




# Influence of IS256 on Genome Variability and Formation of Small-Colony Variants in *Staphylococcus aureus*

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**ABSTRACT** *Staphylococcus aureus* has acquired resistance to nearly all antibiotics used in clinical practice. Whereas some resistance mechanisms are conferred by uptake of resistance genes, others evolve by mutation. In this study, IS256 has been shown to play a role, e.g., in *S. aureus* strains displaying intermediate resistance to vancomycin (VISA). To characterize the IS256 insertion sites in the genomes of two closely related sequence type 247 (ST247) VISA strains, all insertions were mapped in both VISA and a susceptible control strain. The results showed that the three ST247 strains contained the highest number so far of IS256 insertions for all sequenced *S. aureus* strains. Furthermore, in contrast to the case with the other IS elements in these genomes, the IS256 insertion sites were not identical in the closely related strains, indicating a high transposition frequency of IS256. When IS256 was introduced into a laboratory strain which was then cultured in the presence of antibiotics, it was possible to isolate small-colony variants (SCVs) that possessed IS256 insertions in *guaA* and *hemY* that displayed increased resistance to vancomycin and aminoglycosides, respectively. For these clones, a very rapid reversion to the wild type that resembled the fast reversion of clinical SCVs was observed. The reversion was caused by excision of IS256 in a small number of fast-growing clones that quickly outcompeted the SCVs in broth cultures. In conclusion, the presence of IS256 confers a strong genomic plasticity that is useful for adaptation to antibiotic stress.

**KEYWORDS** *Staphylococcus aureus*, transposition, phase variation, insertion element IS256, vancomycin, aminoglycosides, *hemY*, *guaA*, small-colony variants (SCVs), VISA

IS256 was first described as a part of the transposon Tn4001, which harbors the gene *aacA-aphD* and mediates resistance to several aminoglycosides in the nosocomial pathogen *Staphylococcus aureus* (1, 2). It has a length of 1,324 bp, and the single gene *tnpA* encodes a DDE-transposase that contains two Asp (D) and one Glu (E) residue in its active site. (3). The ends of IS256 are formed by two imperfect inverted repeats (IR<sub>L</sub> and IR<sub>R</sub>). In addition, the insertions of IS256 are framed by two 8-bp direct repeats which are formed by duplication of the target sequence during transposition (4). IS256 transposes by a “copy and paste” mechanism (5), thereby leading to an accumulation of copies in the genome, since every transposition will increase the copy number by one. The integration of a single insertion sequence (IS) element into a gene or its promoter may result in an inactivation or overexpression of the affected gene. This way, IS256 confers phase variation phenomena concerning biofilm formation or sigma factor B activity on *S. aureus* and *Staphylococcus epidermidis* (4, 6–9). In addition, in *S. aureus* it has been shown to be involved in modulation of antibiotic resistance (10–13) and virulence; e.g., an insertion into the promoter of *rot* led to an increased virulence in *S. aureus* USA500 (14).

Here we present the genome sequences of three methicillin-resistant *Staphylococcus*

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*aureus* (MRSA) strains that contain a very high number of IS256 insertions. All strains belong to sequence type 247 (ST247), which is a multiresistant ancient sublineage of clonal complex 8 (CC8). The very first MRSA strain that was discovered in the United Kingdom in the early 1960s, strain COL (15, 16), also belongs to CC8 (ST250) and is the closest relative of these strains in the database. ST247 was most widespread in the early 1990s, when it caused hospital-associated MRSA outbreaks in several European countries and was referred to as Iberian strain or the Northern German Epidemic type as well as UK-EMRSA-5, -8, and -17, Rome clone, Spanish pulsed-field gel electrophoresis (PFGE) type E1, Irish AR22, and Irish New02 (16). Strain *S. aureus* SA137/93A was originally isolated in 1993 from a tracheal secretion in Germany, and during a retrospective screening in 1997, it was discovered that this isolate showed intermediate resistance to vancomycin (17). After subculturing of strain SA137/93A in the presence of vancomycin (6  $\mu\text{g/ml}$ ), a variant with an even higher resistance to vancomycin was isolated and designated *S. aureus* SA137/93G (18). Further studies of the vancomycin resistance mechanism showed that the presence of IS256 seemed to be important for the evolution of resistance, since it had inserted upstream of the two-component regulatory system *walRK*, upregulating its expression in strain SA137/93A, and inactivated the gene *tcaA* in strain SA137/93G (10, 12).

In this study, in order to learn more about the role of IS256 in resistance development, selection in the presence of antibiotics was performed with plasmids harboring IS256 elements that had been tagged with antibiotic resistance genes. These studies showed that IS256 may be involved in the generation of unstable small-colony variant (SCV)-like phenotypes. SCVs are slowly growing cells that are able to cause chronic infections and often have lost susceptibility to antibiotics such as aminoglycosides or trimethoprim-sulfamethoxazole. Clinical SCV isolates are often formed during intracellular growth (19) or prolonged antibiotic therapy (20); they are often unstable and therefore very difficult to handle, especially in broth culture, and most laboratory experiments have been performed with stable genetically engineered variants that are inactivated in *hemB* and *menD* or that possess a deletion in *hemH* (21–23). Recently it was shown that in infection models, long-term persistence induced SCV phenotypes, which rapidly reverted to the wild-type phenotype when the organisms were subcultured in the laboratory (24). The mechanism of this switch is still unclear (20), but it was hypothesized that the stress factors encountered during infection might activate the transition and that SigB is involved in this mechanism (19, 24). Here we show that the formation and fast reversion of SCV-like phenotypes may also be mediated by transposition and subsequent excision of IS256.

## RESULTS

**Genomes of three ST247 strains.** Table 1 provides an overview of the mobile and accessory genetic elements of the three ST247 strains. The number of mobile genetic elements and resistance genes was striking, especially in strain SA137/93A, which was susceptible only to netilmicin, fusidic acid, and mupirocin. All three strains contained multiple copies of IS elements (IS1272, IS1272-related elements, IS1181, IS431*mec*, and IS256) which were all mapped by PCR (Table 1; for all insertion sites, see Tables S1 and S2 in the supplemental material).

In total, 67 different IS256 insertions were detected in the three strains. Unlike the other IS elements (IS1272, IS1272-related elements, IS1181, and IS431*mec*), which were mostly located between the coding sequences, 46% of the IS256 insertions were intragenic, inactivating hypothetical genes (58%) and genes involved in processing of genetic information (23%), in metabolism (13%), and in regulatory processes (6%) (Table S2).

In spite of the close relationship between strains SA137/93A and SA137/93G, at least four transpositions of IS256 had occurred (Fig. 1A), indicating that its transposition frequency is rather high in these strains. This is also stressed by the fact that all insertions that were found for the other IS elements were conserved between the strains (Table S2). The inactivation of *sarU* was among the 13 IS256 insertions detected

**TABLE 1** Overview of mobile genetic elements, pathogenicity islands, and amino acid exchanges conferring antibiotic resistance for 3 ST247 strains

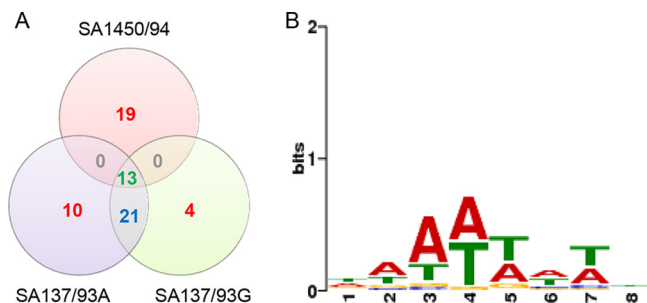
Feature	Datum for strain:		
	SA1450/94	SA137/93A	SA137/93G
Total no. of IS elements	46	63	53
No. of copies of IS elements			
IS256	33	44 (chromosome) + 1 (pSA93A)	39
IS1181	5	7	7
IS431mec	1	2 (chromosome) + 2 (pSA93A)	1
IS1272	1	1	
IS1272 related	6	6	6
Free plasmid pSA93A		pSA93A: <i>merA</i> , <i>merB</i> , <i>cadA</i> , <i>blaZ</i> , <i>qacA</i>	
Integrated pUB110		pUB110: <i>aadD</i> , <i>bleO</i>	
Transposons	Tn4001: <i>aac(6')-aph(2'')</i> Tn5801 variant: <i>tetM</i> Tn554: <i>ermA</i> , <i>ant9-la</i>	Tn4001: <i>aac(6')-aph(2'')</i> Tn5801 variant: <i>tetM</i> Tn554: <i>ermA</i> , <i>ant9-la</i>	Tn4001: <i>aac(6')-aph(2'')</i> Tn5801 variant: <i>tetM</i> Tn554: <i>ermA</i> , <i>ant9-la</i>
SCCmec cassette	Type I	Type IA	Deleted (18)
No. of prophages (complete)	4	3	3
No. of prophages (incomplete)	2	2	2
No. of pathogenicity islands ( <i>νSaα</i> , <i>νSaβ</i> , <i>νSaγ</i> )	3	3	3
Exchanges in RpoB <sup>a</sup>	H481N, L875S, V798A	H481N, L875S, V798A, S529L	H481N, L875S, V798A, S529L
Exchange in GyrA <sup>b</sup>	S84L	S84L	S84L
Exchange in ParC <sup>b</sup>	S80F	S80F	S80F

<sup>a</sup>Exchanges in RpoB confer resistance to rifampin (H481N, L875S, and V798A) and the S529L exchange was previously observed in a VISA strain (58).

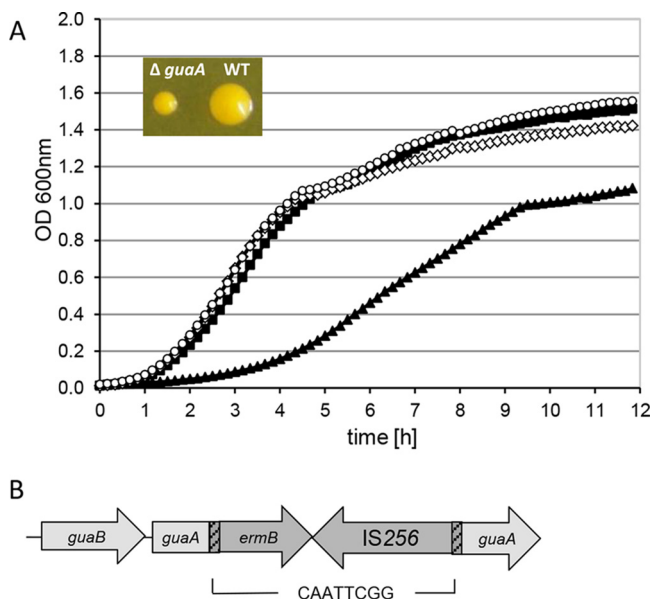
<sup>b</sup>Exchanges in in GyrA and ParC confer resistance to quinolones.

in all three isolates. Strains SA137/93A and SA137/93G carried 21 common IS256 insertions, among others in the promoter of *rot* and in the open reading frame (ORF) of *comGB*. The insertion in the promoter of *rot* was at nearly the same place as the insertion described previously for *S. aureus* USA500 (14). However, IS256 is inserted in the opposite direction from that in *S. aureus* USA500; i.e., in strain SA137/93 the strong C-terminal -35 promoter sequence of IS256 is located in the vicinity of the promoter of *rot*, which might alleviate any negative effects of this insertion on transcription. Indeed, previous microarray experiments comparing strain *S. aureus* SA137/93A to strain SA1450/94, which does not contain this insertion, had not indicated any differences concerning transcription of *rot* or any toxins (25). Although IS256 was integrated in the promoter region of *fosB* in SA137/93A and SA137/93G, the strains showed a fosfomycin resistance phenotype.

Three insertions led to recombinations between neighboring IS256 elements and deletion of the sequence between the two copies; e.g., the deletions of SACOL1333 and parts of SACOL1859 were most probably caused by homologous recombination of two IS elements inserted in the same orientation (see Fig. S1A and B in the supplemental



**FIG 1** IS256 insertion sites. (A) Distribution of 67 different IS256 insertion sites in three ST247 strains. (B) Weight matrix of 67 different direct repeats of IS256 in ST247 strains.



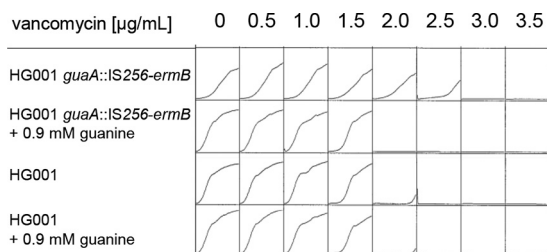
**FIG 2** Characterization of the SCV *S. aureus* HG001 *guaA*::*IS256-ermB*. (A) Growth kinetics of *S. aureus* HG001 *guaA*::*IS256-ermB* in the presence (■) and in the absence (▲) of 0.9 mM guanine as well as *S. aureus* HG001 in the presence (○) and in the absence (◇) of 0.9 mM guanine in BHI medium. The inset shows the colony size after 48 h on BHI agar at 37°C (WT, wild type;  $\Delta$ *guaA*, *guaA*::*IS256-ermB*). (B) Localization of *IS256-ermB* in *guaA*. The inverted repeats are shown in the hatched area, and the 8-bp direct repeat is shown below the diagram.

material). Most notably, if two *IS256* elements are inserted in a head-to-head manner, a 9-bp direct repeat (TGTAAGT) is formed. Illegitimate recombination at this direct repeat most probably led to deletion of the largest parts of two *IS256* elements inserted in a head-to-head manner (at bp 1754695 in the reference genome of *S. aureus* COL) (Fig. S1C).

A comparison of all insertion sites showed that AT-rich sequences were dominant, especially in positions 3 to 5 and 7; the median GC content of the insertion sites was 23.5%. Figure 1B shows the weight matrix of all 67 direct repeats.

**Insertion into *guaA* yields an *in vitro* SCV phenotype with slightly increased resistance to vancomycin.** In order to evaluate whether the presence of *IS256* confers a selective advantage in the presence of vancomycin, *S. aureus* HG001 containing plasmid pA3, which contains a composite *IS256* (*IS256-ermB* [26]), and strains that harbored one insertion of *IS256-ermB* in their genomes (HG001 W5 and HG001 G7) were passaged in the presence of increasing concentrations of vancomycin for at least 10 days. Controls contained the empty vector pBT2, no vector, or pBT2-*ermB*. After 10 days, all strains were able to grow at vancomycin concentrations of 10  $\mu$ g/ml. Therefore, we could not show that a selective advantage is always conveyed by the presence of multiple copies of *IS256 per se*. However, a mutant was isolated from the culture of *S. aureus* HG001 pA3 that contained an insertion of *IS256-ermB* in the gene *guaA*. This mutant was present after day 7 as well as after day 24, showed an MIC of 4 to 8  $\mu$ g/ml for vancomycin, and formed rather small colonies. *guaA* encodes the basic metabolic gene GMP synthetase.

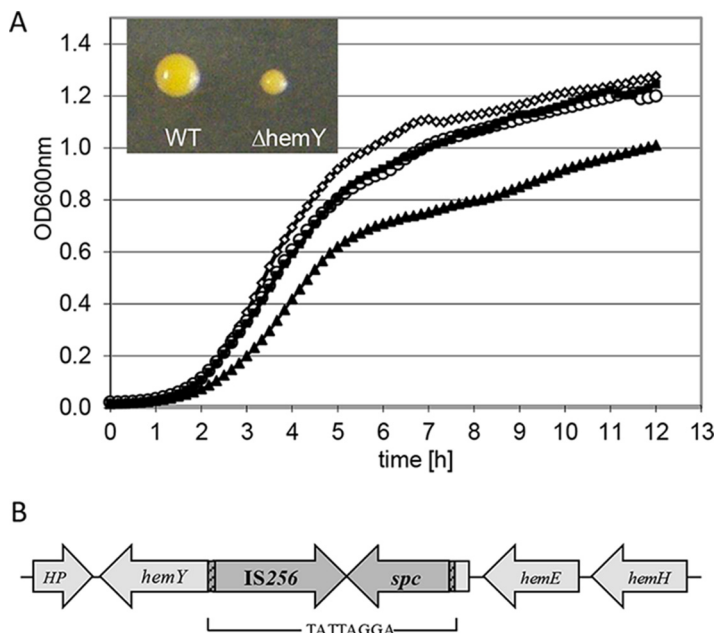
In order to evaluate the effect of this insertion on vancomycin susceptibility and exclude the effects of further point mutations in the chromosomal background of the strains, the insertion was transduced into *S. aureus* HG001 via phage 85 and was verified by PCR analysis. The resulting mutant showed very slow growth with trace amounts of guanine present in tryptic soy broth (TSB) and brain heart infusion (BHI) medium (Fig. 2) and virtually no growth in the absence of guanine in modified RPMI medium (Fig. S2), indicating guanine auxotrophy. On BHI medium, small colonies, reminiscent of SCVs, were formed. Furthermore, there was a slight rise in resistance to vancomycin, from 2



**FIG 3** Vancomycin susceptibility of the SCV *S. aureus* HG001 *guaA::IS256-ermB*. Growth experiments employed *S. aureus* HG001 *guaA::IS256-ermB* and *S. aureus* HG001 in the presence of different concentrations of vancomycin in BHI medium in the presence and absence of guanine; growth was determined using a microplate reader. Shown are the results of one of three replicates.

$\mu\text{g/ml}$  (*S. aureus* HG001) to 3  $\mu\text{g/ml}$  (*S. aureus* HG001 *guaA::IS256-ermB*). This increase in the MIC was observed only in the absence of exogenous guanine; the addition of guanine reversed the effect (Fig. 3). In conclusion, the strain displayed the phenotype of a guanine auxotrophic SCV.

**Insertion into *hemY* yields an SCV with increased resistance to aminoglycosides.** In another experiment, plasmid pA6, which does not replicate at 45°C and contains a genetically engineered IS256 element harboring the resistance gene *spc* or *ant(9)-Ia* (27), which confers resistance to spectinomycin but not to streptomycin (28), was used for selection of spectinomycin-resistant colonies. After culturing on agar containing spectinomycin (150  $\mu\text{g/ml}$ ) at 45°C, one of the colonies contained an insertion of IS256-*spc* into the 3' end of *hemY* (*S. aureus* HG001 *hemY::IS256-spc*) as shown in Fig. 4. The colony showed slow growth, and the wild-type phenotype was reconstituted by growth in the presence of hemin (Fig. 4). In the absence of oxygen, the phenotype was indistinguishable from that of the wild type. Hemin auxotrophic SCVs with mutations in the heme biosynthesis genes are often selected in the presence of



**FIG 4** Characterization of the SCV *S. aureus* HG001 *hemY::IS256-spc*. (A) Growth kinetics of *S. aureus* HG001 *hemY::IS256-spc* in the presence (■) and in the absence (▲) of 2  $\mu\text{g/ml}$  of hemin as well as *S. aureus* HG001 G2 in the presence (○) and in the absence (◇) of 2  $\mu\text{g/ml}$  of hemin in TSB medium containing 64  $\mu\text{g/ml}$  of spectinomycin. The inset shows the colony size after 48 h on TSA agar with 150  $\mu\text{g/ml}$  of spectinomycin at 37°C (WT, *S. aureus* HG001 G2;  $\Delta\text{hemY}$ , *hemY::IS256-ermB*). (B) Localization of IS256-*spc* in *hemY*. The inverted repeats are shown in the hatched area, and the 8-bp direct repeat is shown below the diagram.

**TABLE 2** MICs of drugs for *S. aureus* HG001 *hemY::IS256-spc* in comparison to *S. aureus* HG001 G2 and the revertant *S. aureus* HG001 R1, which had lost the *IS256-spc* element, in MH broth

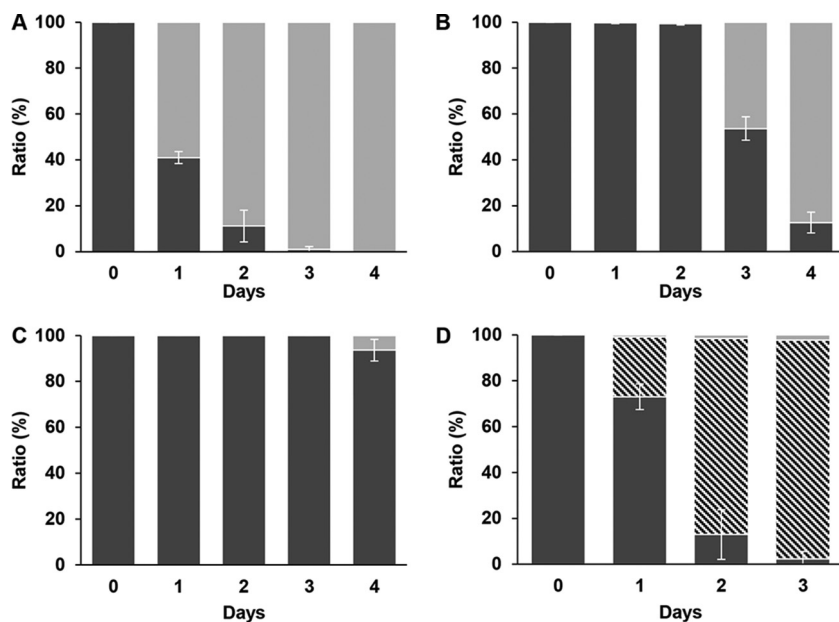
Strain	MIC ( $\mu\text{g/ml}$ ) of:		
	Kanamycin	Gentamicin	Spectinomycin
<i>S. aureus</i> HG001 G2	3.6	0.5	>128
<i>S. aureus</i> HG001 <i>hemY::IS256-spc</i>	58	4	>128
<i>S. aureus</i> HG001 R1	4	0.5	64

aminoglycosides (29). This strain also displayed resistance to kanamycin and gentamicin, which is not conveyed by *spc* and was not present in another mutant, *S. aureus* HG001 G2, which contained an intergenic insertion of *IS256-spc* between SAOUHSC\_0967 and SAOUHSC\_0968, and a revertant that had lost the insertion (Table 2). In conclusion, *S. aureus* HG001 *hemY::IS256-spc* showed the phenotype of an aminoglycoside-selected SCV. The insertion *hemY::IS256-spc* harbored a deletion of the last bases of the direct repeat (GAT). However, a 9-bp direct repeat was still formed, as the first 3 bases of *IS256* (GAT) complemented this deletion. Any further mutations in the heme biosynthesis operons, *hemEHY* and *hemAXCDBL*, in this clone were excluded by sequencing.

The *hemY* mutant still produced some staphyloxanthin (Fig. 4), which was particularly noticeable after 40 h of incubation. Typically, clinical SCVs and  $\Delta\textit{hemB}$  mutants show no or decreased pigment production compared to that of the wild type (20). However, when CtaB, the enzyme that catalyzes the very last step of the pathway and couples the farnesyl diphosphate chain to protoheme IX, is removed in *S. aureus*, an increased pigmentation is observed, indicating that farnesyl diphosphate is fed into the pigment production pathway (30). Since the insertion into *hemY* interrupts the pathway between the steps catalyzed by HemB and CtaB, the *hemY* mutant was supplemented with hemin on an agar plate to assess staphyloxanthin production in the absence and presence of hemin. The result clearly showed that the *hemY* mutant produced less color in the absence of hemin (Fig. S3).

**SCV phenotypes that are caused by *IS256* insertions revert quickly to the normal phenotype.** *IS256* replicates by a copy-and-paste mechanism (5). Loss of an insertion is mediated by illegitimate recombination via the 8-bp direct repeats and is a rare event (3). In order to test the stability of the SCV, the *guaA* and the *hemY* mutants were passaged in BHI (data not shown) or TSB (Fig. 5A). The selective antibiotic was either absent or present only in the very first culture, which had been inoculated by resuspending an SCV colony (day 0), or present from day 0 to day 4. Every day, aliquots of the cultures were plated on BHI or TSB agar plates (no addition of antibiotics) and SCV colonies and wild-type colonies were counted. In the absence of erythromycin, the reversion of the *guaA* mutant occurred quickly, with 60% revertants on day 1 after 24 h of incubation (Fig. 5A). The presence of erythromycin (4  $\mu\text{g/ml}$ ) in the first culture delayed the reversion (Fig. 5B), and this effect was even more pronounced when the antibiotic was present during all passages (Fig. 5C), indicating that the presence of erythromycin selected for strains that had kept the *IS256-ermB* element in their genome. Large-colony-forming revertants were checked for the insertion of *IS256-ermB* in *guaA* by PCR using the primer pair *guaA\_forMH* and *guaA\_revMH* or *guaA\_for* and *guaA\_rev*. The IS element was lost in most cases, but there were also revertants that showed the large-colony phenotype and still harbored the insertion sequence in *guaA*. The uptake of guanine or its precursors in *S. aureus* has not been investigated, but our growth experiments in the presence of guanine in RPMI medium demonstrated that such an uptake must exist. For *Escherichia coli*, several purine transporters have been identified (*purP*, *ygfO*, and *yicE*) (31) which according to protein BLAST resemble SA2050, *pbuX*, and SA1042 (*pyrP*) in *S. aureus*. However, sequencing (promoters and structural genes of SA1041 [*pyrR*], SA1042 [*pyrP*], SA2050, and the riboswitch upstream of *xpt-pbuX-guaB-guaA* [32]) did not reveal any mutations.





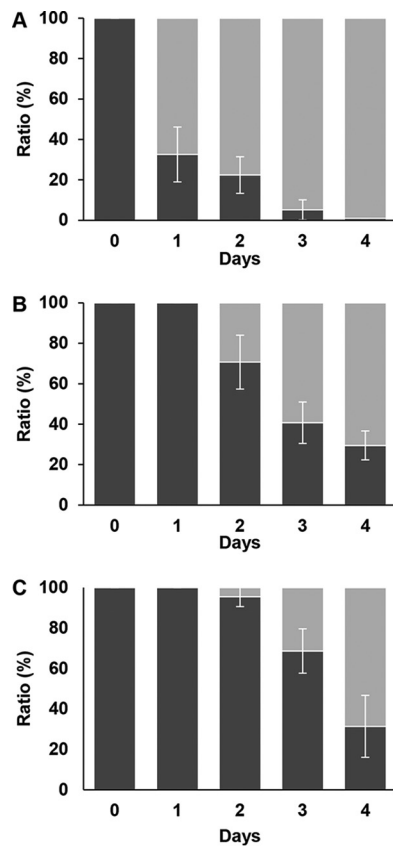
**FIG 5** Reversion of *S. aureus* *guaA::IS256-ermB* to the wild-type colony phenotype. (A) *S. aureus* *guaA::IS256-ermB* (black) reverting to the large-colony phenotype (gray) during daily serial passages in TSB medium without antibiotic. (B and C) Same experiment as for panel A performed in TSB containing antibiotic (4  $\mu\text{g/ml}$  of erythromycin) in the first culture (inoculated on day 0) only (B) and in TSB containing 4  $\mu\text{g/ml}$  of erythromycin from day 0 to day 4 (C). (D) Appearance of surrogate revertants, i.e., blue colonies harboring pMAD (oblique striped) during serial passages in TSB medium containing 25  $\mu\text{g/ml}$  of erythromycin from day 0 to day 3. For this experiment, the initial inoculum was approximately 1 CFU/ml of *S. aureus* HG001 pMAD and one SCV colony ( $2 \times 10^5$  to  $6 \times 10^5$  cells/ml). Error bars indicate the standard deviations from at least 3 experiments.

The phenotype of *S. aureus* HG001 *hemY::IS256-spc* was also lost very quickly in TSB and BHI (Fig. 6A). As described above, the reversion could be delayed by the addition of spectinomycin (Fig. 6B) and the delay could be prolonged, but not inhibited, if spectinomycin (150  $\mu\text{g/ml}$ ) was always present (Fig. 6C). Characterization of 12 randomly chosen revertant colonies from several experiments with *S. aureus* HG001 *hemY::IS256-spc* showed that *IS256-spc* was never retained in *hemY*; i.e., the reversion was always accompanied by loss of *IS256-spc* from *hemY*. Four out of eight *hemY* revertants chosen on day 4 from experiments that had been conducted in the presence of antibiotics at least at day 0 were still able to grow on antibiotic-containing agar, indicating that the resistance gene was still present in these strains. In order to localize the *spc* gene in two such mutants, the insertion sites of *IS256-spc* were analyzed by inverse PCR and sequencing, and it could be shown that *IS256-spc* had inserted into SAOUHSC\_00997 and SAOUHSC\_00950, respectively. Both revertants had been isolated on day 4 from the same culture incubated with antibiotic on day 0 only.

This result indicated that in the presence of antibiotics, replicative transposition of *IS256* with the associated resistance gene into another locus of the chromosome had enabled subsequent loss of the IS element from *hemY*. This way, reversion of the phenotype even in the presence of the selective antibiotic was possible; however, two successive mutational events, i.e., transposition of *IS256* using the copy-and-paste mechanism and the subsequent illegitimate recombination that excised *IS256* from *hemY*, were needed.

#### Reversion may be mediated by a very low number of quickly growing cells.

As a control, the reversion experiments were repeated with both auxotrophic SCVs in TSB and BHI after supplementation with guanine (0.9 mM) or hemin (2  $\mu\text{g/ml}$ ) in the absence of antibiotics, respectively. These conditions conferred a fast-growing phenotype on the SCV cells; therefore, possible revertants could not outcompete

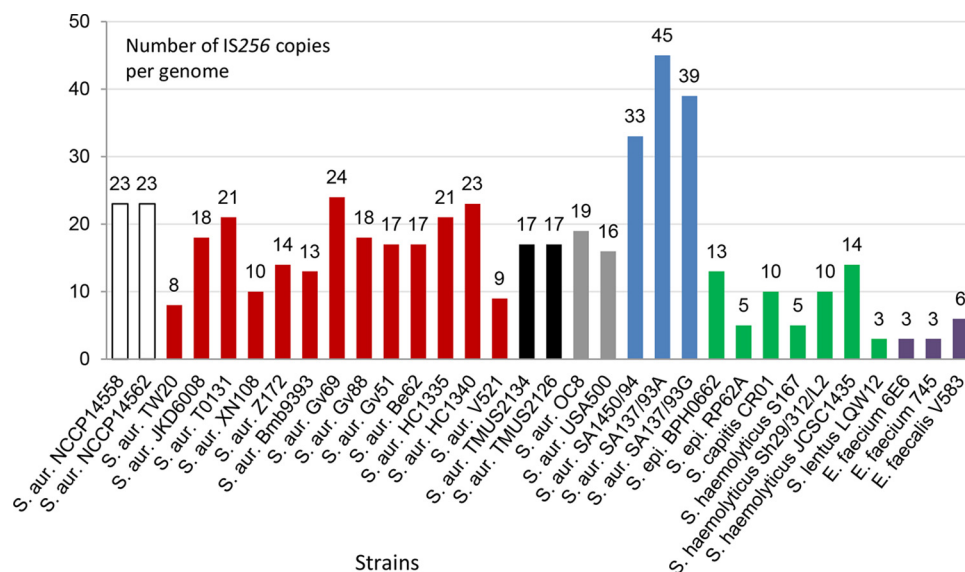


**FIG 6** Reversion of *S. aureus hemY::IS256-spc* to the wild-type colony phenotype. (A) *S. aureus hemY::IS256-spc* (black) reverting to the large-colony phenotype (gray) during daily serial passages in TSB medium without antibiotic. (B and C) Same experiment as for panel A performed in TSB containing antibiotic (150  $\mu\text{g/ml}$  of spectinomycin) in the first culture on day 0 only (B) and in TSB containing 150  $\mu\text{g/ml}$  of spectinomycin from day 0 to day 4 (C). Error bars indicate the standard deviations from at least 3 experiments.

the SCV genotype cells. When culture aliquots were plated on guanine-free or hemin-free agar, all cells had retained the SCV phenotype and no revertant colonies were visible after 4 days. This result indicates that the reversion might be mediated by only a few mutational events that occur in a very small subpopulation and is not accomplished by simultaneous mutation of the majority of the cells. We therefore hypothesized that only a few revertant cells would be able to outcompete the SCV by their fast growth. In our media, the doubling time of the SCV cells was twice as long as that of the wild type, and both strains grew more quickly in TSB than in BHI.

In order to visualize the competition between SCV and a fast-growing revertant, we inoculated 5 ml of TSB containing a high concentration of the selective antibiotic (25  $\mu\text{g/ml}$  of erythromycin) with one SCV colony of *S. aureus* HG001 *guaA::IS256-ermB*, yielding  $2 \times 10^5$  to  $6 \times 10^5$  CFU/ml, and added approximately 1 CFU/ml of the parent strain *S. aureus* HG001 (displaying the large phenotype) harboring plasmid pMAD. This strain is also resistant to erythromycin at 30°C and forms blue colonies on agar containing 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal). Thus, it served as an easily detectable surrogate “revertant” in this experiment. Figure 5D shows the appearance of blue colonies harboring pMAD in the culture after passages at 30°C. Indeed, the progeny of the plasmid-bearing clone represented the majority of the culture after 2 days, indicating that a very low number of mutants was sufficient for a reversion of the SCV genotype. In BHI the same phenomenon was observed but with a slower appearance of blue colonies.





**FIG 7** Distribution of IS256 in different cocci. Sequence types (ST) of *S. aureus* are indicated by colors as follows: ST5, white; ST239, red; ST72, black; ST8, gray; and ST247, blue. Strains with fewer than three IS256 copies are not shown. Coagulase-negative staphylococci are green, and *Enterococcus* species are purple. IS256 was localized by NCBI BLAST and *S. aureus* MLST types were determined by MLST 1.8. *S. aur.*, *S. aureus*; *S. epi.*, *Staphylococcus epidermidis*.

## DISCUSSION

This paper describes the genomes of three strains of the Iberian clone (ST247). The high number of IS256 elements present in the ST247 genomes is striking; it is the highest number found by NCBI BLAST using all complete staphylococcal and enterococcal genomes deposited in the databases so far (Fig. 7). The IS density of *S. aureus* 137/93A (number of IS elements per kilobase of nucleotides) is 0.021 and is at the upper limit for free-living organisms (33). The highest number of IS elements in prokaryotes has been described for *Wolbachia* strains (52 to 171 elements per genome); however, more than 70% of these ISs constitute “fossil insertions” that contain inactivating mutations (34). The different insertion sites of IS256 encountered in two closely related isolates indicate a high transposition activity. This might be specific for these isolates, as the copy number of the transposase gene will increase with the number of IS elements; therefore, the transposition activity might increase along with the number of *tnpA* copies in these strains. In the end, such an “IS expansion” may run out of control and result in genome reduction by favoring homologous recombination (34). Two examples of such deletions are present in the strains described here (Fig. S1), and interestingly, a large IS256-based genomic inversion that contains 19 copies of IS256 has been described for *S. aureus* OC8 (35). IS256 itself is present only in some successful MRSA lineages, most of which belong to clonal complex 8 (i.e., ST239, ST8, ST247, and ST72 [16]) (Fig. 7). Other staphylococcal IS elements, like IS257/431*mec*, are found in more lineages and show lower copy numbers and transposition activity. In our hands, IS257/431*mec* was nearly inactive when we tested its transposition frequency (data not shown). However, IS257/431*mec* might be involved in recombination events like the integration of pUB into SCC*mec* in strain SA137/93A or the large deletion present in strain SA137/93G (18) and other VISA strains (36).

Plasmids pA3 and pA6 contain the pE194*ts* replicon, which gives rise to about 14 copies of the plasmid (37), which would be equivalent to 14 copies of IS256 in the genome and is in the range of the average copy number of IS256 in clinical CC8 isolates (Fig. 7). These plasmids were used to introduce a recombinant IS element into *S. aureus* HG001 in the presence of antibiotics; most intriguingly, unstable SCV-like phenotypes were generated during these experiments.

The insertion into *guaA* led to slow growth and a slight increase in resistance to

vancomycin, if guanine was not added to the BHI serving as culture medium. The absence of GuaA activity elicits the stringent response in staphylococci (38). This phenomenon, which is also triggered by mutations in *relA* (*rsh*), *relP*, or *relQ*, has already been described in connection with intermediate vancomycin resistance and slow growth in *Staphylococcus* and *Enterococcus* (39–41). Indeed, an *rsh* mutant with an SCV phenotype was also isolated from a patient with a recurrent infection (42). In addition, knockout of *guaA* leads to thickened cell walls (43). However, *guaA* deletion mutants are not viable in human serum (43) and therefore represent an *in vitro* phenotype only. In contrast, inactivation of *hemY* led to a heme-auxotrophic phenotype, and recently, HemY has been shown to be an essential enzyme in the heme biosynthesis pathway in *S. aureus* (44). Heme auxotrophy is also observed *in vitro* after treatment with aminoglycosides (45). Interestingly, recently a clinical *S. aureus* SCV isolate has been published to possess a truncated *hemY* gene (46) (called *hemG* here; this is the *E. coli* locus designation for *hemY*).

Both genotypes reverted quickly to the wild-type phenotype in the absence of antibiotics. Whereas IS256 was always lost from the *hemY* insertion mutants, the *guaA* mutants seemed also to be rescued by other mutations. The molecular details of this mechanism are still unclear, as sequencing showed no alterations (e.g., insertion of another copy of IS256, forming a stronger promoter) in the promoters of SA2050, *pbuX*, or SA1042, which represent ORFs with similarity to *E. coli* purine base transporters (31). However, the regulation of guanine uptake in *S. aureus* is also unclear, and a mutation in a regulatory gene might still stimulate import of guanine in BHI and TSB medium which still contain trace amounts of this metabolite.

IS256 is excised by illegitimate recombination (slipped-strand mispairing) of the direct repeats (3). The inverted repeat that is flanked by the direct repeats will facilitate such a deletion since it is able to form a hairpin structure that places the direct repeats close to each other (47, 48). The experiment with the pMAD-bearing surrogate revertant clearly demonstrated that outcompetition of the SCV by a revertant may be fast. The doubling times of the SCV in our experiments were about twice as long as those of the wild type. If one revertant was present or arose in a culture containing approximately  $10^5$  SCVs, a calculation of the cell numbers indicates that under these conditions (with assumed mean doubling times of 44 min for the wild type and 88 min for the *guaA* insertion mutant, as seen, for example, in TSB), 24.44 h of exponential growth would be needed to reach equal cell numbers of SCVs and the wild type in the culture. However, the very high cell numbers at this time (about  $2 \times 10^{10}$  CFU per ml) would probably not be supported by the growth medium, and therefore, a second passage is normally needed. This confirms the *in vitro* data shown in Fig. 5. The speed of the reversion of the *hemY* mutants in BHI reproducibly exceeded the speed that can be reached after one mutational event. Here several, possibly preexisting, mutants would be required for the observed high reversion rate. Indeed, such revertants already arose during the storage of the SCV colonies on agar plates, because in the first experiments, large colonies were sometimes detected during inspection of agar plates that had been plated directly after resuspension of a *hemY::IS256-spc* SCV colony, when the colony had been taken from an agar plate stored in the refrigerator for over 1 week. These experiments were not evaluated for this study but served to demonstrate the high excision frequency, which might in part depend on the fact that a 9-bp direct repeat could be used for excision of this insertion.

So far, there seem to be several mechanisms that generate the SCV phenotype *in vivo* and *in vitro* (20). There is sound evidence that indicates the presence of regulatory mechanisms, since SigB seems to be essential if SCVs are formed intracellularly *in vivo* (19) and a high percentage of intracellular cells seems to be able to switch to the SCV phenotype (24). On the other hand, mutations may also play a role since stable *hemH* mutants have been isolated after treatment with gentamicin *in vitro* from *S. aureus* NCTC 8325, which is an *rsbU*-negative strain and therefore displays very low SigB activity (23), and stable *thyA* mutants were isolated after long-term exposure to trimethoprim-sulfamethoxazole *in vivo* (49). Here, we show for the first time that via

**TABLE 3** Strains and plasmids used in this study

Strain or plasmid	Description <sup>a</sup>	Reference
<i>Staphylococcus aureus</i> strains		
HG001	<i>rsbU</i> -repaired laboratory strain NCTC 8325	59
SA137/93A	Clinical VISA/MRSA isolate	18
SA137/93G	Spontaneous mutant of clinical VISA isolate SA137/93A; $\Delta$ SCCmec (Met <sup>s</sup> )	18
SA1450/94	MRSA, Northern German epidemic strain; <i>mutS</i> ::IS256	18
HG001 <i>hemY</i> ::IS256- <i>spc</i>	<i>hemY</i> ::IS256- <i>spc</i> insertion mutant; DR: TATTAGGAT	This study
HG001 G2	Intergenic IS256- <i>spc</i> insertion mutant (SAOUHSC_0967/SAOUHSC_0968); DR: TTTTATAGC	7
HG001 G7	Intergenic IS256- <i>ermB</i> insertion mutant (SACOL0573/ <i>gltX</i> )	This study
HG001 W5	<i>rsbU</i> ::IS256- <i>spc</i> insertion mutant; DR: TTTAATTA	7
HG001 <i>guaA</i> ::IS256- <i>ermB</i>	<i>guaA</i> ::IS256- <i>ermB</i> insertion mutant; DR: CAATTCGG	This study
HG001 R1	Revertant of HG001 <i>hemY</i> ::IS256- <i>spc</i>	This study
Plasmids		
pBT2	<i>E. coli</i> - <i>S. aureus</i> shuttle vector with thermosensitive origin of replication for Gram-positive bacteria; <i>cat</i>	7
pBT2- <i>ermB</i>	pBT2 harboring the erythromycin resistance-mediating cassette <i>ermB</i> as insert	26
pA3	pBT2 carrying a recombinant IS256 element containing an erythromycin resistance-mediating cassette	26
pA6	pBT2 carrying a recombinant IS256 element containing a spectinomycin resistance-mediating cassette, <i>spc</i> from <i>S. aureus</i> Mu50 (GenBank accession no. <a href="#">BA000017.4</a> )	7
pMAD	<i>E. coli</i> - <i>S. aureus</i> shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria; <i>ermC</i> <i>bgaB</i>	60

<sup>a</sup>VISA, vancomycin-intermediate *S. aureus*; DR, direct repeat.

insertions of IS elements, mutations may also give rise to unstable auxotrophic SCVs. Other genetic mechanisms that can confer fast phenotypic switching, like insertion or deletion of single bases or short repeats by slipped-strand mispairing, have also been discussed previously for phase variation in *E. coli* and biofilm formation of *S. aureus* (50, 51).

In conclusion, the isolates of the ST247 lineage sequenced in this study are burdened by a huge number of IS256 insertion elements, which are actively transposing and confer a strong genomic plasticity on these strains. As an example, the presence of IS256 may give rise to unstable SCV phenotypes that are reminiscent of phase variation phenomena but which are nonetheless caused by mutations.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 3.

*S. aureus* strains were shaken in tryptic soy broth (TSB) (Merck KGaA, Darmstadt, Germany), brain heart infusion (BHI) medium (Oxoid, Wesel, Germany), Mueller-Hinton (MH) broth (Oxoid, Wesel, Germany), or lysogenic broth (LB [52]) at 37°C or 45°C. For solid media, tryptic soy agar (TSA) or BHI agar was used. Growth experiments under anaerobic conditions were performed at 37°C using the GasPak EZ container system (Becton Dickinson, Heidelberg, Germany). *S. aureus* strains containing plasmids with a temperature-sensitive origin of replication were incubated at 34°C in TSB or BHI medium supplemented with 20 µg/ml of chloramphenicol, 25 µg/ml of erythromycin, or 150 µg/ml of spectinomycin.

**Growth kinetic measurements and antibiotic resistance testing.** For growth experiments, an overnight culture was diluted to an optical density at 600 nm (OD<sub>600</sub>) of 0.1 in TSB, BHI, or MH medium supplemented with selective concentrations of 4 µg/ml of erythromycin or 64 µg/ml of spectinomycin. The growth curves were measured over a period of 12 h at 37°C in a microplate reader (Sunrise; Tecan, Crailsheim, Germany) at intervals of 12 min, and the microtiter plate was shaken for two periods of 3 min within each interval. Data were analyzed by Magellan data analysis software (Tecan, Crailsheim, Germany).

Some growth experiments with the *S. aureus* HG001 *guaA*::IS256-*ermB* insertion mutant were performed in modified RPMI 1640 medium R7509 (Sigma-Aldrich, Taufkirchen, Germany) (53). Before each experiment, 500 ml of RPMI medium was supplemented with glutamine (end concentration, 2 mM) and 10 ml of a sterile solution of Wolfe's mineral salts [500 mg/liter of Titriplex I, 180 mg/liter of ZnSO<sub>4</sub>·7 H<sub>2</sub>O, 50 mg/liter of CuSO<sub>4</sub>·5 H<sub>2</sub>O, 170 mg/liter of CoCl<sub>2</sub>·6 H<sub>2</sub>O, 130 mg/liter of CaCl<sub>2</sub>·2 H<sub>2</sub>O, 11 mg/liter of NaMoO<sub>4</sub>·H<sub>2</sub>O, 26 mg/liter of Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>, 10 mg/liter of H<sub>3</sub>BO<sub>3</sub>, 5 ml/liter of 1 M NaOH]. In order to show the guanine auxotrophy of the *S. aureus* HG001 *guaA*::IS256-*ermB* insertion mutant, the main cultures were inoculated with 2 or 3 colonies from a fresh TSA plate resuspended in RPMI medium (OD<sub>600</sub>, 0.05 to 0.1) to prevent contamination with trace amounts of guanine present in the TSB overnight culture.

Guanine was added to the RPMI medium at different concentrations between 0.005 mM and 0.5 mM. To demonstrate the effect of the *guaA* insertion on vancomycin resistance, the strains were incubated in the presence of different concentrations of vancomycin in BHI growth medium in the microplate reader for 12 h exactly as described above; also, MICs were determined in BHI.

Fosfomicin MICs were determined by Etest (bioMérieux, Mercy-l'Étoile, France). For the MIC determinations for spectinomycin and aminoglycosides, fresh colonies of *S. aureus* HG001 G2, *S. aureus* HG001 *hemY::IS256-spc*, and the revertant *S. aureus* HG001 R1, which had lost the insertion in *hemY*, were grown on TSA agar plates (150 µg/ml of spectinomycin for the strains containing *IS256-spc*). The colonies were resuspended in MH broth (no antibiotic) and diluted to 10<sup>6</sup> cells per ml. A total of 100 µl of this suspension was mixed with 100 µl of the antibiotics dissolved in MH broth in the wells of a microtiter plate. The MICs were read after 24 and 48 h of incubation at 37°C.

**Mapping of all IS256 insertions in three ST247 strains by 454 sequencing.** The genomic sequences of the ST247 strains *S. aureus* SA1450/94, SA137/93A, and SA137/93G were determined by next-generation sequencing (NGS) using 454 sequencing technology (54). In the first *de novo* assembly of the NGS reads with the GS De Novo Assembler (Roche Applied Science GmbH, Mannheim, Germany), the sequences of the IS elements *IS256*, *IS431mec*, and *IS1181* were recognized as repetitive sequences and were summarized as three single contigs. Only the first or last bases of the IS elements were present in the neighboring contigs. To identify all insertion sites of *IS256* and other insertion sequences and to close the gaps between the neighboring contigs, the nucleotide sequences of *IS256*, *IS431mec*, and *IS1181* were compared via a BLAST search against all reads of strain SA137/93G using Geneious 6.1.8 (Biomatters Limited, Auckland, New Zealand). The resulting hits were assembled *de novo* and generated contigs, which then were analyzed via BLAST against the reference strain *S. aureus* COL (GenBank accession no. NC\_002951.2), identifying the precise insertion sites. In BLAST analyses of SA137/93A and SA1450/94, the contigs, which were joined by an *IS256* sequence *in vivo*, were identified by the 8-bp direct repeat that frames every *IS256* insertion. All insertion sites of the IS elements were confirmed for all three strains by PCR and Sanger sequencing and integrated into the draft genome sequences.

**Mapping of single IS256, IS256-spc, and IS256-ermB insertions.** The localizations of *IS256* and recombinant *IS256* elements in different insertion mutants of *S. aureus* HG001 were determined by inverse PCR as previously described (12) (Table 4). In brief, the genomic DNA was digested with XmnI (New England BioLabs, Frankfurt am Main, Germany) or AluI (Roche Applied Science GmbH, Mannheim, Germany). After circularization of the fragments using the T4 ligase (Roche Applied Science GmbH, Mannheim, Germany), the insertion sites were detected by PCR analysis using the outgoing primers IS91rev and IS141for, which anneal within the 5' end of *IS256* (Table 4). Afterwards, the insertion site of the insertion element was detected by Sanger sequencing (SEQLAB Sequence Laboratories Göttingen GmbH, Göttingen, Germany).

**Passaging with increasing vancomycin concentrations.** In order to select vancomycin-resistant *IS256* insertion mutants, different strains (see Results) were passaged in the presence of increasing vancomycin concentrations. Each strain was inoculated in 5 ml of BHI medium with three different vancomycin concentrations and was shaken at 170 rpm and 34°C. After 24 h, the culture that had yielded visible bacterial growth and contained the highest vancomycin concentration was used to inoculate three new BHI cultures supplemented with the same vancomycin concentration as the preculture and two higher vancomycin concentrations (preculture plus 0.5 µg/ml and preculture plus 1 µg/ml). The passaging was performed for at least 10 days. In these tests, the appropriate antibiotics were used to maintain the different plasmids and several inoculum volumes were tested.

**Selection of an IS256-mediated SCV in the presence of spectinomycin.** The *spc* gene employed in this study encodes the spectinomycin adenylyl transferase ANT (9)-Ia, which confers resistance to spectinomycin but not to streptomycin (28). In order to select *IS256-spc* insertion mutants, the strain *S. aureus* HG001 containing the temperature-sensitive plasmid pA6 (7) was shaken overnight in TSB medium supplemented with 20 µg/ml of chloramphenicol at 30°C. Then the culture was diluted 1:100 in 20 ml TSB medium without chloramphenicol and was shaken for 6 h at 30°C. Aliquots of the culture were diluted and plated onto TSA containing 150 µg/ml of spectinomycin. After incubation of the plates for 48 h at 45°C, a nonpermissive temperature for plasmid replication, a yellow small-colony variant, which exhibited spectinomycin resistance and chloramphenicol susceptibility (indicating the presence of the IS element and loss of the plasmid), was selected from an agar plate containing less than 200 CFU.

**Phage transduction.** Phage transduction was used to transfer the *guaA::IS256-ermB* insertion of the *S. aureus* HG001 insertion mutant (which had been isolated after passaging for 24 days in the presence of vancomycin) to the wild-type strain *S. aureus* HG001 employing phage 85 as previously described (55). The transductants were selected on LB agar containing 25 µg/ml of erythromycin. The chromosomal *guaA::IS256-ermB* insertion in wild-type *S. aureus* HG001 was then verified by PCR using primers *guaA*for and *guaA*rev and sequencing.

**Determination of revertants of the SCV phenotype.** To analyze the appearance of revertants of the SCV phenotype, a single SCV colony (yielding  $2 \times 10^5$  to  $6 \times 10^5$  CFU/ml as determined by plating immediately after inoculation) from a fresh agar plate was resuspended in 5 ml of TSB or BHI medium and was shaken at 37°C. In some experiments the medium was also supplemented with 4 µg/ml of erythromycin (*S. aureus* HG001 *guaA::IS256-ermB*) or 150 µg/ml of spectinomycin (*S. aureus* HG001 *hemY::IS256-spc*). Every 24 h, the culture was diluted to an OD<sub>600</sub> of 0.05 with fresh medium and an aliquot was plated in dilution on TSA or BHI agar. After incubation at 37°C for 24 to 48 h, the small and large colonies (phenotypic revertants) were counted on agar plates containing less than 200 CFU. All experiments were performed at least in triplicate, and mean values are presented. After 4 days, phenotypic revertants were isolated and analyzed by PCR to monitor the loss of the *IS256*

**TABLE 4** Oligonucleotides used in this study

Oligonucleotide	Sequence (5'–3') <sup>a</sup>
ISIRLfor	GTA <u>GAA TTC</u> GGA TAA AGT CCG TAT AAT TGT GT
ISIRRrev	TGT <u>TCT AGA</u> TGC TAT ACA ATG TTT TTA CCA TTT C
IS91rev	CTT TTT ACT TTT ACA CAA TTA TAC GG
IS141for	CCC AGG AGG ACT TTT ACA TGA C
Spc-for_HindIII	ACG <u>AAG CTT</u> AGT CAA GTC CAG ACT CCT GTG TAA AAT CGT CCA ATC TAG GGT A
Spc-rev_XbaI	AAG <u>TCT AGA</u> TGA ATT TCA CAA GAG GAC
guaAfor	CAT GTA TCT TTG TAG ACC ATG
guaArev	AGG ATC TGA AAC ACC TTT TAA T
guaA_forMH	TTC GTG AAA TGG GCG TTT AT
guaA_revMH	TAG ACT ACG CGG TTG ACG TG
hemY_forMH	GTG ACT AAA TCA GTG GCT ATT
hemY_revMH	TGC GAT TAC TTC TTC AGC
hemLBDCA_for	GTT GAT CCG ATT TGC ACA C
hemLBDCA_int1	CCT AAT GGT GCT ATA TGA TCC
hemLBDCA_int2	CGT ACT GGA CTA TTT ACA CC
hemLBDCA_int3	GTT GCC TGT TGA ATA ACA CC
hemLBDCA_int4	GCG CTG TCT TAC TTC CTA TC
hemLBDCA_int5	CAA TAA TAC TGC CTT CTG GC
hemLBDCA_int6	AAC TTA ACA CAG CTA GAC CG
hemLBDCA_int7	ATC CAC TCA TTG TGT GCA TG
hemLBDCA_rev	GGA TGT TCC AGG GTA TGG AT
hemQ_for	ATT GTA TAG AGT AGC GAC TGT
hemQ_rev	GAA CCT TAG TCT CAT CAC CT
hemN_for	TGT CAA TGC TGA TGA TAC ATC
hemN_rev	AGG ACA TAT TAC ATC TGG CT
hemYHE_for	CAT CTG TCA TCT CAT TCT CTC C
hemYHE_int1	ATG CCA ATA CAA CAG TTG C
hemYHE_int2	ATT CAG GCC CTA GTT CAA TC
hemYHE_int3	ATT GCT TCA GTA ATG CCA TC
hemYHE_rev	GTT GAT GGG TCG GGT TAT TG
hemYHE_int2.5.1	GGT ACG GAA CTC GTT ATT TC
hemYHE_int2.5.2	CAC AGG AGT CTG GAC TTG AC
SA2050_for	TAT CGC TTT ATG TTA TTT CG
SA2050_int1	TCC TAT ACC TGC TGA AAC AG
SA2050_int2	TAC CGC TTC TTT CCC TTT AC
SA2050_int3	GCG TGC TTG GTC TTT ATA TG
SA2050_rev	CCT CAA CAG TAC AAT GAA ATA G
377504 F	TGT CAC GAA CCG CTA CAA CT
378517R	TGC GAT ACC GGA AGC TTC AA
1,112,585 F	GTT CAT CCA TCA CCA GGG CA
1,116,051 R	TAC CAT CAC CAG CAT TCG CA
1113415_int1	CCA GAG AGA CGT CAA AGA C
1114264_int2	CTT GTA GCA ACT TGT CAC AC
1114821_int3	CCT GGA GAT ATG AAA GGT TAC

<sup>a</sup>Restriction sites used for cloning are underlined.

insertion in *guaA* and *hemY*. The primer pairs *guaA\_forMH/guaA\_revMH* and *hemY\_forMH/hemY\_revMH* (Table 4) yielded a large band (>3.5 kb) if the recombinant IS256 was inserted into the gene and a small band (about 1.5 kb) after loss of the recombinant IS256. Colonies used for the inoculum of the culture were also tested for the correct IS256 insertions for both insertion mutants and only the large PCR band was visible, indicating that the IS256 element was still present in *hemY* or *guaA*, respectively.

Competition experiments with *S. aureus* HG001 *guaA::IS256-ermB* and *S. aureus* HG001 pMAD were performed at 30°C in the presence of 25 µg/ml of erythromycin. For the inoculum of the mixed culture, successive 10-fold dilutions were prepared from an *S. aureus* HG001 pMAD preculture. Then 0.1 ml of each dilution was plated on TSA and another 0.1-ml aliquot was used as an inoculum for a separate main culture that already contained one resuspended colony of *S. aureus* HG001 *guaA::IS256-ermB*. The CFU of *S. aureus* HG001 pMAD were counted after 24 h, and only the main culture that had been inoculated with approximately 1 CFU/ml was incubated further for the competition experiment.

**Identification of the number of IS256 elements in sequenced genomes in NCBI.** The numbers of IS256 elements in sequenced complete genomes were identified in December 2016 by NCBI Nucleotide BLAST search employing the IS256 sequence (accession no. [AF535088.1](https://www.ncbi.nlm.nih.gov/nuclot/AF535088.1) [56]) and to this end, all hits were counted, i.e., the insertion that was formed by the recombination of two IS256 elements after head-to-head insertion was counted as two elements. In Fig. 1B, this counts as one insertion site, however, since



both IS256s were inserted at the same site. Multilocus sequence types of *S. aureus* were determined from the published sequences using MLST 1.8 (<https://cge.cbs.dtu.dk/services/MLST/>) (57).

**Accession number(s).** The sequences have been deposited in GenBank with the following accession numbers: for *S. aureus* SA137/93A, [JWMH01000001](https://ncbi.nlm.nih.gov/nucl/JWMH01000001) to [JWMH01000036](https://ncbi.nlm.nih.gov/nucl/JWMH01000036); for *S. aureus* SA137/93G, [JWMG00000000](https://ncbi.nlm.nih.gov/nucl/JWMG00000000) ([JWMG01000001](https://ncbi.nlm.nih.gov/nucl/JWMG01000001) to [JWMG01000041](https://ncbi.nlm.nih.gov/nucl/JWMG01000041)); and for *S. aureus* SA1450/94, [JWMI00000000](https://ncbi.nlm.nih.gov/nucl/JWMI00000000) ([JWMI01000001](https://ncbi.nlm.nih.gov/nucl/JWMI01000001) to [JWMI01000029](https://ncbi.nlm.nih.gov/nucl/JWMI01000029)) (54).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00144-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 3.4 MB.

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