Antibiotic-Induced Autoactivation of IS256 in *Staphylococcus aureus*

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The 3' end of *rsbU*, encoding the positive regulator of the stress factor sigma B, was identified as a hot spot for spontaneous IS256 insertion in *Staphylococcus aureus* SA137/93G. Interestingly, subinhibitory concentrations of chloramphenicol in combination with heat stress, as well as linezolid and spectinomycin at physiological temperatures, selected for such *rsbU*::IS256 insertion mutants. In consequence of the inactivation of *rsbU*, the IS256 transposition frequency was increased 4-fold in *S. aureus* HG001.

Staphylococcus aureus is a major human pathogen and has been able to acquire resistance to nearly all clinically used antibiotics. Insertion sequences (IS elements) have been instrumental in the development of resistance, since they represent the active component of transposons and, thus, mobilize resistance genes for horizontal gene transfer (1) or modulate the expression of resistance-conferring genes (2). Therefore, investigation of the transposition process of IS elements, especially in the presence of subinhibitory concentrations of antibiotics, will help us to understand and ultimately, perhaps, interfere with resistance development.

IS256 is a highly active insertion sequence that, as a component of the composite transposon Tn4001, has played a pivotal role in the development of aminoglycoside resistance in *S. aureus* (3, 4). IS256 has been detected in the genome of several clinical isolates of enterococci and staphylococci (5, 6), e.g., in the clinical methicillin-resistant *S. aureus* (MRSA) isolates of sequence type 228 (ST228) (1 copy), ST239 (8 to 21 copies), and ST247 (at least 14 copies) (7–11). The accumulation of multiple IS256 copies in the genomes is due to its transposition process, which is mediated by a copy-and-paste mechanism (12). The effect of multiple IS256 elements on genomic flexibility and resistance development is exemplified by *S. aureus* SA137/93G (more than 14 copies), an ST247 strain that displays homogeneous vancomycin-intermediate resistance (vancomycin-intermediate *S. aureus* [VISA] phenotype) (13), which is mainly caused by the insertion of IS256 into the *tcaA* gene (11, 14). A variety of intrinsic and extrinsic mechanisms regulate the transposition activity of mobile elements in bacteria (15). Whereas subinhibitory concentrations of ciprofloxacin and vancomycin lead to the activation of IS256 transposition (16), the alternative sigma factor B, which controls the expression of genes involved in stress response (17), is a negative regulator of IS256 transposition in *S. aureus* (18). In the present study, we demonstrate that frequent insertion of IS256 into the *rsbU* gene, which encodes a positive regulator of SigB (19), resulted in the autoactivation of IS256 transposition in *S. aureus*.

***rsbU* is a hot spot for spontaneous IS256 insertions.** All strains, plasmids, and oligonucleotides are shown in Tables 1 and 2. Spontaneous insertion of IS256 into *rsbU* was first detected during attempts to integrate temperature-sensitive plasmids harboring a chloramphenicol resistance gene into the genome of *S. aureus* SA137/93G. This *rsbU*-positive strain showed strong staphyloxanthin production and little hemolysis. However, after incubation of the recombinant strains on tryptic soy agar (TSA) (Merck KGaA, Darmstadt, Germany) containing 20 µg/ml chlor-

amphenicol at 45°C, many colonies appeared white (Fig. 1A). Additionally, these white colonies displayed large hemolysis zones on blood agar, thus resembling a SigB-negative phenotype (19). PCR and sequencing (SEQLAB Sequence Laboratories Göttingen GmbH, Göttingen, Germany) of the complete *sigB* operon of the white colonies with primers SigBfor and SigBrev yielded insertions of IS256 into the *rsbU* gene, flanked by 8-bp direct repeats generated during the transposition process and designated wg1, wg2, and wg3 in Fig. 1B (20). Interestingly, after long-term incubation of *S. aureus* SA137/93G pMGS100 at 45°C (up to 6 days) on chloramphenicol-containing TSA, white papillae became visible on *rsbU*-positive yellow colonies (Fig. 1A). Two papillae that were sequenced harbored the insertions wg4 and wg5.

To investigate the appearance of white insertion mutants in the presence of other antibiotics, at lower temperatures, and in the absence of resistance-conferring plasmids, *S. aureus* SA137/93G and a vancomycin-sensitive *S. aureus* (VSSA) control strain, SA1450/94, both of which contain multiple IS256 copies, were incubated in tryptic soy broth (TSB) without antibiotics to an optical density at 600 nm (OD₆₀₀) of 1.0. Afterwards, aliquots of the culture were plated in dilutions onto TSA or brain heart infusion (BHI) agar containing subinhibitory concentrations of different antibiotics ($\leq 0.5 \times$ MIC) (Table 3). In three independent experiments, the numbers of white and yellow colonies were counted on agar plates containing less than 200 CFU after incubation at 37°C or 43°C for 36 h. Interestingly, among all antibiotics tested (Table 3), only chloramphenicol at 43°C and linezolid and spectinomycin at 37°C and 43°C selected for a SigB-negative phenotype of both *S. aureus* strains. With other antibiotics, including inhibitors of protein synthesis (tigecycline and erythromycin), cell wall biosynthesis (vancomycin and oxacillin), DNA replication (ciprofloxacin), and RNA polymerase (rifampin), no effect was visible. In the presence of chloramphenicol and heat stress, as well as linezolid at both temperatures, the appearance of white colonies in VSSA strain SA1450/94 was reduced 2- to 4-fold

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TABLE 1 Strains and plasmids used in the study

Strain or plasmid	Description	Reference or source
<i>Escherichia coli</i> JM109	Laboratory strain	25
<i>Staphylococcus aureus</i> strains		
RN4220	Restriction-negative derivative of NCTC 8325	26
HG001	<i>rsbU</i> -repaired laboratory strain NCTC 8325	27
HG001 W5	<i>rsbU</i> ::IS256- <i>spc</i> insertion mutant	This study
HG001 G2	Intergenic IS256- <i>spc</i> insertion mutant (SAOUHSC_0967/SAOUHSC_0968)	This study
SA137/93G	Spontaneous mutant of clinical VISA isolate SA137/93A; ΔSCCmec (Met ^r), <i>rsbU</i> -positive	13
SA1450/94	MRSA, Northern German epidemic strain, <i>mutS</i> ::IS256	13
Plasmids		
pBT2	<i>E. coli</i> - <i>S. aureus</i> shuttle vector with thermosensitive origin of replication for Gram-positive bacteria, <i>cat</i>	28
pA3	pBT2 carrying a recombinant IS256 element containing an erythromycin resistance-mediating cassette	16
pA6	pBT2 carrying a recombinant IS256 element containing an spectinomycin resistance-mediating cassette, <i>spc</i>	This study
pMGS100	<i>E. coli</i> - <i>S. aureus</i> shuttle expression vector, <i>cat</i>	29

compared to the number in strain SA137/93G, which might indicate that the VISA chromosomal background might be more sensitive to the selection stress. The decreased occurrence of white colonies after spectinomycin treatment in strain SA137/93G compared to their occurrence in SA1450/94 might be due to the fact that for strain SA137/93G spectinomycin concentrations of $\leq 0.25\times$ MIC had to be used in this experiment, since higher concentrations led to precipitation of the antibiotic. Interestingly, in the presence of lower concentrations of chloramphenicol ($0.33\times$ MIC), linezolid ($0.25\times$ MIC), and spectinomycin ($\leq 0.125\times$ MIC), only yellow colonies appeared, indicating that a particular threshold concentration of the antibiotic is required to select for white mutants. During these experiments, additional insertion sites of IS256 into the 3' end of *rsbU* were discovered after selection with chloramphenicol at 43°C (wG6), with linezolid (wG7, wG9, and wN2), and with spectinomycin (wG8, wG10, wG11, and wN1) (Fig. 1B). In strain SA137/93G, the *sigB* operon was affected by IS256 insertions in 94% (17 out of 18) of white mutants, and from these mutants, 93% (16) showed *rsbU* insertions and only 1 (7%) showed an insertion into the *sigB* gene, indicating that *rsbU* is a hot spot for IS256 insertion in *S. aureus*. In contrast to *Staphylococcus epidermidis*, where the 5' end of *rsbU* was identified as a chromosomal hot spot for IS256 insertion (21), the 3' end of *rsbU* was primarily affected in *S. aureus*.

The selection of white colonies was driven by a higher fitness of the *rsbU*-negative cells than of the wild type: pure cultures of the *rsbU*::IS256 mutant with the wG6 insertion and the *rsbU*-positive wild type were plated in different dilutions onto linezolid- or chloramphenicol-containing ($0.5\times$ MIC) agar and antibiotic-free agar for determination of the total CFU. Seventy-one percent of wG6-containing cells were able to form colonies on linezolid agar, compared to 0.03% of the wild type. On chloramphenicol agar, 98% of cells containing wG6 and 0.07%

TABLE 2 Oligonucleotides used in the study

Oligonucleotide	Sequence	Purpose
ISIRLfor	GTAGAATTCGGATAAAGTCCGTA TAATTGTGT	Amplification of IS256 for pA6
ISIRRev	TGTTCTAGATGCTATACAATGTTTT TACCATTTC	
IS91rev	CTTTTACTTTTACACAATTATACGG	Inverse PCR, primers anneal to IS256
IS141for	CCCAGGAGGACTTTTACATGAC	
Spc-for_HindIII	ACGAAGCTTAGTCAAGTCCAGAC TCCTGTGTAAATCGTCCAATCTA GGGTA	Amplification of <i>spc</i> for pA6
Spc-rev_XbaI	AAGTCTAGATGAATTCACAAAGAG GAC	
SigBfor	GTTTGCCGCGAATCTATCAT	Amplification of the whole <i>sigB</i> operon
SigBrev	GCAAATTCTATTATGTGCTGCTT	
SigBforII	CAGCAAAATTGCAAACGACA	Amplification of <i>rsbU</i>
SigBrevII	GGACCTATTTTTGATTAGTTGTTTC	

of wild-type cells grew to colonies. Competition experiments in TSB containing chloramphenicol (3 μg/ml) at 43°C with an inoculum of 50% white wG6-containing and 50% yellow wild-type cells confirmed this result; after two passages (24 h each), only white colonies were detected on antibiotic-free TSA, indicating that a population of SigB-negative phenotypes (wG6-containing strain and *sigB*-negative mutants of the wild type) had outcompeted the wild type.

Activation of IS256 transposition frequency in *rsbU*::IS256 insertion mutants. It has previously been reported that the transposition frequency of IS256 is increased in a *sigB* mutant strain of *S. aureus* (18). In order to study the influence of the integration of IS256 into *rsbU* on transposition frequency, the plasmid pA6 carrying a recombinant IS256 element (IS256-*spc*) with a spectinomycin resistance cassette (amplified from the plasmid pBPsy1Prom) located downstream from the transposase gene was constructed largely as previously described (18) and was transformed into *S. aureus* HG001 by electroporation (22).

Then, chromosomal IS256-*spc* insertion mutants of *S. aureus* HG001 pA6 were selected on TSA containing 150 μg/ml spectinomycin at 45°C largely as previously described (16). Next, nine white and one yellow chromosomal IS256-*spc* insertion mutants displaying spectinomycin resistance and chloramphenicol sensitivity were selected. PCR amplification and sequencing of the complete *sigB* operon of the nine white mutants revealed that 78% (7 mutants) harbored an insertion of IS256 in the *sigB* operon, and from these mutants, 43% (3 mutants) were *S. aureus* HG001 IS256::*rsbU* mutants with insertions W1, W5, and W7 (Fig. 1B). Four other mutants (57%) harbored insertions in other parts of the *sigB* operon. In the yellow *rsbU*-positive IS256-*spc* insertion mutant *S. aureus* HG001 G2, which served as a control in later experiments, the IS256-*spc* insertion site was determined by inverse PCR and sequencing (11). The IS256-*spc* element was integrated between SAOUHSC_01967, a putative ABC transporter, and SAOUHSC_01968 (*hit*). The downregulation of SigB-dependent genes in the *rsbU*::IS256-*spc* insertion mutant HG001 W5 compared to their expression in the *rsbU*-positive control HG001 G2 was confirmed by whole-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (23). To determine whether the transposition of IS256 is autoactivated by the insertion of IS256-*spc* into the *rsbU* gene in strain HG001 W5, the transposition frequency

FIG 1 (A) Top, appearance of white mutants of *S. aureus* SA137/93G at 43°C on TSA containing chloramphenicol; bottom, white papilla on an *rsbU*-positive colony of *S. aureus* SA137/93G, as observed for 3% of the colonies after 6 days at 45°C. (B) Localization of IS256 and IS256-*spc* insertions in the *rsbU* gene of *S. aureus* SA137/93G (wG1 to wG11), *S. aureus* HG001 (W1, W5, and W7), and *S. aureus* SA1450/94 (wN1 and wN2). The orientation of the transposase gene is shown by arrows, and the 8-bp direct repeats created during the transposition process are underlined. Of note, one of these insertions (wG5) was located at the same base pair as the insertion characterized in clones with wG1 and wN2, but IS256 was oriented in the opposite direction. The start and stop codons of *rsbU* are printed in boldface.

of another recombinant IS256 element (IS256-*ermB*), carrying an erythromycin resistance cassette, in strain HG001 W5 was compared to its transposition frequency in strain HG001 G2. To this end, the vector pA3 containing IS256-*ermB* (16) was introduced into strains HG001 W5 and HG001 G2 by electroporation. Subsequently, the transposition frequencies of IS256-*ermB* were determined as previously described (16). Figure 2 shows the results of three independent experiments for each strain. The transposition activity of IS256-*ermB* in the *rsbU*::IS256-*spc* insertion mutant HG001 W5 was increased 4-fold on average compared to its transposition activity in the *rsbU*-positive strain HG001 G2. Our previous studies had shown that the transposition of IS256-*ermB*, harboring an inactivated transposase gene (mutated DDE motif), was not mea-

surable (16), indicating that the transposition frequency measured here is due to the enzymatic activity of the functional transposase. Thus, these data suggest that the insertion of IS256 into *rsbU* leads to the autoactivation of IS256 transposition in *S. aureus*. In particular, the indirect activation of transposition of IS256 by linezolid at physiological temperatures may be relevant for the development of resistance. In this context, it is remarkable that two IS256-like elements were found to be framing the *cfr* gene, which conveys cross-resistance to linezolid, the phenicols, and MLS_B antibiotics, on a plasmid in an *Enterococcus faecalis* isolate (24).

Conclusions. We identified *rsbU* as a hot spot for IS256 insertion in ST247 *S. aureus* strains and showed that selection of these *rsbU* mutants by subinhibitory concentrations of three antibiotics

TABLE 3 Antimicrobial susceptibilities and occurrence of white colonies for two *S. aureus* strains after incubation in the presence of particular antibiotics

Antibiotic	MIC (μg/ml) for:		Concn (μg/ml) tested in:		Mean % (SD) ^b of white colonies at indicated temp in:			
					A		B	
	A ^a	B ^a	A	B	37°C	43°C	37°C	43°C
Chloramphenicol	6	6	3	3	0	35.9 (22.4)	0	3.3 (1.3)
Linezolid	1.5	1.5	0.75	0.75	51.6 (18.1)	56.8 (16.1)	20.9 (13.7)	22.5 (7.2)
Spectinomycin	>32,768	8,192	16,384	4,096	9.6 (4.9)	22.4 (16.5)	14.7 (13.6)	36.8 (24.1)
Tigecycline	0.3	0.075	0.15	0.0375	0	0	0	0
Erythromycin	8,192	8,192	4,096	4,096	0	0	0	0
Vancomycin	4–8	2	2	1	0	0	0	0
Oxacillin	1	ND ^c	0.5	ND	0	0	ND	ND
Ciprofloxacin	16	16	8	8	0	0	0	0
Rifampin	512	ND	256	ND	0	0	ND	ND

^a A, *S. aureus* SA137/93G; B, SA1450/94. MIC determination was performed in half-concentrated Mueller-Hinton broth (except for vancomycin MIC determination, which was performed in BHI).

^b *n* = 3 independent experiments.

^c ND, not determined.

FIG 2 Transposition frequencies of IS256-*ermB* in *S. aureus* HG001 W5 harboring pA3 (■) and *S. aureus* HG001 G2 harboring pA3 (▲). Each graph represents the mean values of three independent experiments, and error bars represent the standard deviations.

(chloramphenicol, linezolid, and spectinomycin) relevant in human and veterinary medicine will trigger a cascade that, in the end, upregulates the activity of transposition of IS256, which is one of the factors contributing to a genetic flexibility that, in turn, may accelerate the development of resistance in *S. aureus*.

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