcription could not be restored to wild-type levels when only *rsp* (p*rsp*) or SSR42 (pSSR42) was complemented in *trans*. Introduction of SSR42 (pSSR42) in *trans* could not successfully complement SSR42 transcription. (E) ncRNA SSR42 is required for efficient *hla* transcription. Quantitative real-time PCR analysis was performed of *hla* levels in wild-type *S. aureus* 6850, a ΔSSR42-*rsp* double-knockout mutant, and a mutant complemented with AHT-inducible transcription of either SSR42 (pAHT-SSR42) or *rsp* (pAHT-*rsp*). Only when SSR42 transcription was induced via

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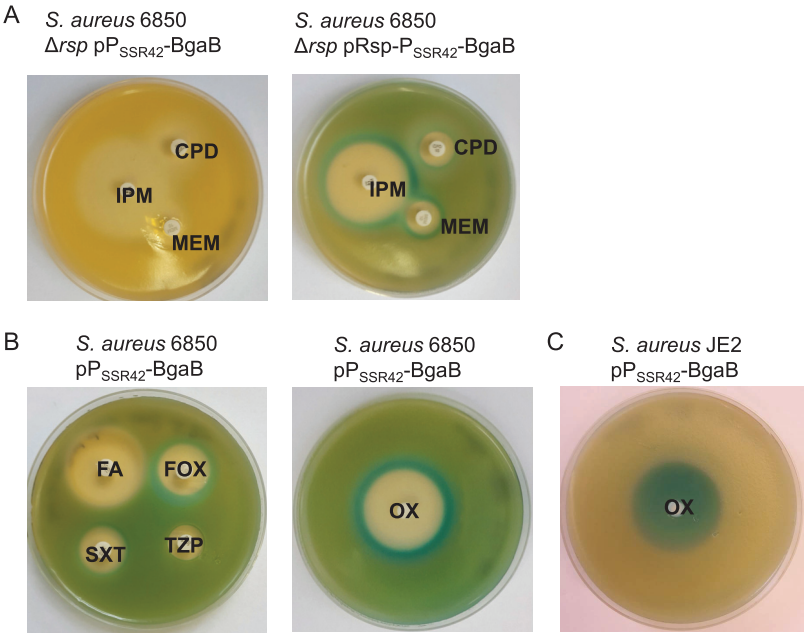

FIG 2 Promoter P_{SSR42} is *rsp*-dependently activated upon exposure to subinhibitory concentrations of antibiotics. (A and B) Disk diffusion assays with *S. aureus* 6850 Δ*rsp* harboring a transcriptional fusion of P_{SSR42} with β-galactosidase BgaB in the presence or absence of *rsp* (pP$_{SSR42}$-BgaB or pRsp-P$_{SSR42}$-BgaB) demonstrate activation of the promoter by production of blue dye on X-Gal agar plates. IPM, imipenem; CPD, cefpodoxime; MEM, meropenem; OX, oxacillin; FA, fusidic acid; FOX, cefoxitin; SXT, trimethoprim-sulfamethoxazole; TZP, piperacillin-tazobactam. agar plates. (C) Induction of SSR42 promoter activity with oxacillin in the MRSA strain JE2 harboring plasmid pP$_{SSR42}$-BgaB.

the control of an anhydrous tetracycline (AHT)-inducible promoter. Only upon induction of SSR42 transcription did we also observe a significant increase in *hla* transcript levels, suggesting that SSR42 transcription is involved in *hla* transcription, whereas Rsp mainly serves as a regulator for SSR42 transcription (Fig. 1E). Thus, effects of Rsp on *S. aureus* hemolysis are mediated via SSR42.

Next, we investigated if the complete SSR42 region was required for regulation of hemolysis. We therefore deleted various fragments of approximately 70 nt from the molecule in pSSR42 (deletions Δ1 through Δ8) and tested hemolysis of *S. aureus* ΔSSR42 complemented with the resulting plasmids (Fig. S3A to D). A 65-nt deletion (deletion Δ7; bp 2352858 to 2352794 according to the genome sequence of *S. aureus* 6850; NCBI RefSeq accession number NC_022222) spanning the region previously identified as the potential 5′ end of SSR42 (37) resulted in significantly reduced hemolysis and accumulation of RNA degradation products, as evidenced by Northern blotting. Another 63-nt region (Δ6; bp 2352688 to 2352742) was also important for SSR42 stability since its deletion resulted in reduction of RNA abundance. Deletion of 62 nucleotides upstream of a predicted RNase Y cleavage site (Δ8; bp 2352917 to 2352978) had no impact on hemolysis, whereas deletion of a 76-nt region (Δ1; bp 2352023 to 2352099) had a strong impact on hemolysis of *S. aureus* but not on stability of SSR42 (Fig. S3A to D).

FIG 1 Legend (Continued)
treatment with AHT (+) did *hla* transcript levels show a significant increase and reach wild-type levels. (F) Growth curve and *hla* promoter (P_{hla}) activity profile in wild-type *S. aureus* 6850 (WT) and an isogenic ΔSSR42 mutant. *hla* promoter activity is significantly reduced in ΔSSR42 at 9 h postinoculation ($P = 7.34 \times 10^{-5}$ normalized to optical density). (G) Hemolysis of *S. aureus* ΔSSR42 culture supernatants is complemented by SSR42 (pAHT-SSR42) upon AHT-induced (+ AHT) expression only in stationary growth phase and not in the exponential phase. For quantification of hemolysis, sheep erythrocytes were exposed to stationary-growth-phase culture supernatants, and absorbance of liberated heme at 405 nm was determined. Hemolytic level of wild-type supernatants was set to 100%. Statistical analysis was performed using Student's *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Introducing small mutations in SSR42 revealed a broad sequence range which was not implicated in the hemolysis regulation. A total of 437 nt encompassing this region (bp 2351972 to 2352410) were deleted from the SSR42 complementation plasmid, creating minimal SSR42 version 1. By deletion of another 128 nt (bp 2353043 to 2352915) of the 5′ end of SSR42, a complementation plasmid for minimal SSR42 version 2 was created. Secondary structure prediction showed the conservation of previous observed structural motifs (Fig. S3E and F). Due to the length of the molecule, the secondary structure predictions are only for visualization and rather incorrect. Only when a ΔSSR42 mutant was complemented with minimal version 1 were the hemolytic activity deficiencies partially complemented (75.64%, $P > 0.001$). Expression of minimal version 2 did not restore the hemolytic activity of a ΔSSR42 mutant (Fig. S3G). Analysis of transcript levels via Northern blotting revealed a complete lack of SSR42 transcripts, indicating the requirement of the deleted sequence in stabilizing SSR42 (Fig. S3H).

Sequence Δ1 (bp 2352023 to 2352099) was thought to regulate hemolysis since its absence did not affect the stability of the molecule as did the absence of sequences Δ6 and Δ7. To investigate whether region Δ1 would be sufficient to restore hemolysis in a ΔSSR42 mutant, the stem-loop structure encompassing this region was introduced in *trans* (pstemloop). Transcript levels of both SSR42 and *hla* were still significantly reduced compared to the wild-type level after introduction of pstemloop (Fig. S3I).

We next tested effects of SSR42 deletion on promoter activity of the *hla* transcript (P_{hla}). We generated a transcriptional fusion of P_{hla} and green fluorescent protein (GFP) (Fig. 1F and S4A) and introduced the reporter in the wild-type and ΔSSR42 backgrounds. As expected, *hla* promoter activity in wild-type bacteria was strongest in the stationary growth phase of wild-type *S. aureus* when transcription of *hla* is induced and stabilized by factors such as SaeR and SarA (37, 41). In contrast, in the ΔSSR42 mutant, P_{hla} promoter activity was significantly reduced during stationary growth phase, thereby corroborating that transcriptional activation of *hla* requires SSR42 (37). By monitoring P_{hla} activity over time in both the wild-type and ΔSSR42 backgrounds, we further show that *hla* transcription begins during transition from the exponential to the stationary growth phase and is highest during late stationary growth of the bacteria. Whereas we observed overexpression of *hla* message in *S. aureus* ΔSSR42 harboring pAHT-SSR42 after AHT addition already during exponential growth of the bacteria (Fig. S4B), hemolysis was restored only upon induction of SSR42 transcription during the stationary growth phase (Fig. 1G). Since the quorum sensing-controlled RNAIII is required for translation initiation of *hla* mRNA (30) yet only is transcribed during stationary phase, functional alpha-toxin can be formed only whenever RNAIII is present.

SSR42 transcription is modulated in response to subinhibitory concentrations of antibiotics. Subinhibitory concentrations of antibiotics have been shown to alter the toxin expression of *S. aureus* (42–45); however, the molecular details of this regulation are not known. Since SSR42 is required for transcript abundance of the alpha-toxin mRNA, we generated *S. aureus* reporter strains. These harbored a reporter plasmid in which the promoter P_{SSR42} was transcriptionally fused to the open reading frame *bgaB* encoding a β-galactosidase. We tested the effect of antibiotics on SSR42 transcription using disk diffusion assays, and activation of P_{SSR42} was detected by production of blue indigo dye on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

P_{SSR42} promoter activity was induced by antibiotics such as oxacillin, imipenem, meropenem, or cefpodoxime, and also by the DNA-damaging agent mitomycin C (Fig. 2 and S5; Table 1). This induction again was strongly dependent on functional Rsp (Fig. 2A). Further, we observed by disk diffusion as well as MIC strips (Fig. S5C) that the β-galactosidase activity was active in distinct zones beyond the zones of growth inhibition in which subinhibitory concentrations of the substances were present. We also tested P_{SSR42} promoter activation by a GFP reporter plasmid in *S. aureus* JE2. Planktonic growth in tryptic soy broth (TSB) of *S. aureus* JE2 was inhibited by 10 and 64 μg/ml oxacillin, and accordingly P_{SSR42} promoter activity was not detected. Upon exposure of the bacteria to 0.05 μg/ml oxacillin, which is only slightly inhibitory to growth of *S. aureus* JE2 in TSB,

TABLE 1 The SSR42 promoter P_{SSR42} is induced by various antibiotics

Drug (amt)	Description and/or effect(s)	Influence on P_{SSR42}[a]
Cefpodoxime (10 μg)	β-Lactam antibiotic; inhibition of transpeptidation, cell wall disruption	+
Cefoxitin (30 μg)		+
Oxacillin (10 μg)		+
Ampicillin (10 μg)		NA
Piperacillin-tazobactam (30 μg/60 μg)		NA
Imipenem (10 μg)		+
Meropenem (10 μg)		+
Amoxicillin-clavulanate (20 μg/10μg)		NA
Mitomycin C (10 μg)	DNA double-strand breaks, inhibition of DNA synthesis	+
Fusidic acid (10 μg)	Inhibition of protein synthesis, prevents turnover of elongation factor G from the ribosome	NA
Colistin (600 μg)	Disruption of cell membrane, amphiphilic interaction with cell membrane	−

[a]An increase (+) or decrease (−) in promoter activity is indicated. NA, not applicable (activity was not observed).

P_{SSR42} activity decreased initially, rose after 11 h, and eventually exceeded activity of untreated bacteria (Fig. S6B). In the methicillin-susceptible *S. aureus* (MSSA) strain 6850, subinhibitory concentrations of oxacillin (0.025 μg/ml) led to an overall increase in P_{SSR42} activity (Fig. S6C). In contrast, treatment of *S. aureus* JE2 with colistin reduced P_{SSR42} promoter activity in a GFP promoter activity assay (Fig. S6D).

Since antibiotics as well as the DNA-damaging agent mitomycin C induced the promoter of SSR42 (Fig. S5D) and since the substances previously had been shown to mediate the *S. aureus* SOS response (4, 46, 47), we tested if P_{SSR42} activation was part of the SOS response in *S. aureus*. However, mitomycin C-induced activation of P_{SSR42} was still observed at wild-type levels in an *S. aureus lexA*-G94E strain, a mutant in which the autoproteolytic cleavage of LexA and thus induction of the SOS-response are prevented (Fig. S5D) (48). Thus, SSR42 transcription is not affected by lack of functional LexA. However, involvement of the SOS response in regulation of transcriptional control cannot be completely excluded and needs further investigation.

***hla* transcription is modulated in response to subinhibitory concentrations of antibiotics in an SSR42-dependent manner.** We next investigated if oxacillin would induce upregulation of *hla* transcription in an SSR42-dependent manner. We therefore spotted bacterial cultures of wild-type *S. aureus* and an isogenic ΔSSR42 mutant on sheep blood agar and placed an oxacillin-containing disk (1 μg) between the two spotted strains (Fig. 3A). Wild-type bacteria close to the oxacillin disk displayed stronger hemolysis than distant bacterial colonies. We also recorded bacterial growth and GFP fluorescence in wild-type *S. aureus* as well as in an isogenic ΔSSR42 mutant equipped with a P_{hla}-GFP transcriptional fusion. Again, upregulation of *hla* promoter activity in response to oxacillin was observed only in wild-type *S. aureus* and not in the ΔSSR42 mutant (Fig. 3B). Our results thereby indicated that upregulation of hemolysin transcription in response to subinhibitory concentrations of oxacillin required the noncoding RNA SSR42.

SSR42 transcription is controlled by multiple global regulators and is required upstream of SaeRS. Since SSR42 was required for oxacillin-induced hemolysis of *S. aureus*, we sought to identify factors involved in transcription of the ncRNA. For that purpose, we used a transcriptional fusion of the SSR42 promoter (P_{SSR42}) and GFP and transduced the resulting reporter plasmid (pP_{SSR42}-GFP) into several different *S. aureus* strains and recorded P_{SSR42} activity over time courses of 23 h. We found two distinct promoter activity curves in all of the tested strains. Whereas the MRSA strains JE2 and MW2 as well as MSSA strain Newman exhibited sigmoidal activity profiles, the strains 6850, RN4220, COL, Cowan I, and HG003 displayed only one peak of P_{SSR42} activity (Fig. S6A).

We next transduced the reporter plasmid in a variety of insertional mutants of *S. aureus* JE2 (49) (Fig. 4 and S7). Figure 4 depicts a heat map of the differences in GFP

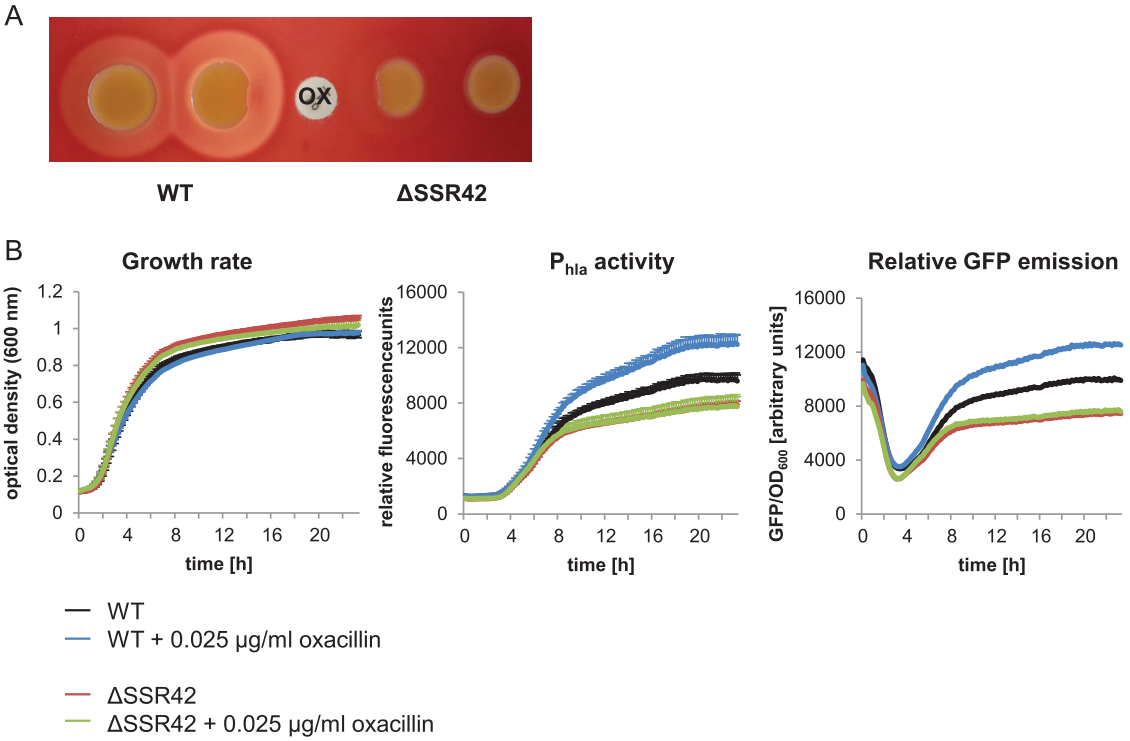

FIG 3 Oxacillin enhances hemolysis in an SSR42-dependent fashion. (A) Hemolysis of wild-type *S. aureus* and the ΔSSR42 mutant on sheep blood agar in the presence of oxacillin (OX). Wild-type bacteria display enhanced hemolysis when grown in close proximity to the 1-μg oxacillin disk. (B) Promoter P_{hla} shows strong activity in the presence of 0.025 μg/ml oxacillin in wild-type *S. aureus* 6850 but not in an isogenic SSR42 knockout mutant. Promoter activity was recorded over 23 h using a strain harboring a transcriptional fusion of P_{hla} with GFP (pP_{hla}-GFP) and measuring fluorescence as well as the OD_{600}. Depicted is GFP emission normalized to the OD (GFP/OD_{600}).

fluorescence levels between the wild type and the respective mutant strains. Activity of P_{SSR42} was completely lost in an *rsp* mutant (Network on Antimicrobial Resistance in *Staphylococcus aureus* [NARSA] Strain Repository number NE1304) (Fig. 4 and S7A), corroborating our findings on the strict Rsp dependency of SSR42 transcription (36). We also observed strongly reduced P_{SSR42} activity in mutants of the alternative sigma factor σB (*sigB* or *rpoF*; strain NE1109) and the σ^B-regulatory protein RsbU (NE1607) (Fig. S7B), whereas inactivation of the alternative sigma factor σ^S by gene deletion resulted in only a minute decrease in P_{SSR42} activity (Fig. 4 and S7I)

Insertional transposon mutants within genes for the carbon catabolite repressor CcpE (strain NE1560) as well as the global repressor CodY (NE1555) and a knockout of RpiRc (50) also demonstrated reduced P_{SSR42} promoter activity (Fig. S7C and D). Interestingly, strains carrying mutations in the CodY and CcpE genes exhibited strongly reduced P_{SSR42} activity completely lacking the second peak of P_{SSR42} activation (Fig. S7C).

Insertional inactivation of the gene encoding the quorum sensing response regulator AgrA (strain NE1532) and inactivation of a positive regulator of the *agr* system, SarU (NE96), drastically altered the activation profile by delaying the second peak of promoter activity from 9 h to approximately 17 h after inoculation. At these later time points, however, GFP fluorescence levels exceeded the level of isogenic wild-type bacteria (Fig. S7E and F). SarU is a MarR-type transcriptional regulator and part of the multigene *sarA* family of regulators in *S. aureus*. However, mutations within *sarA* and *sarT* did not significantly alter the expression profile of P_{SSR42}, and disruption of the gene encoding repressor of toxins, *rot* (strain NE386), led only to a slightly reduced activity of P_{SSR42} starting approximately at 11 h after inoculation (Fig. S7J). Insertional disruption of histidine kinase gene *saeS* (NE1296) led to stronger promoter activation starting 11 h after inoculation, whereas disruption of *saeR* (NE1622) did not change the

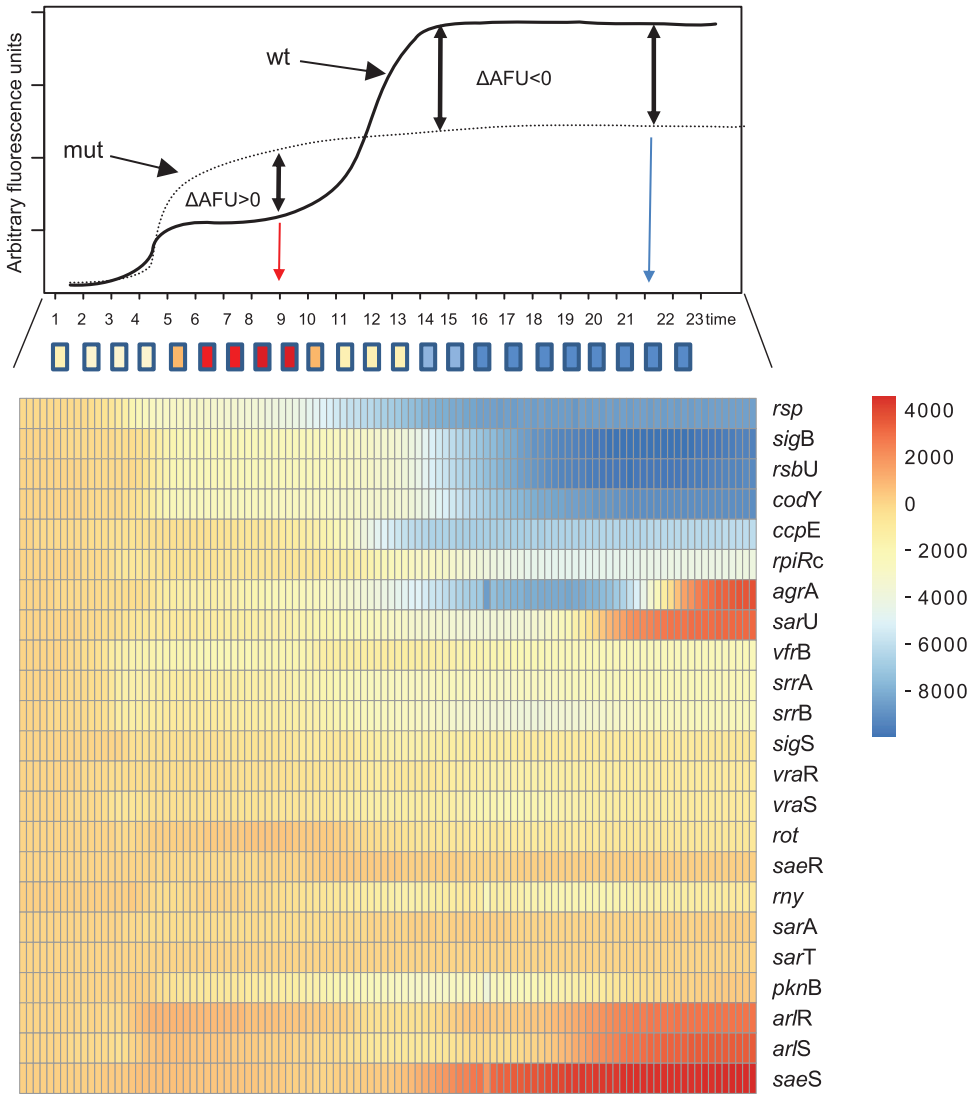

FIG 4 Dependency of SSR42 promoter activity on global regulators. Activity of the SSR42 promoter (P_{SSR42}) was measured by a transcriptional fusion with GFP in *S. aureus* JE2 and insertional mutants from NTML. GFP emission was recorded over 23 h. Shown are the promoter activity curve of wild-type bacteria (upper panel) and a heat map of differential activities of P_{SSR42} in mutants from the NTML (lower panel) compared to P_{SSR42} activity in wild-type *S. aureus* JE2. AFU, arbitrary fluorescence units.

activity of P_{SSR42} (Fig. S7P). Further, mutations in the two-component system ArlRS (strains NE1684 and NE1183) (Fig. S7N) led to overall higher activity of P_{SSR42}, whereas strains carrying mutations within SrrAB (NE1309 and NE588) (Fig. S7L) as well as VraRS (NE554 and NE823) (Fig. S7M) displayed only slightly reduced GFP fluorescence levels. Although insertional inactivation of *sigS*, *pknB*, *srrAB*, and *vraR* resulted in only minor effects on P_{SSR42} activity, SSR42 transcript levels were significantly decreased, whereas the *arlR* mutant demonstrated significantly enhanced SSR42 levels, as observed by quantitative reverse transcription-PCR (qRT-PCR), thereby corroborating the promoter activity data (Fig. S7R).

We next tested *rsp* promoter activities (P_{rsp}) in selected insertional mutants within *S. aureus* JE2 and recorded time courses of GFP fluorescence as well as bacterial growth. Strains carrying mutations within each of the genes *agrA*, *rpiRc*, *codY*, and *arlR* demonstrated P_{rsp} promoter activity profiles similar to the ones recorded for P_{SSR42}. This suggests that P_{SSR42} activities in these mutants indirectly resulted from altered expression of Rsp. However, this was not the case for the other regulators tested, *ccpE*, *rsbU*, and *sigB* (Fig. S8).

Thus, expression of the Rsp/SSR42 system is dependent on several global regulators, two-component systems, and alternative σ factors, thereby highlighting the central role of the molecules in the virulence regulatory circuit of *S. aureus*.

We next tested transcript levels of key regulators of P_{SSR42}. Whereas mRNA levels of *sigB*, *codY*, RNAII (*agrB*), and RNAIII were unaltered in *S. aureus* ΔSSR42 compared to the wild-type levels, *saeS* transcript levels were significantly reduced in the ΔSSR42 background (Fig. 5A). We further found *saeS* and *saeP* mRNAs at significantly elevated levels upon AHT induction of SSR42 transcription in a ΔSSR42 mutant, whereas the genes were significantly reduced in noninduced samples (Fig. 5B). Since we observed a significant increase in *hla* transcription upon inducible transcription of SSR42 (Fig. 1F and S4B), we also tested the transcription of class I SaeRS target genes for their dependency on SSR42. Whereas significantly elevated mRNA levels of *coa*, *eap*, and *emp* were detected upon induction of SSR42 transcription during exponential growth, mRNA levels in noninduced controls were significantly reduced compared to those of wild-type bacteria (Fig. 5C).

Our data thus suggested that SSR42 regulates the SaeRS two-component system. In order to test if SSR42 is functional upstream of the SaeRS two-component system, we analyzed transcription of *hla* in a genetic background deficient in the response regulator SaeR (Fig. 5D). We therefore introduced pAHT-SSR42 in wild-type *S. aureus* JE2 as well as an insertional *saeR* mutant (NE1622) (49). Whereas induction of SSR42 transcription resulted in significantly elevated *hla* mRNA levels in wild-type bacteria, *hla* mRNA levels were unaltered in the *saeR* mutant despite overexpression of SSR42 (Fig. 5D). Similar results were found for class I target genes of the SaeRS system, *coa*, *eap*, and *emp* (Fig. S9). We next monitored *sae* transcript stability by a rifampin assay. Our data show that the stability of *sae* transcripts T1 and T2 was significantly lower in a ΔSSR4 mutant background than the level in wild-type bacteria at 20 min after addition of rifampin (Fig. 5E).

DISCUSSION

SSR42 serves as an effector of Rsp in expression of alpha-toxin. We previously identified repressor of surface proteins (Rsp) as a global regulator of *S. aureus* hemolysis, cytotoxicity, and virulence and found that Rsp is required for transcription of SSR42, which is located directly upstream of Rsp in an antiparallel orientation (36). Loss of hemolysis has been described for mutants of *rsp* (36, 49, 51), and functional Rsp is a requirement for transcription of SSR42 (36). SSR42 is essential for wild-type hemolysis in *S. aureus* (Fig. 1). Upon deletion of arbitrarily selected regions of the ncRNA, SSR42 RNA stability was found diminished, and accordingly hemolysis of bacterial culture supernatants was reduced (see Fig. S2E to H in the supplemental material). This illustrated the requirement of a full-length SSR42 molecule for RNA stability as well as phenotypic hemolysis. Since we observed both hemolysis and SSR42 transcription only in the presence of the *rsp* gene, this demonstrates that Rsp-dependent hemolysis is regulated by way of the SSR42 transcript. Therefore, the ncRNA is the effector of the transcriptional regulator (Fig. 1). A previous study demonstrated SSR42 involvement in hemolysis yet determined SSR42 to encompass 891 nt in strain UAMS-1 (37). Incidentally, the construct used to complement the SSR42 deletion in the previous study contained a genomic fragment, which consisted of the entire SSR42 transcript, thereby explaining the efficient complementation of the mutant phenotype (37). We can exclude the notion that UAMS-1 produces a truncated version of the RNA since our Northern blots demonstrate an RNA of a size similar to that of JE2 but at low abundance (Fig. S2C).

By inducible expression of SSR42 in a ΔSSR42 mutant, we produced the ncRNA either during stationary phase or ectopically during exponential growth phase. Whereas high *hla* mRNA levels were obtained after induction of SSR42 transcription during exponential phase, *S. aureus* hemolysis was restored only upon SSR42 induction during the stationary growth phase (Fig. 1G and S4B). Since the *agr* quorum sensing effector RNAIII is required for translation of the *hla* message (30) and is expressed only during stationary growth (29, 52–54), our data thus show that alpha-toxin production

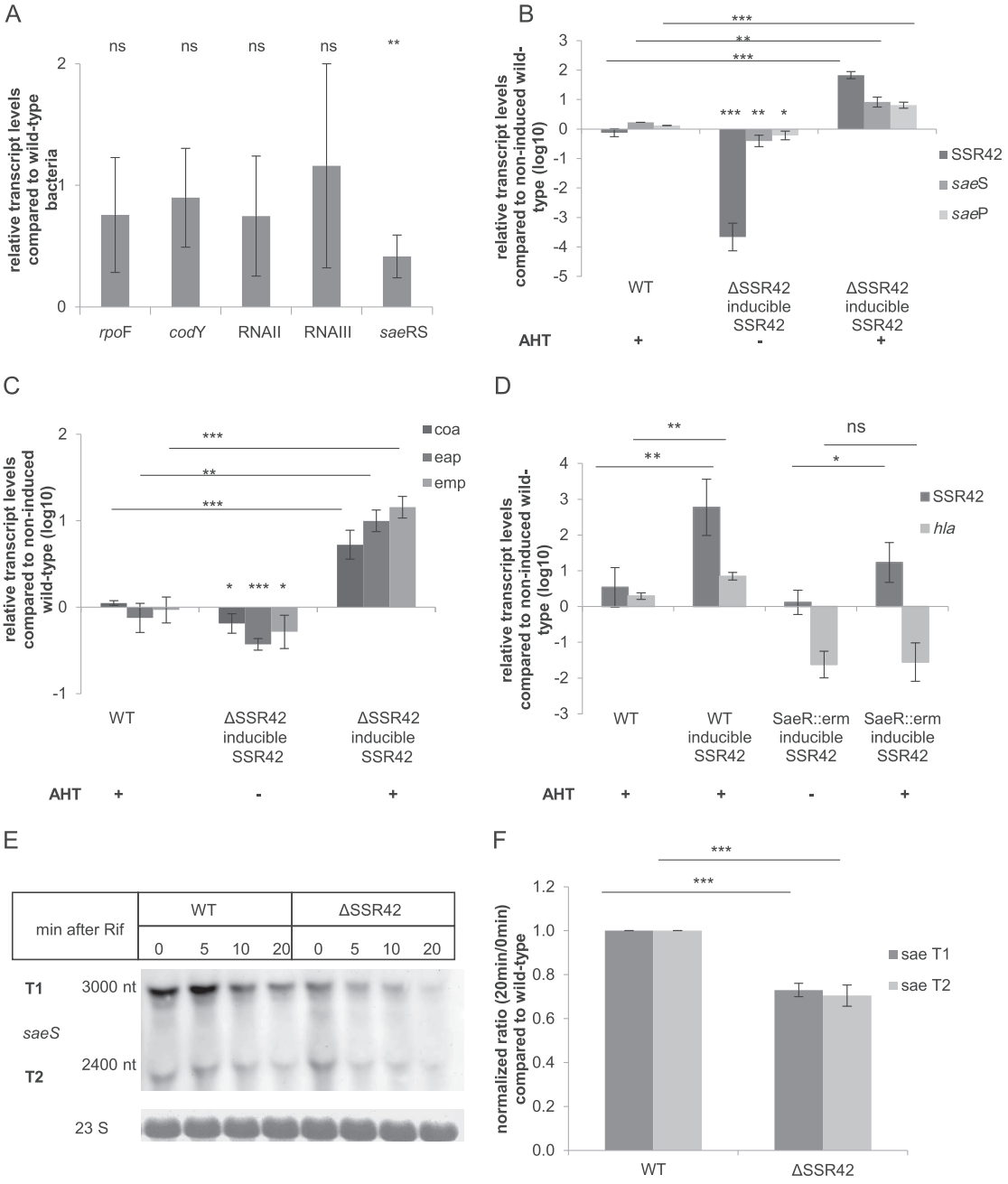

FIG 5 SSR42 regulates *hla* transcription upstream of SaeRS. (A) In *S. aureus* ΔSSR42, *saeRS* is transcribed at significantly decreased levels, whereas other genes of global regulators such as *rpoF* (*sigB*), *codY*, *agrB*, and RNAIII are unaltered. (B) Upon induction of SSR42 transcription by AHT in *S. aureus* ΔSSR42 pAHT-SSR42, transcriptional upregulation of *saeP* and *saeS* is observed in contrast to levels in noninduced samples. Without induction *saeP* and *saeS* are present at significantly reduced levels compared to the level of *S. aureus* wild type. (C) AHT induction of SSR42 transcription results in significantly elevated transcript levels of the class I targets *coa*, *eap*, and *emp* compared to levels in the noninduced complemented mutant and AHT-treated wild-type bacteria (WT). (D) SSR42-dependent upregulation of *hla* transcription is dependent on a functional SaeRS system. *hla* transcript levels are elevated upon AHT induction of SSR42 transcription *in S. aureus* JE2 (WT) yet are absent in an isogenic *saeR* insertional mutant strain. (E and F) *sae* T1 and T2 transcripts are less stable in a ΔSSR42 mutant of *S. aureus* 6850. Stability was assayed by addition of rifampin and assaying transcripts by Northern blotting. Chemiluminescence signals were quantified using ImageJ and normalized to transcript levels of wild-type bacteria. Statistical analysis was performed using Student's *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

involves two ncRNAs: the 1,232-nt SSR42 is required for transcription of the *hla* message, whereas the 514-nt RNAIII renders the Shine-Dalgarno sequence of the mRNA accessible to ribosomes.

Hemolysis induction by antibiotics is dependent on the ncRNA SSR42. Enhanced alpha-toxin production upon exposure of *S. aureus* to subinhibitory concentrations of

antibiotics has been previously described (44, 55). Especially β-lactam antibiotics such as oxacillin significantly altered toxin expression profiles of the bacteria (45) and enhanced *hla* transcription in *S. aureus* (44) or altered disease progression (56). During the course of treatment of bacterial infections, the pathogens may encounter subinhibitory concentrations of antibiotics, e.g., during low-dosage therapy, at the beginning and end of a treatment (57), or within biofilms (58). However, the underlying molecular mechanisms by which antibiotics would increase *S. aureus* hemolysis are only incompletely understood. The *saeRS* (55, 59), alternative σ factor σ^S (60), and VraRS (59) were found involved; however, the last two are not directly linked to hemolysin production. We show that SSR42 promoter activity of P_{SSR42} as well as transcription of the ncRNA is increased by various antibiotics at subinhibitory concentrations (Fig. S5 and 6B to D; Table 1). Unexpectedly, P_{SSR42} was also activated when *S. aureus* reporter strains were exposed to mitomycin C (Fig. S5D). Both β-lactam antibiotics and mitomycin C were implicated in activation of the *S. aureus* SOS response (4). However, in a *lexA* mutant, the P_{SSR42} promoter was still activated by mitomycin C exposure, thus demonstrating that P_{SSR42} induction is independent of LexA derepression. Similarly, subinhibitory concentrations of oxacillin induced the *hla* promoter (P_{hla}) in a process that depended on SSR42 (Fig. 3). Thus, β-lactam-enhanced *S. aureus* hemolysis is regulated via the Rsp/SSR42 axis, thereby identifying a hitherto undescribed role for both factors.

Multiple global regulators integrate SSR42 transcription. *hla* expression is regulated by a variety of factors and conditions (29, 61–65). In order to assess the position of SSR42 in this complex regulatory network, we assayed the activity of P_{SSR42} promoter in a variety of regulator mutants. Aside from *rsp* (Fig. 2A, 4, and S7A), mutation of *agrA*, *sarU*, *codY*, σ^B, *saeS*, *arlRS*, *ccpE*, and *rpiRc* significantly altered P_{SSR42} activity, whereas the effect of *saeR*, σ^S, *vraRS*, or *srrAB* mutation was negligible (Fig. 4 and S7). Insertional disruption of *agr* and *sarU*, an activator of *agr*, resulted in similar P_{SSR42} profiles with a long phase of inactivity and a late stationary-phase boost eventually exceeding P_{SSR42} activity of the wild type (Fig. 4 and S7E and F). In contrast, insertional mutations within *sarA*, *sarT*, and *rot* did not drastically alter P_{SSR42} activity over time. ArlRS was shown to positively regulate expression of *agr* (66); however, its influence on hemolysis is controversial (66–70). Here, we observed increased P_{SSR42} activity in insertional *arlR* and *arlS* mutants. In contrast, two other two-component systems implicated in altered β-lactam susceptibility, *srrAB* and *vraRS* (71), did not significantly contribute to P_{SSR42} activity.

In the *codY* mutant, P_{SSR42} activity was strongly reduced, completely lacking the second peak of activation. The second phase of promoter activation commenced about 9 h after inoculation and thus far into the stationary growth phase. The resulting sigmoidal P_{SSR42} activity was strongly dependent on the strain background and was observed in *S. aureus* JE2, MW2, and Newman strains but not in the remainder of the strains (6850, COL, Cowan I, HG003, and RN4220) (Fig. S6A), indicating that the peculiar profile does not correlate with methicillin resistance, virulence, or hemolysis. Since the SSR42 promoter lacks a typical CodY-binding motif (data not shown) and is not bound by CodY (72), regulation of SSR42 by CodY likely is indirect. For instance, *rsbU* as well as *rpiRc* is activated by CodY (72). Consistently, inactivation of *sigB*, *rsbU*, and *rpiRc* led to P_{SSR42} inactivation (Fig. 4 and S7) (73, 74). Our data hence suggest that CodY indirectly regulates SSR42 by way of σ^B and RpiRc activity. σ^B itself positively regulates approximately 120 genes in response to various conditions (75, 76). However, a consensus σ^B-binding site is absent in P_{SSR42} (data not shown). RpiRc reacts to the metabolic state of the bacteria and was reported to control pentose phosphate pathway genes as well as RNAIII, likely via σ^B and SarA (50). However, in our analyses *sarA* did not influence P_{SSR42} activity.

CcpE, another virulence regulator monitoring metabolic levels, represses not only tricarboxylic acid (TCA) cycle genes but also RNAIII, *hla*, *psmα*, and *capA* in the presence of glucose (77, 78). The *ccpE* mutant demonstrated a drastically altered P_{SSR42} profile in which the second activation peak was lost.

Since alpha-toxin transcription depended on SSR42, we also investigated SaeRS, the major transcriptional regulator of *hla*. SaeR induces *hla* transcription predominantly in the postexponential growth phase (19, 61), and SSR42-dependent promoter activity of P_{hla} begins at the transition to the stationary growth phase (Fig. 1F). Deletion of SSR42 resulted in a significant decrease in expression of *sae* as well as class I and class II target genes (Fig. 5). Together, these data suggested that SSR42 is required upstream of SaeRS. We thus found that SSR42 modulates the stability of the *sae* transcripts T1 and T2 (Fig. 5E). However, it has been shown previously that small amounts of SaeRS are sufficient to initiate transcription of the class II SaeR target *hla* (17) and that only a complete lack of SaeR activity results in loss of P_{hla} activity (79). The mechanism by which SSR42 stabilizes *sae* mRNA is currently unknown. Since both transcripts, SSR42 (40) and *saePQRS* (26), are processed by the endoribonuclease RNase Y, SSR42 interaction with RNase Y thereby may affect the stability of *sae* mRNA by influencing *sae* T1 cleavage. Whereas the stability of *sae* is affected by RNase Y cleavage (26), SSR42 stability remains unaltered in the absence of *rny* (Fig. 2E). We also observed that transcriptional activation of SSR42 levels is attenuated by SaeS at time points of high SSR42 levels (Fig. S7H). This interdependency may illustrate a negative-feedback loop between the two molecules. Most strikingly, the effects were not observed in an *saeR* response regulator mutant. It is questionable if the observed loss of *saeRS* transcript stability in the SSR42 mutant is sufficient for the strong phenotypic decrease in hemolysis (79). In addition, knockout of Rsp, the direct regulator of SSR42, resulted in an increase in transcription of *sae* (36). The precise role of the interplay of SSR42, *saePQRS*, and RNase Y (Fig. 6) therefore remains elusive.

Staphylococcal alpha-toxin is controlled by a variety of virulence regulators on the transcriptional as well as posttranscriptional level. We show that SSR42 contributes to *hla* transcription in a process upstream of SaeRS. SSR42 transcription itself is modulated by global regulators such as Rsp, AgrA, SarU, ArlRS, SaeS, CodY, σ^B, CcpE, and RpiRc, thereby demonstrating that the ncRNA is involved in integrating nutritional as well as environmental signals during cytolysin production and virulence. By this pathway, *S. aureus* hemolysis is enhanced SSR42 dependently upon exposure to subinhibitory β-lactam concentrations. Thus, exposure to antibiotics eventually can alter *S. aureus* virulence.

MATERIALS AND METHODS

Bacterial culture conditions. *Staphylococcus aureus* strains were grown on tryptic soy agar (TSA) or in tryptic soy broth (TSB) (lot number BCBP7262V; Sigma) supplemented with 0.25% glucose and appropriate antibiotics. *Escherichia coli* strains were grown on LB using appropriate antibiotics. Broth cultures were grown aerobically at 37°C overnight at 180 rpm.

Bacterial growth curves. *S. aureus* strains were grown in TSB at 37°C at 180 rpm in air. Triplicates of the cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.1 in 400 μl of fresh TSB and were grown for 23 h in a 48-well microwell plate. Absorbance was measured automatically at 600 nm every 10 min using a Tecan Infinite M200 plate reader.

Construction of bacterial strains and plasmids. For all strains, plasmids, and oligonucleotides used, see Table S1 in the supplemental material. All *S. aureus* insertional transposon mutants available through the Nebraska Transposon Mutant Library (NTML) Library were transduced via phage 11 into the erythromycin-sensitive genetic background of wild-type *S. aureus* JE2 in order to avoid secondary-site mutations. Markerless targeted gene deletions of SSR42 and SSR42-*rsp* were generated using the vectors pBASE6-SSR42 and pBASE6-SSR42-*rsp*. Gene deletions were performed as described previously (80). For inducible complementation of SSR42 or Rsp, plasmids pAHT-SSR42 and pAHT-*rsp* were used, respectively. For complementation of ΔSSR42 and ΔSSR42-*rsp* mutants, plasmids pSSR42, p*rsp*, pSSR42-*rsp*, and p2216-2218 were used. Small deletions in SSR42 were investigated using complementation plasmids pSSR42Δ1, pSSR42Δ2, pSSR42Δ3, pSSR42Δ4, pSSR42Δ5, pSSR42Δ6, pSSR42Δ7, and pSSR42Δ8. Promoter activities of P_{SSR42} and P_{hla} were monitored using reporter plasmid pRsp-P_{SSR42}-BgaB, p-P_{SSR42}-BgaB, pP_{SSR42}-GFP, or pP_{hla}-GFP. The construction of all strains and plasmids is described in Text S1 in the supplemental material.

RNA isolation. Bacterial RNA was extracted using a previously described TRIzol method (81) and treated with DNase I.

qRT-PCR. Reverse transcription of total isolated RNA was performed using RevertAID reverse transcriptase (Thermo Scientific). A 10-ng sample of cDNA was used to perform qRT-PCR in a one-step reaction using Sybr Green master mix (2×; Genaxxon) on a StepOne Plus real-time PCR system (Applied Biosystems). For primers used for qRT-PCR see Table S1 in the supplemental material. Analysis was performed using the $2^{-\Delta\Delta CT}$ (where C_T is threshold cycle) method. Relative gene expression was

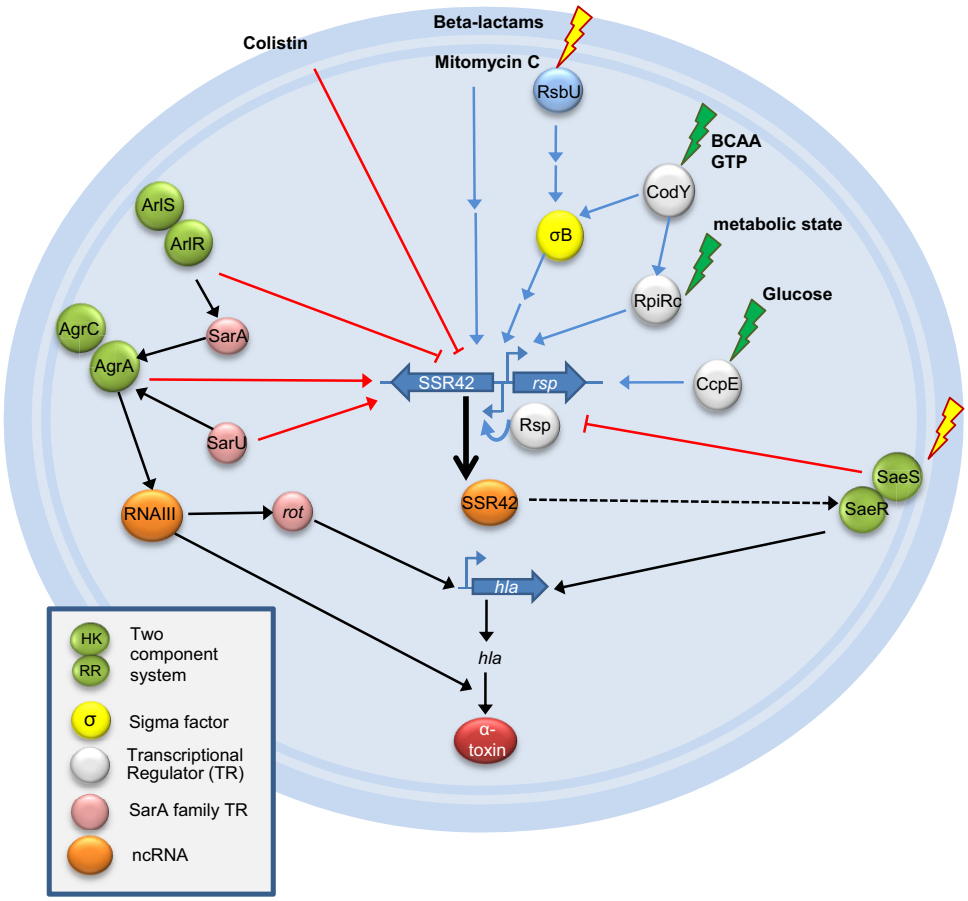

FIG 6 Overview of SSR42-dependent alpha-toxin expression. Disruption of the *agr* quorum sensing system results in complete lack of hemolysis due to the absence of RNAIII as well as reduced *sae* mRNA levels and also strongly delays SSR42 transcription with a dynamic similar to that of an *sarU* insertional mutant strain. In *codY*, σ^B, and *rsbU* mutants, P_{SSR42} activity was drastically reduced, illustrating positive regulation of SSR42 transcription by these factors. CodY acts presumably via RsbU and RpiRc. Further, in a *ccpE* mutant the second peak of the P_{SSR42} activity profile activation was lost. In contrast, loss of the ArlRS two-component system led to induction of P_{SSR42} activity. Similarly, this also was observed in an *saeS* mutant, illustrating feedback between SSR42 and *sae*, since SSR42 acts via SaeRS and the effects of ectopic ncRNA expression are lost in an *saeR* mutant. The exact mode of action of SSR42 on *sae* is unknown. However, both the SSR42 and *sae* transcripts are processed by RNase Y, which therefore suggests that RNA stability is involved. The β-lactam-dependent induction of *hla* transcription thereby is dependent on SSR42, whereas colistin reduces P_{SSR42} promoter activity. For details, refer to the text. Black arrows, known interactions; red arrows, downregulation; blue arrows, upregulation of SSR42 by factors shown in this study.

normalized to expression of the housekeeping gene of gyrase subunit B (*gyrB*) and to the corresponding expression in wild-type cells.

Northern blotting. Northern blotting of RNA was performed as previously described (82) using digoxigenin-labeled probes. Primers for the generation of probes are listed in Table S1. For detailed information on RNA methods, see Text S1 in the supplemental material.

Rifampin assay. *S. aureus* strains were grown in TSB at 37°C in air until cultures reached stationary growth phase. Rifampin was added to 500 μg/ml, and bacteria were harvested at time points indicated within the figures by flash freezing. RNA was isolated and analyzed by Northern blotting. ImageJ was used for quantification of signals (83).

Hemolysis assay. Bacteria were grown overnight in TSB at 37°C. Hemolysis of *S. aureus* was determined by spotting 10 μl of 100-fold-diluted culture on Columbia agar (BD Biosciences) supplemented with 5% defibrinated sheep blood (Fiebig Nährstofftechnik, Germany). For quantitative analysis, sheep erythrocytes (Fiebig Nährstofftechnik, Germany) were washed with 0.9% NaCl and then diluted to a final concentration of 1% in the same buffer. Bacteria were grown overnight in TSB at 37°C and harvested, and supernatant was collected and sterile filtered (0.45-μm pore size). Sterile-filtered supernatant (5%, vol/vol) of *S. aureus* was added to a 1% erythrocyte solution and incubated for 1 h at 37°C. Thereafter, the suspension was centrifuged, and supernatants were analyzed in technical replicates for heme release by measuring absorbance at 405 ± 9 nm using a Tecan Infinite M200 plate reader.

β-Galactosidase assay. SSR42 promoter activity upon treatment with chemicals and antibiotics was analyzed using a β-galactosidase reporter construct. Strains harboring this reporter plasmid were grown

overnight at 37°C in TSB with 10 μg/ml chloramphenicol. One hundred microliters of bacteria was added to 5 ml of soft agar containing 40 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and plated on agar plates containing 40 mg/ml X-Gal. Bacteria were either streaked on the agar or plated within soft agar. Diffusion disks containing different antibiotics were placed upon the agar plates, which were incubated overnight for 37°C.

Fluorescence-based promoter activity assay. Promoter activities during bacterial growth were assessed by monitoring GFP fluorescence (excitation, 488 $\pm$ 9 nm; emission, 518 $\pm$ 20 nm) as well as optical density (600 nm) using a Tecan Infinite M200 multiplate reader. For this, bacteria were grown in TSB overnight at 37°C at 180 rpm in air. The cultures were diluted in 400 μl of fresh TSB to an OD_{600} of 0.1 in triplicates. Concentrations of supplemented antibiotics are indicated within the figures. Bacteria were grown for 23 h in a 48-well microwell plate. Absorbance and GFP fluorescence were measured automatically every 10 min using a Tecan Infinite M200 multiplate reader.

Heat maps. Heat maps were generated by calculating the difference in fluorescence units between each mutant and respective wild-type strain for each time point. The resulting matrix was visualized using the R library application pheatmat.

Statistics. If not stated otherwise, statistical analyses were performed using Student's *t* test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00252-18.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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We declare that we have no competing interests.

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