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Teicoplanin Stress-Selected Mutations Increasing σ^B Activity in *Staphylococcus aureus*

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A natural *rsbU* mutant of *Staphylococcus aureus,* unable to activate the alternative transcription factor σ^B via the RsbU pathway and therefore forming unpigmented colonies, produced first-step teicoplanin-resistant mutants upon selection for growth in the presence of teicoplanin, of which the majority were of an intense orange color. By using an *asp23* promoter-luciferase fusion as an indicator, the pigmented mutants were shown to express increased σ^B activity. Increased σ^B activity was associated with point mutations in *rsbW,* releasing σ^B from sequestration by the anti-sigma factor RsbW, or to promoter mutations increasing the σ^B/RsbW ratio. Genetic manipulations involving the *sigB* operon suggested that the mutations within the operon were associated with the increase in teicoplanin resistance. The upregulation of σ^B suggests that a σ^B-controlled gene(s) is directly or indirectly involved in the development of teicoplanin resistance in *S. aureus*. Carotenoids do not contribute to teicoplanin resistance, since inactivation of the dehydrosqualene synthase gene *crtM* abolished pigment formation without affecting teicoplanin resistance. The relevant σ^B-controlled target genes involved in teicoplanin resistance remain to be identified.

Teicoplanin and vancomycin are the drugs of choice against multidrug-resistant methicillin-resistant *Staphylococcus aureus*. Their antibacterial activity is based on the ability to bind the terminal D-alanyl-D-alanine present in the lipid-II-linked peptidoglycan precursor and in peptidoglycan intermediates, thereby inhibiting transglycosylation and transpeptidation of the cell wall. Though both drugs interact with the same target, teicoplanin anchors to the membrane while vancomycin forms dimers to increase its activity (1). Even though teicoplanin is more active than vancomycin against staphylococci (8), resistance to teicoplanin is more easily acquired than resistance to vancomycin. Teicoplanin resistance is believed to precede vancomycin resistance (reviewed in reference 13). In clinical isolates of *S. aureus,* teicoplanin resistance was found to emerge during extended teicoplanin treatment (15), suggesting an in vivo selection for resistant mutants. In contrast to the *van* gene-mediated glycopeptide resistance in enterococci, resistance in *S. aureus* is not due to acquisition of foreign elements but formed endogenously. Analogously, teicoplanin-resistant mutants can be obtained in vitro by step selection for growth on increasing concentrations of the drug. Such in vitro-selected teicoplanin-resistant mutants may have characteristics similar to those of clinical teicoplanin-resistant isolates, allowing their use to study the genes involved in the resistance mechanism. Except for the work of Shlaes et al. (23), who identified a site in the *Sma*I-I fragment of the *S. aureus* chromosome responsible for increase in a 35-kDa protein and PBP 2 production in teicoplanin-resistant *S. aureus,* few genetic studies of teicoplanin resistance have been done.

In the process of infection and disease, *S. aureus* has to adapt to variable external surroundings. One of the triggers responding to environmental stimuli is alternate transcription factors, such as σ^B. The *S. aureus sigB* operon comprises the genes *rsbU, rsbV, rsbW*, and *sigB* (17, 26), which modulate σ^B activity in a sequential fashion (Fig. 1). RsbW acts as an anti-sigma factor by sequestering σ^B through protein-protein interactions, and RsbU controls, via RsbV phosphorylation, the availability of free RsbW to interact with σ^B. The widely used pigmentless laboratory strain NCTC8325 and its descendants are natural *rsbU* mutants (17). They are unable to activate the RsbU-initiated cascade leading to σ^B activity, resulting in low σ^B activity (10). This may have consequences for the mode of stress response. One of the properties of *S. aureus* influenced by the *sigB* operon is pigment formation. The yellow-to-orange color of *S. aureus* colonies stems from triterpennoid carotenoids. Pigment production, although chromosomally encoded, is an unstable characteristic. It is usually found in strains freshly isolated from natural sources or those which are multiply resistant and tends to be lost in stored organisms. Pigmented variants are more resistant to desiccation than nonpigmented ones (11). Pigment formation in *S. aureus* is a multistep procedure, involving regulatory genes and several biosynthetic genes (20), of which *crtMN* catalyze early steps in carotenoid biosynthesis (25). Both the *sigB* operon and the uncharacterized *pig* mutation in NCTC8325 derivatives map in the chromosomal *Sma*I-I fragment (14).

In this study, we identified the *sigB* operon as one of the preferred mutation sites associated with first-step teicoplanin resistance in a pigmentless *S. aureus* strain.

MATERIALS AND METHODS

General methods. All DNA manipulations, basic molecular methods, and handling of *Escherichia coli* were performed in accordance with standard protocols (21). Genetic manipulation of *S. aureus* was done as described earlier (17). The general transducing phage 80α was used for transductions. Sequence data were obtained from the website of The Institute for Genomic Research (http://www.tigr.org).

Strains and growth conditions. The strains used in this study are listed in Table 1. Growth was on Luria-Bertani (LB) agar (Difco) plates at 35°C unless

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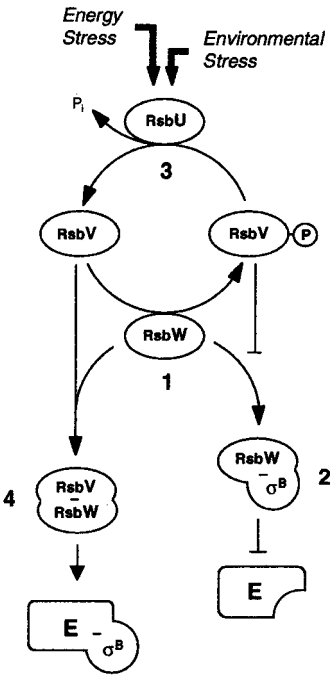

FIG. 1. Proposed model for the regulation of σ^B in *S. aureus* (adapted from references 22 and 24). Based on the known functions of the RsbUVW homologues from *B. subtilis* (reviewed in reference 12), it is assumed that the anti-σ^B protein RsbW from *S. aureus* can form mutually exclusive complexes with either σ^B or its antagonist, RsbV (step 1). RsbV is normally inactive (RsbV-P) due to phosphorylation by RsbW and is thus unable to complex with RsbW, leaving the latter free to interact with σ^B (step 2). When bound to RsbW, σ^B is unable to aggregate with the RNA polymerase core enzyme (E) to form an active holoenzyme (E-σ^B). Upon stress, the RsbV-P-specific phosphatase activity of RsbU, a positive activator of σ^B, becomes activated and thus reactivates RsbV (step 3). Unphosphorylated RsbV interacts and complexes highly specifically with RsbW (step 4), thereby releasing σ^B. RsbW, if complexed with RsbV, is unable to bind to σ^B, leaving the latter free to form an active σ^B-holoenzyme (E-σ^B). Even though the exact mode of activation of RsbU in *S. aureus* remains unclear, there is evidence that its activation differs substantially from that of the RsbU homologue in *B. subtilis*.

otherwise specified. MIC determinations for antibiotics were performed with the Etest from AB-Biodisk (Solna, Sweden) in accordance with the NCCLS guidelines on Mueller-Hinton agar (Difco) plates with an inoculum of 0.5 McFarland standard. Additionally, MICs of teicoplanin were determined on brain heart infusion (BHI) (Difco) plates as recommended by AB-Biodisk (Etest Technical Guide 11: Etest Application Sheets) with an inoculum of 2 McFarland standard and incubation at 35°C for 48 h. Resistance levels were compared on rectangular plates containing an antibiotic gradient by swabbing a 0.5-McFarland-standard suspension of an overnight culture along the gradient. The antibiotic used for selection of transductants was either erythromycin (20 μg ml^{-1}) or tetracycline (5 μg ml^{-1}). Population analysis profiles were done by plating 0.1 ml of appropriate dilutions of overnight cultures on LB agar plates containing increasing concentrations of teicoplanin and incubating them for 48 h.

Selection of first-step teicoplanin-resistant mutants. First-step teicoplanin-resistant mutants were selected by plating 0.1 ml of serial 10-fold dilutions of an overnight culture in Luria broth on LB agar plates containing increasing concentrations of the antibiotic. After a 48-h incubation, single colonies appearing at the highest concentration were purified on sheep blood agar without a selective agent and kept for further use.

Generation of GP266. Electroporation of plasmid pPG11 (Table 1), carrying a functional *sigB* operon and a *tet*(L) gene cassette downstream of the *sigB* operon (10), into RN4220 Δ*rsbUVWsigB* and screening for double-crossover transformants sensitive to erythromycin and resistant to tetracycline yielded, among others, the unpigmented strain GP266. Strain GP266 carries an amber mutation due to a 2-bp deletion in the 5′ part of *sigB* (corresponding to positions 2932 to 2933 [accession number Y07645]), which must have occurred accidentially upon selection. Double-crossover integration of the *sigB* operon, including the *tet*(L) cassette, was confirmed by Southern blot analysis. The inability of GP266 to produce σ^B was confirmed by Western blot analysis using anti-σ^B antibodies as probes (data not shown). The *tet*(L)-tagged *sigB1*(Am) mutation of GP266 was used to transduce *sigB1*(Am) into different genetic backgrounds.

Construction of plasmid pBT*crtM* used for insertional inactivation. An internal 510-bp *crtM* fragment containing a *Hin*dIII restriction site was generated by PCR using the upstream primer 5′-*CAATATAGGAGGACTAGTATGAC*-3′ and the downstream primer (5′-GGAATTC*CAACGATTCACCAAGTCTTCTTGC G*-3′), including an *Eco*RI linker, with the italic nucleotides corresponding to positions 211 to 233 and 696 to 720 of the sequence accession no. X73889, respectively. The PCR product was digested with *Hin*dIII and *Eco*RI and cloned into the suicide plasmid pBT (13). The plasmid obtained was transformed by electroporation into RN4220 and subsequently transduced into the strains MB137 and MB140.

Construction of an *E. coli* vector for overexpression of His-tagged RsbW and generation of anti-RsbW antibodies. A DNA fragment encoding 486 bp of the *rsbW* gene was amplified by PCR using the *Nde*I linker containing the primer 5′-GGAGATATACAT*ATGCAATCTAAAGAAGATTTTATCG*-3′ and the *Xho*I linker containing the primer 5′-GGTGGTGCTC*GCTGATTTCGACTCTTTCG C*-3′, with the italic nucleotides corresponding to positions 2210 to 2234 and 2677 to 2696 of the sequence accession no. Y07645, respectively. The PCR product was cloned into pET24b to obtain pET*rsbW*. The junction regions including the PCR product were sequenced to ensure proper ligation and fidelity of the PCR. *E. coli* strain BL21 (DE3) was transformed with the plasmid obtained. Overexpression and purification of the His-tagged protein was performed using Ni-nitrilotriacetic acid columns (Qiagen, Basel, Switzerland) according to the recommendations of the manufacturer. The purified protein was separated using sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis, and bands containing the protein were cut out of the gels. N-terminal sequencing confirmed the identity of the desired protein. The gel slices containing the protein were injected into rabbits to raise anti-RsbW polyclonal antibodies (BioScience, Göttingen, Germany). The resulting antisera were purified against the immobilized antigen.

Luciferase assay. Bacterial cells from overnight cultures containing the appropriate antibiotic were diluted in fresh drug-free Luria broth to an an optical density at 600 nm (OD_{600}) of 0.01 and grown at 37°C and 200 rpm. The cells were harvested at different time points by centrifugation at 11,000 × *g* for 1 min at room temperature, and the cell pellets were resuspended in 0.1 M sodium phosphate buffer (pH 7.0) to an OD_{600} of 10. Luciferase activity was then determined by rapidly mixing the resuspended cells (10 μl) with an equal volume of luciferase assay reagent (Promega, Madison, Wis.). Luminescence was measured on a Tumer Designs TD-20/20 luminometer (Promega) for 10 s with a delay of 2 s.

RESULTS

Selection of spontaneous first-step mutants with decreased teicoplanin susceptibilities. The susceptible strain MB33 (10), derived from NCTC8325, showed heterogeneous susceptibility on plates containing increasing concentrations of teicoplanin and was able to form a few colonies in the presence of up to 8 μg ml^{-1} of teicoplanin (Fig. 2). While the parental strain was white, more than half of the colonies growing at concentrations over 4 μg ml^{-1} of teicoplanin produced an intense orange pigmentation. Propagation of those colonies on nonselective plates showed that they retained pigment formation and increased teicoplanin resistance (Fig. 2, strain MB128). Pigment formation in NCTC8325 derivatives, which are natural *rsbU* mutants, is known to be restored by overexpression of σ^B (18). Strain MB33, which harbors a chromosomally integrated reporter system that allows monitoring of the activity of σ^B (10), was therefore used in these experiments. First-step teicoplanin-resistant mutants arose with a frequency of 1.3×10^{-7} when MB33 was plated on 7 μg of teicoplanin ml^{-1}. White mutants appeared after 24 h; orange mutants appeared only

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype[a]	Reference or source
Strains		
E. coli		
DH10B	F$^-$ ϕ80d*lacZ*ΔM15 *recA1*	Gibco
BL21 (DE3)	F$^-$ *ompT gal [dcm] [lon] hsdS*$_B$ (r_B^- m_B^-); with DE3	Novagen
S. aureus		
RN4220	NCTC8325-4 r m$^+$ (restriction minus; modification plus); *rsbU*	16
MSSA1112	Clinical isolate; *bla rsbU*$^+$	7
Newman	ATCC25904; clinical isolate; high level of clumping factor; *rsbU*$^+$	6
GP266	RN4220; *rsbU*$^+$ *sigB1*(Am) Tc^r	This study
GP268	BB255; *rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$ Tc^r	10
MB33	BB255; *rsbU asp23*$^+$ *asp23p*::pEC*asp23p-luc*+ Em^r	10
MB49	GP268; *asp23*$^+$ *asp23p*::pEC*asp23p-luc*+ Em^r Tc^r	10
MB51	RN4220; *crtM*::pBT*crtM* Tc^r	This study
MB118	MB33; *rsbU rsbW1* Em^r	This study
MB119	MB33; *rsbU rsbW2*(Am) Em^r	This study
MB127	MB33; *rsbU rsbW3* Em^r	This study
MB128	MB33; *rsbU rsbW4* Em^r	This study
MB130	MB33; *rsbU rsbW5*(Am) Em^r	This study
MB132	MB33; *rsbU rsbW6* Em^r	This study
MB137	MB33; *rsbU sigB2* Em^r	This study
MB138	MB33; *rsbU rsbW7* Em^r	This study
MB140	MB33; *rsbU chrX1* Em^r	This study
MB148	MB33; *rsbU rsbW8* Em^r	This study
MB158	MB137; *rsbU*$^+$ *sigB1*(Am) Em^r Tc^r	This study
MB159	MB137; *rsbU crtM*::pBT*crtM* Em^r Tc^r	This study
MB161	MB140; *rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$ Tc^r Em^r	This study
MB162	MB140; *rsbU*$^+$ *sigB1*(Am) Em^r Tc^r	This study
MB163	MB140; *rsbU crtM*::pBT*crtM* Em^r Tc^r	This study
MB213	MB137; *rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$ Em^r Tc^r	This study
MB215	MB119; *rsbU*$^+$ *sigB1*(Am) Em^r Tc^r	This study
Plasmids		
pBC SK(+)	Cm^r; *E. coli* cloning vector	Stratagene
pBT	Tc^r; 1.6-kb PCR fragment of *tet*(L) from pHY300PLK into *Alw*261-digested pBC SK(+)	10
pEC1	Ap^r Em^r; 1.45-kb *Cla*I *erm*(B) fragment of Tn*551* in pUC18	4
pET-24b(+)	Km^r; *E. coli* expression vector	Novagen
pSP-*luc*+	Ap^r; firefly luciferase casette vector	Promega
pPG11	Ap^r Tc^r; 6.6-kb *Pst*I-*Eco*RI *sigB* fragment from strain BB255, including a 252-bp *Mlu*I-*Bst*XI fragment of *rsbU* from strain COL replacing the corresponding region of the *sigB* operon from strain BB255 and carrying a 1.6-kb PCR fragment of *tet*(L) from pHY300PLK integrated into a blunted *Bsp*1191 site downstream of *sigB* in pUC19	10
pET*rsbW*	Ap^r; 486-bp PCR fragment of *rsbW* from strain BB255 in pET24b(+)	This study
pSP*asp23p*	Ap^r; 1.1-kb fragment of the *asp23* promoter from strain COL into pSP-*luc*+	10
pEC*asp23p-luo*+	Em^r; 2.7-kb *Kpn*I-*Eco*RI *asp23p-luc*+ fragment of pSP *asp23p* in pEC1, *S. aureus* integration vector that inserts into the *asp23* promoter *(asp23p)*	10
pBT*asp23p-luc*+	Tc^r; 2.7-kb *Kpn*I-*Eco*RI *asp23p-luc*+ fragment of pSP*asp23p* in pBT, *S. aureus* integration vector that inserts into the *asp23* promoter *(asp23p)*	10
pBT*crtM*	Tc^r; 510-bp PCR fragment of *crtM* in pBT, *S. aureus* integration vector that inserts into *crtM*	This study

[a] Abbreviations are as follows: Am, amber mutation; Ap^r, ampicillin resistant; *chrX1*, unmapped mutation; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant.

after 48 h. The ratio of white to orange mutants was approximately 1:3. The teicoplanin MICs for those mutants ranged between 6 and 12 μg ml^{-1} on Mueller-Hinton agar and between 24 and 32 μg ml^{-1} on BHI agar (Table 2). The pigmented mutants were generally slightly more resistant than the white ones, as shown on the teicoplanin gradient plate in Fig. 3. Increase in resistance was specific for glycopeptides and more pronounced for teicoplanin than for vancomycin (Table 2). The MICs of other, unrelated antibiotics, such as methicillin, oxacillin, cefoxitin, imipenem, ciprofloxacin, kanamycin, erythromycin, rifampin, and tetracycline, seemed not to be affected (data not shown).

Genetic analyses of first-step mutants. Sequencing of the *rsbVWsigB* gene region of orange-pigmented mutants revealed nine independent point mutations (Table 3). Two mutants, MB128 and MB148, carried mutations in the putative ribosome-binding site of *rsbW,* while four mutants, MB118, MB127, MB138, and MB139, had single-nucleotide exchanges in the *rsbW* gene, resulting in amino acid exchanges. One mutation, *rsbW2*(Am) of mutant MB119, resulted in a stop codon, while another mutation, *rsbW5*(Am) of mutant MB130, led to a frame shift within the *rsbW* open reading frame, introducing a premature stop codon. A single orange mutant, MB137, had an intact *rsbW* gene but a point mutation in *sigB,*

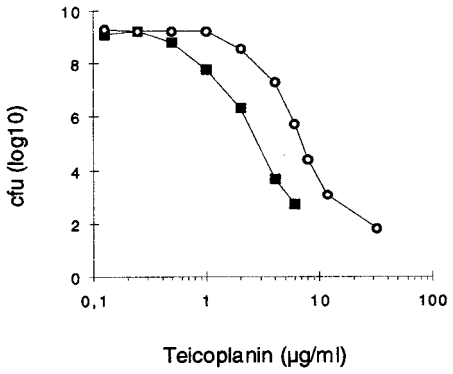

FIG. 2. Population analysis profiles. Colonies formed from overnight cultures of the parent, MB33 (squares), and its teicoplanin first-step mutant MB128 (circles) were plated on LB agar plates containing increased concentrations of teicoplanin.

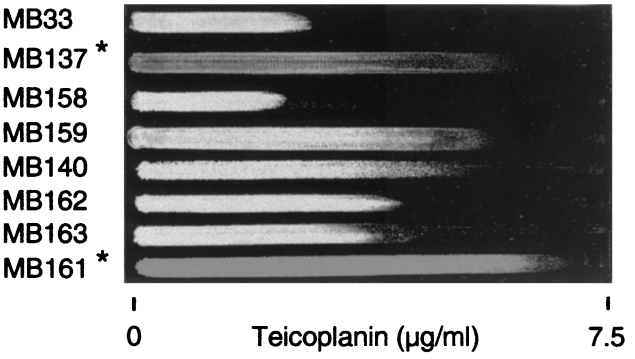

FIG. 3. Teicoplanin gradient plate. Suspensions (0.5 McFarland standard) of overnight cultures were swabbed on an LB agar plate along an antibiotic gradient as indicated. Growth was monitored after 24 h of incubation. The asterisks indicate pigmented strains. For a detailed description of the strains, refer to Table 1.

leading to an amino acid exchange that did not, however, affect the ability of σ^B to interact with the RNA polymerase core enzyme. White first-step mutants had intact *rsbVW* and *sigB* genes.

σ^B activity. The *asp23* gene, encoding a protein of yet-unknown function that was shown to be highly expressed upon alkaline stress and heat shock (9, 19), is preceded by three tandem σ^B consensus promoters that are known to be under the sole control of σ^B (10). The *asp23* promoters were found to be suitable for monitoring σ^B activity by reporter gene fusion experiments using the firefly luciferase gene *luc*+ as a reporter gene (10). Experiments with derivatives of wild-type strains, such as Newman and MSSA1112, carrying this reporter system integrated in their genomes, as well as strain MB49, showed that σ^B activity increased during the exponential growth phase, reaching a maximal activity during the late exponential growth phase (OD_{600}, 2 to 4), which was followed by a significant decrease thereafter (10). In contrast, in *rsbU* strains, such as MB33, σ^B activity remained low throughout the growth cycle (10) (Fig. 4). All pigmented teicoplanin-resistant mutants derived from MB33, represented in Fig. 4 by strains MB130 and MB137, showed σ^B to be highly induced during the early exponential growth phase, reaching a maximal activity at an OD_{600} of 1, followed by a strong decrease thereafter. In all orange teicoplanin-resistant mutants, the maximal activities of σ^B were drastically higher than in the *rsbU*-negative parent,

TABLE 2. Phenotypic properties of different *S. aureus* strains

Strain	Relevant genotype[a]	MIC (μg/ml)[b]				σ^B activity[c]	Color
		Teicoplanin		Vancomycin			
		MH	BHI	MH	BHI		
MB33	BB255; *asp23p*::pEC*asp23p-luc*+	2	6	2	4	34.0 ± 3.00	White
MB49	MB33; *rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$	4	8–12	3	4–6	301.3 ± 23.8	Orange
MB118	MB33; *rsbW1*	8	24	3	6	632.5 ± 13.8	Orange
MB119	MB33; *rsbW2*(Am)	12	38	4–6	8	609.0 ± 43.5	Orange
MB127	MB33; *rsbW3*	12	32	4–6	8	760.3 ± 52.8	Orange
MB128	MB33; *rsbW4*	6	24	3	6	933.8 ± 99.5	Orange
MB130	MB33; *rsbW5*(Am)	8	24	4	8	869.5 ± 74.5	Orange
MB132	MB33; *rsbW6*	8	32	4	8	804.0 ± 70.5	Orange
MB137	MB33; *sigB2*	12	32	4	8	671.0 ± 35.5	Orange
MB138	MB33; *rsbW7*	12	32	3–4	8	1,084.0 ± 46.5	Orange
MB140	MB33; *chrX1*	8	16	3	6	31.2 ± 2.80	White
MB148	MB33; *rsbW8*	12	32	4	8	883.8 ± 70.3	Orange
MB158	MB137; *sigB1*(Am)	2	4	2	4	0.59 ± 0.08	White
MB159	MB137; *crtM*::pBT*crtM*	12	32	4	8	704.8 ± 43.8	White
MB161	MB140; *rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$	18	32	3	8	315.3 ± 24.3	Orange
MB162	MB140; *sigB1*(Am)	8	16	3	6	0.66 ± 0.07	White
MB163	MB140; *crtM*::pBT*crtM*	8	16	3	6	41.6 ± 3.80	White
MB213	MB119; *rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$	4	8–12	3	4–6	313.0 ± 12.8	Orange
MB215	MB119; *sigB1*(Am)	2	4	2	4	0.57 ± 0.09	White
Newman	*rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$	2	6	2	4	354.5 ± 31.5	Yellow
MSSA1112	*rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$	1.5	4	1.5	6	272.4 ± 27.3	Yellow

[a] Detailed relevant genotypes and phenotypes are listed in Table 1; exact mutation sites are listed in Table 3. *chrX1,* unmapped mutation.
[b] MICs were obtained using Etest as outlined in Materials and Methods. MH, Mueller-Hinton agar.
[c] σ^B transcriptional activity (relative light units) was determined from cells grown to an OD_{600} of 1.5 by measuring the luciferase activity of Luc+, the product of the *luc*+ reporter gene fused to the σ^B-dependent promoters of *asp23 (asp23p)*. The values shown are the results of four independent assays.

TABLE 3. Mutation sites in orange-pigmented first-step mutants

Strain	Allele	Mutation[a]	Remarks
MB118	*rsbW1*	A2350T	Amino acid exchange K44M[b] in RsbW
MB119	*rsbW2*(Am)	C2525G	Amino acid exchange Y102stop[b] in RsbW
MB127	*rsbW3*	C2375G	Amino acid exchange T52R[b] in RsbW
MB128	*rsbW4*	G2210A	Mutation in putative ribosome-binding site
MB130	*rsbW5*(Am)	2527T	Frame shift mutation by T insertion in RsbW
MB132	*rsbW6*	G2222T	Amino acid exchange M1I[b] in RsbW
MB137	*sigB2*	G3115A	Amino acid exchange E148K[c] in σ^B
MB138	*rsbW7*	G2287A	Amino acid exchange R23H[b] in RsbW
MB148	*rsbW8*	G2209T	Mutation in putative ribosome-binding site

[a] Position of mutation in the *sigB* operon (accession number Y07645).
[b] Position of the amino acid exchange in RsbW (accession number CAA68931).
[c] Position of the amino acid exchange in σ^B (accession number CAA68932).

MB33, irrespective of the mutation site (Table 2). σ^B activities were still over twofold higher and were induced in an earlier growth phase than in strains carrying the *rsbU* wild-type allele (Table 2 and Fig. 4). In contrast, the σ^B activities of white first-step mutants, represented by MB140, were as low as those of their parental strains.

Western blot analyses of RsbW and σ^B. The content of RsbW and σ^B was determined by analysis of Western blots from cytoplasmic extracts of cells harvested at an OD_{600} of 1.5 (Fig. 5). The differences either in the content or in the ratio of σ^B and RsbW were remarkable. The parent, MB33, as well as the white teicoplanin-resistant mutant MB140 produced RsbW and σ^B in significantly smaller amounts than their *rsbU*$^+$ relative MB49. Strains such as MB138, harboring a point mutation in RsbW, produced both proteins in a ratio comparable to that of their parent. Strain MB128, carrying a mutation in the proposed ribosome-binding site of *rsbW,* also produced both proteins but showed a significantly higher σ^B/RsbW ratio than the parent. Only very little RsbW was found in the protein fraction of MB132, which had an isoleucine instead of the start methionine, and no RsbW at all could be seen in the protein fractions of MB119 and MB130, harboring mutations in *rsbW* that lead to premature stop codons. Those mutants produced significantly smaller but still detectable amounts of σ^B. Finally, the σ^B in the *sigB* mutant MB137 was abundant and migrated slightly faster than wild-type σ^B but was still active, as seen in Table 2.

Genetic manipulations showing the influence of the *sigB* operon on teicoplanin resistance. Transfer of the *rsbU* wild-type allele into the white parent, MB33, resulted in the orange-pigmented strain MB49 with an intact *sigB* operon (10). This strain possessed a slightly increased teicoplanin resistance, though not as high as in the first-step mutants. Teicoplanin MICs obtained for other *rsbU* wild-type strains, such as Newman and MSSA1112, were in the range of MB33 (Table 2).

Replacement of the active *sigB2* allele in the orange first-

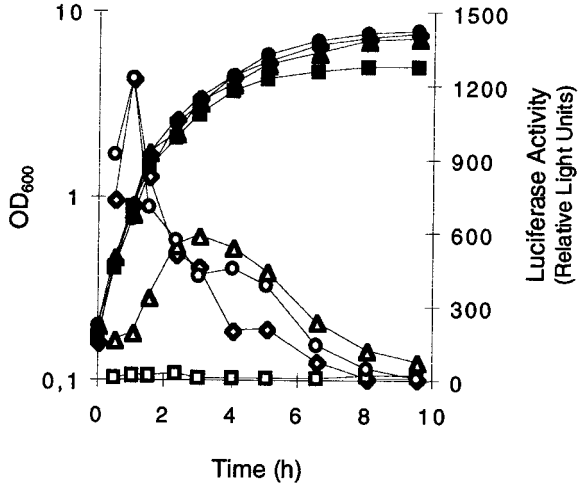

FIG. 4. σ^B activities of different *S. aureus* strains during growth. The expression profiles of *asp23p*::*luc*+ during growth of different *S. aureus* strains, grown in LB medium at 37°C, are shown. Bacterial growth was measured as the OD_{600} (solid symbols). σ^B transcriptional activity was determined by measuring the luciferase activity of Luc+ (open symbols), the product of the *luc*+ reporter gene fused to the σ^B-dependent promoters of *asp23 (asp23p)*. Squares, parental strain MB33 *(rsbU);* triangles, MB49 (MB33 *rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$); circles, MB130 [MB33 *rsbW5*(Am)]; diamonds, MB137 (MB33 *sigB2*).

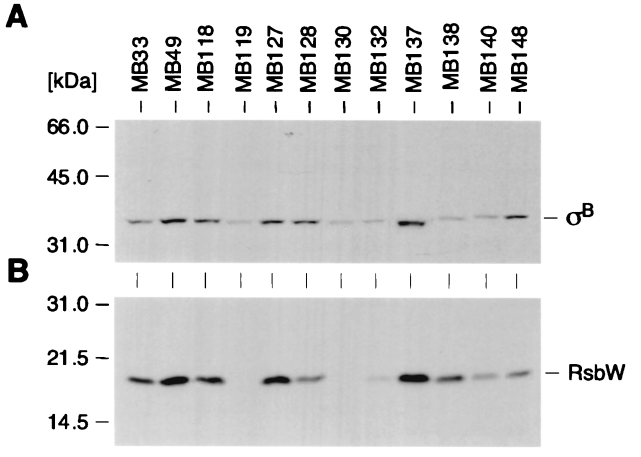

FIG. 5. Western blot analyses of RsbW and σ^B. Cytoplasmic protein fractions (10 μg/lane) of different *S. aureus* strains, obtained from cells grown to an OD_{600} of 1.5, were separated using sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and blotted onto nitrocellulose. The blotted proteins were stained with amido black prior to hybridization to ensure equal loading and were subjected to Western blot analyses using either antigen-purified anti-σ^B antibodies (A) or anti-RsbW antibodies (B). The broad-range molecular-weight marker (Gibco-BRL) was used as a size marker. Relevant protein signals are indicated. For a detailed description of the strains, refer to Table 1.

step mutant MB137 by the defective *sigB1*(Am) allele, as well as transduction of *sigB1*(Am) into the orange mutant MB119, yielded the white teicoplanin-susceptible strains MB158 and MB215, respectively, showing virtually no σ^B activity (Table 2). In contrast, inactivation of *sigB* in the white teicoplanin-resistant mutant MB140 resulted in the white mutant MB162, for which the teicoplanin MIC was the same as that of its parent, as expected (Table 2 and Fig. 3).

Introduction of an intact *sigB* operon into MB140 enhanced the resistance and increased σ^B activity 10-fold, as seen in the resulting orange mutant, MB161 (Table 2 and Fig. 3). However, when the wild-type *sigB* operon was introduced into the resistant orange mutant MB119, which overproduced σ^B, the resulting strain, MB213, with a wild-type *sigB* operon, possessed a resistance level and σ^B activity corresponding to those of MB49 (Table 2).

Inactivation of carotenoid biosynthesis genes. To analyze the impact of carotenoids on teicoplanin resistance and to distinguish it from the effects of the *sigB* operon, *crtM,* encoding an early step in *S. aureus* carotenoid biosynthesis, was inactivated by insertional inactivation in the pigmented first-step mutant MB137, as well as in the white mutant MB140, resulting in strains MB159 and MB163, respectively. The inactivation of *crtM* resulted in loss of pigment formation in MB137. Resistance to teicoplanin was not affected by *crtM* in either of the strains (Fig. 3), showing that the increase in σ^B activity was the primary responsible effector in teicoplanin resistance.

DISCUSSION

In staphylococci, teicoplanin resistance is assumed to be acquired endogenously in a stepwise manner through mutation and selection after exposure to the glycopeptide (15). Here, we identified the *sigB* operon as the preferred mutation site leading to first-step teicoplanin resistance in an *rsbU* mutant. Mutations within the *sigB* operon were associated with excessively high σ^B activities and resulted in teicoplanin MICs two- to sixfold-higher than that for the parental strain (Table 2), classifying all first-step mutants as teicoplanin intermediate resistant according to the interpretive standards of the NCCLS.

Increased resistance was associated in most cases with a single-base-pair mutation (Table 3). All mutants, regardless of the individual mutation site or kind of mutation within the *sigB* operon, showed essentially the same approximately 20-fold increase in σ^B activity in addition to the increase in the MIC. The increase in resistance was significantly higher for teicoplanin than for vancomycin, suggesting that the relevant σ^B-controlled gene product(s) interfered more efficiently with teicoplanin than with vancomycin. The σ^B activities and MICs clearly exceeded those of the *rsbU* wild-type strains (Table 2), implying that excessive σ^B activity enhances the teicoplanin resistance level of *S. aureus*. However, the slightly higher teicoplanin MICs observed for MB49 did not reflect the usual teicoplanin susceptibilities found for other *rsbU* wild-type strains, such as Newman or MSSA1112, which ranged between 1.5 and 2 μg/ml on Mueller-Hinton agar (Table 2). This may suggest that the original *rsbU* deletion gave rise to second-site mutations or that transduction brought in other genes that may affect susceptibility to teicoplanin. Analogously, in strain BB938, a teicoplanin-resistant transformant, the *sigB* operon was also identified as the causative agent for increased resistance, but a second cotransducible locus involved in teicoplanin resistance could not be ruled out completely (3).

Conceivable ways to increase σ^B activity in *S. aureus* are mutations resulting in the uncoupling of σ^B from its antagonistic protein, RsbW. Accordingly, we identified most of the mutations within the *rsbW* structural gene (Table 3), which either (i) suppressed the translation of RsbW, (ii) significantly altered the length of the open reading frame of RsbW, or (iii) resulted in the loss of function of RsbW. Inactivation of *sigB* in orange-pigmented first-step mutants resulted in unpigmented strains that, along with loss of σ^B activity, lost the increased resistance against teicoplanin. Transfer of an intact *sigB* operon into such an orange mutant yielded an orange-pigmented strain possessing a teicoplanin resistance level similar to that found for the *rsbU*$^+$ derivative MB49. These data strongly suggest that except for the mutation leading to increased σ^B activity no further unlinked mutation contributed to teicoplanin resistance in those strains. While increased σ^B activity constituted the majority of mutants, other pathways leading to first-step teicoplanin resistance are possible, as shown by the white mutant MB140. Deletion of σ^B in MB140 did not affect the teicoplanin resistance level (Table 2 and Fig. 3), indicating that the mutation conferring teicoplanin resistance in MB140 was not linked to the *sigB* operon. The introduction of an *rsbU* wild-type allele into MB140 had an additive effect on teicoplanin resistance (Table 2 and Fig. 3), implying that a functional *sigB* operon contributes positively to the teicoplanin resistance levels of *S. aureus* NCTC8325 derivatives.

In *S. aureus,* carotenoid biosynthesis is dependent on the activity of σ^B, so that high σ^B activity results in strong pigmentation (10). All mutations in the *sigB* operon leading to increased σ^B activity produced in perfect correlation a strong orange pigmentation. By inactivation of *crtM,* a gene encoding dehydrosqualene synthase, an early step in the carotenoid biosynthesis of *S. aureus*, we could rule out the possibility that the increased pigment content itself enhances teicoplanin resistance, since the unpigmented *crtM* mutant MB159 was found to be as resistant as its orange-pigmented donor (Fig. 3). Among the multiple σ^B-activated genes, those responsible for modulation of teicoplanin resistance still need to be identified. The *sigB* operon may well have been the site in *Sma*I-I identified by Shlaes et al. (23) and postulated to control teicoplanin resistance.

Glycopeptide resistance of *S. aureus* has been associated in some but not all clinical isolates with thickened cell walls and increased proportions of glutamine-nonamidated muropeptides (5), PBP 2 overproduction, and enhanced production of a 35-kDa membrane protein of still-unknown function (13, 23). In none of the *sigB* operon mutants investigated could we identify an increase either in PBP 2 or the 35-kDa protein (data not shown).

Besides their role in teicoplanin resistance, such mutations within the *sigB* operon are of additional interest, as they provide us with new insights and information concerning the regulation and functional sites of σ^B and its regulators in *S. aureus*. Single-base-pair mutations that result in a single-amino-acid exchange and that are sufficient to abolish the ability of

RsbW to regulate σ^B activity suggest that such amino acid residues are important for RsbW function. The finding that mutants carrying an amber mutation in *rsbW* produced only small amounts of σ^B, even though transcription of *rsbUVWsigB* was found to be highly abundant and no alterations in the *sigB* gene itself were uncovered in such strains (data not shown), strongly suggest translational coupling of RsbW and σ^B in *S. aureus*. It is noteworthy that a similar situation has been proposed for the RsbW and σ^B homologues of *Bacillus subtilis* (2). In the absence of its antagonist, RsbW, even small amounts of σ^B are likely to be sufficient to produce high σ^B activity. The strong decrease in σ^B activity observed in orange-pigmented first-step mutants (Fig. 4), beginning from the mid-exponential growth phase, suggests that a further, as-yet-unidentified negative regulator of σ^B is present in *S. aureus*. The unexpected finding that σ^B activity also decreases in MB137 (Fig. 4), harboring the mutation in *sigB,* suggests that the proposed negative regulator interacts with σ^B in a way that is different from that of RsbW, as its ability to interact with σ^B is not inhibited by the *sigB2* mutation.

Taken together, this system provides us with an elegant means for functional studies by generating mutations increasing σ^B activity. These mutations are favored because of the *rsbU* background, and hence low σ^B activity, in the original strain used. It remains an open question if and to what extent such mutations would also occur in *S. aureus* strains harboring a functional *sigB* operon.

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