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Sigma-B, a Putative Operon Encoding Alternate Sigma Factor of *Staphylococcus aureus* RNA Polymerase: Molecular Cloning and DNA Sequencing

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We have identified a gene cluster located on the chromosomal *Sma*I I fragment of a highly methicillin resistant strain of *Staphylococcus aureus*, consisting of four open reading frames (ORFs), named after the number of deduced amino acid residues, in the sequential order orf333-orf108-orf159-orf256. The gene cluster showed close similarities to the *Bacillus subtilis sigB* operon both in overall organization and in primary sequences of the gene products. The complete gene cluster (provisionally named sigma-B or *sigB*) was preceded by an σ^A-like promoter (P_A) and had an internal σ^B-like promoter sequence (P_B) between orf333 and orf108, suggesting a complex regulatory mechanism. The polypeptides encoded by orf333, -108, -159, and -256 showed 62, 67, 71, and 77% homologies, respectively, with the RsbU, RsbV, RsbW, and SigB polypeptides encoded by the *B. subtilis sigB* operon. A Tn*551* insertional mutant, RUSA168 (insert in orf256 of the staphylococcal sigma-B operon), showed drastic reduction in methicillin resistance (decrease in MIC from 1,600 μg ml^{-1} to 12 to 25 μg ml^{-1}).

Bacteria have evolved adaptive networks to face the challenges of changing environment and to survive under conditions of stress (27). One of the stress conditions most frequently encountered by bacteria is a suboptimal nutritional milieu, leading to a stationary phase of growth. Drastic changes in cellular physiology and morphology accompany the onset of bacterial stationary phase: there are structural changes in the cell envelope, differences in DNA supercoiling and compactness, synthesis of storage compounds and protective substances, modification of DNA polymerase core, etc. (12). The molecular mechanisms of these changes require expression of a number of new genes, and the expression of many of these genes is controlled by the association of alternative sigma factors with the catalytic core of RNA polymerase (10, 11, 15, 16). The alternative or minor sigma factors are activated early in the stationary growth phase to confer different promoter recognition specificities on the polymerase holoenzyme and reprogram the pattern of gene expression in response to environmental signals (11). No alternative sigma factor has so far been identified in *Staphylococcus aureus*, and we are not aware of any reports in the literature describing the involvement of stress response genes in the expression of antibiotic resistance. In this report, we describe a chromosomal gene cluster in a methicillin-resistant strain of *S. aureus*, the gene products of which are highly homologous with those of the sigma-B operon in *Bacillus subtilis* (27), an operon that is believed to define an alternative sigma factor regulating bacterial metabolism in response to environmental stress in the stationary phase (3, 4, 8). Insertional inactivation by transposon Tn*551* in this region of the staphylococcal chromosome has generated a group of mutants (RUSA168, RUSA150, and RUSA122) with drastic reduction in resistance to methicillin, suggesting that an intact stress response system is essential for the optimal expression of antibiotic resistance in these bacteria.

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MATERIALS AND METHODS

Bacterial strains, phage, and plasmids. The bacterial strains, phage, and plasmids used in this study are described in Table 1.

Media and growth conditions. *S. aureus* and methicillin-resistant *S. aureus* mutants were grown in tryptic soy broth (Difco Laboratories) with aeration as described previously (18). Luria-Bertani medium was used to propagate *Escherichia coli* DH5α, and ampicillin (100 μg ml^{-1}) was added for selection and maintenance of the plasmids listed in Table 1. *E. coli* XL1-Blue MRA and MRA(P2) were the host cells for Lambda DASHII phage. They were cultured as recommended by the supplier (Stratagene Cloning Systems, La Jolla, Calif.).

Antimicrobial susceptibility testing. Overnight cultures grown in tryptic soy broth in which various dilutions of the bacterial cultures were plated at 37°C with aeration were used for testing the expression of methicillin resistance, using the method of population analysis in which various dilutions of the bacterial cultures are plated on tryptic soy agar containing increasing concentrations of methicillin (5). Colonies were counted after incubation at 37°C for 48 h.

DNA methods. All routine DNA manipulations were performed as described in reference 21 and 1. Restriction enzymes, calf intestine alkaline phosphatase, and T4 DNA ligase were purchased from New England Biolabs, Inc., and used as recommended by the manufacturer. Southern analysis was performed with ECL random prime labeling and detection systems (Amersham Life Science) as recommended by the manufacturer.

DNA sequence analysis. Double-stranded DNA sequencing was accomplished by the dideoxy-chain termination method (22) with templates of DNA fragment cloned in pGEM-3Z. The oligonucleotide primers were synthesized and purified by Genosys Biotechnologies, Inc. Sequenase 2.0 (United States Biochemical) was used for chain elongation, and [^{35}S]dATP-labeled samples were run in 8 M urea–6% polyacrylamide gels. Nucleotide and derived amino acid sequences were analyzed with the Wisconsin Genetics Computer Group software.

RESULTS

Reduction of methicillin resistance in Tn*551* insertional mutants RUSA122, -150, and -168. The insertional mutants had drastically reduced resistance to methicillin. The MICs of Tn*551* mutants RUSA168, -122, and -150 decreased from the MIC of the parental strain (1,600 μg ml^{-1}) to 25, 50, and 50 μg ml^{-1}, respectively (5).

Cloning the Ω727/729 region. The Tn*551* insertion sites Ω727 and Ω729, which generated the insertional mutants

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TABLE 1. Strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Relevant characteristics[a]	Origin or reference
Strains		
E. coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1* ϕ80 d*lacZ*ΔM15	Bethesda Research Laboratories
XL1-Blue MRA	Δ(MCRA) 183Δ(MCRCB-HSD SMR-*mrr*)*173 endA1 supE44 thi-1 gyrA96 relA1 lac*	Stratagene
XL1-Blue MRA(P2)	XL1-Blue MRA(P2 lysogen)	Stratagene
S. aureus		
COL	Homogeneous Mc^r	Laboratory collection
RUSA168	COL Ω727(*sigB*::Tn*551*) Em^r heterogeneous Mc^r	5
RUSA122	COL Ω729(*orf333*::Tn*551*) Em^r heterogeneous Mc^r	5
Phages		
Lambda DASHII	λ*sbh*λ1° b189 KH54 *chiC* srIλ4° *nin5* shndIIIλ6° srIλ5° *red*$^+$ *gam*$^+$	Stratagene
λDII/R168	Lambda DASHII/15-kb *Eco*RI fragment from RUSA168(*sigB*::Tn*551*)	This study
λDII/R122	Lambda DASHII/15-kb *Eco*RI fragment from RUSA122(*orf333*::Tn*551*)	This study
Plasmids		
pGEM-3Z	Subcloning vector Amp^r	Promega Corp.
pRT1	pGEM-1/4.0-kb *Xba*I-*Hpa*I fragment of Tn*551*	17
pSW-2	pGEM-3Z/5.9-kb *Sal*I fragment from λDII/R122(Tn*551*J$_R$::Ω729 right flanking)	This study
pSW-7	pGEM-3Z/4.1-kb *Pst*I-*Ava*I fragment from λDII/R122(Tn*551*J$_L$::Ω729 left flanking)	This study
pSW-7A	pGEM-3Z/1.6-kb *Hin*dIII-*Ava*I fragment from λDII/R122(Tn*551*J$_L$::Ω729 left flanking)	This study
pSW-11	pGEM-3Z/3.3-kb *Sal*I-*Eco*RV fragment from λDII/R168(Tn*551*J$_R$::Ω727 right flanking)	This study

[a] Mc^r, methicillin resistance; Em^r, erythromycin resistance; Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

RUSA168 and RUSA122, respectively, were found to be located on a 9.8-kb *Eco*RI DNA fragment of the COL chromosome (5). The 15-kb *Eco*RI fragments including transposon Tn*551* were purified from strains RUSA168 and RUSA122 and then cloned into the Lambda DASHII/*Eco*RI phage vector as previously described (29). The recombinant lambda phages were named λDII/R168 and λDII/R122, respectively. The physical maps of the DNA inserts in λDII/R168 and λDII/R122, determined by restriction digestions and Southern hybridization (Fig. 1a and e), showed that Ω727 and Ω729 were

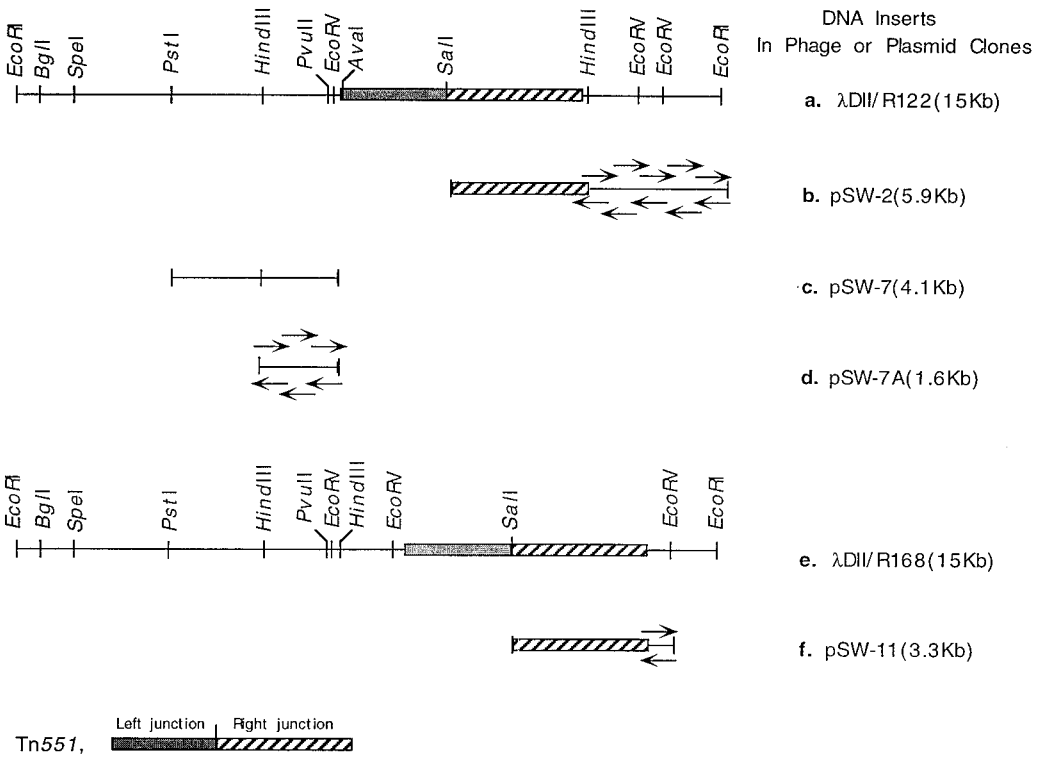

FIG. 1. Restriction map and strategy for sequencing of the Ω727/729 region. (a) Restriction map of the 15-kb DNA insert in λDII/R122. Transposon Tn*551* is shown as a box. (b) The 5.9-kb DNA insert in pSW-2. The striped box represents the Tn*551* right junction, and the arrows indicate the direction of sequencing. (c) The 4.1-kb DNA insert in pSW-7. (d) The 1.6-kb DNA insert in pSW-7A. (e) Restriction map of the 15-kb DNA insert in λDII/R168. (f) The 3.3-kb DNA insert in pSW-11.

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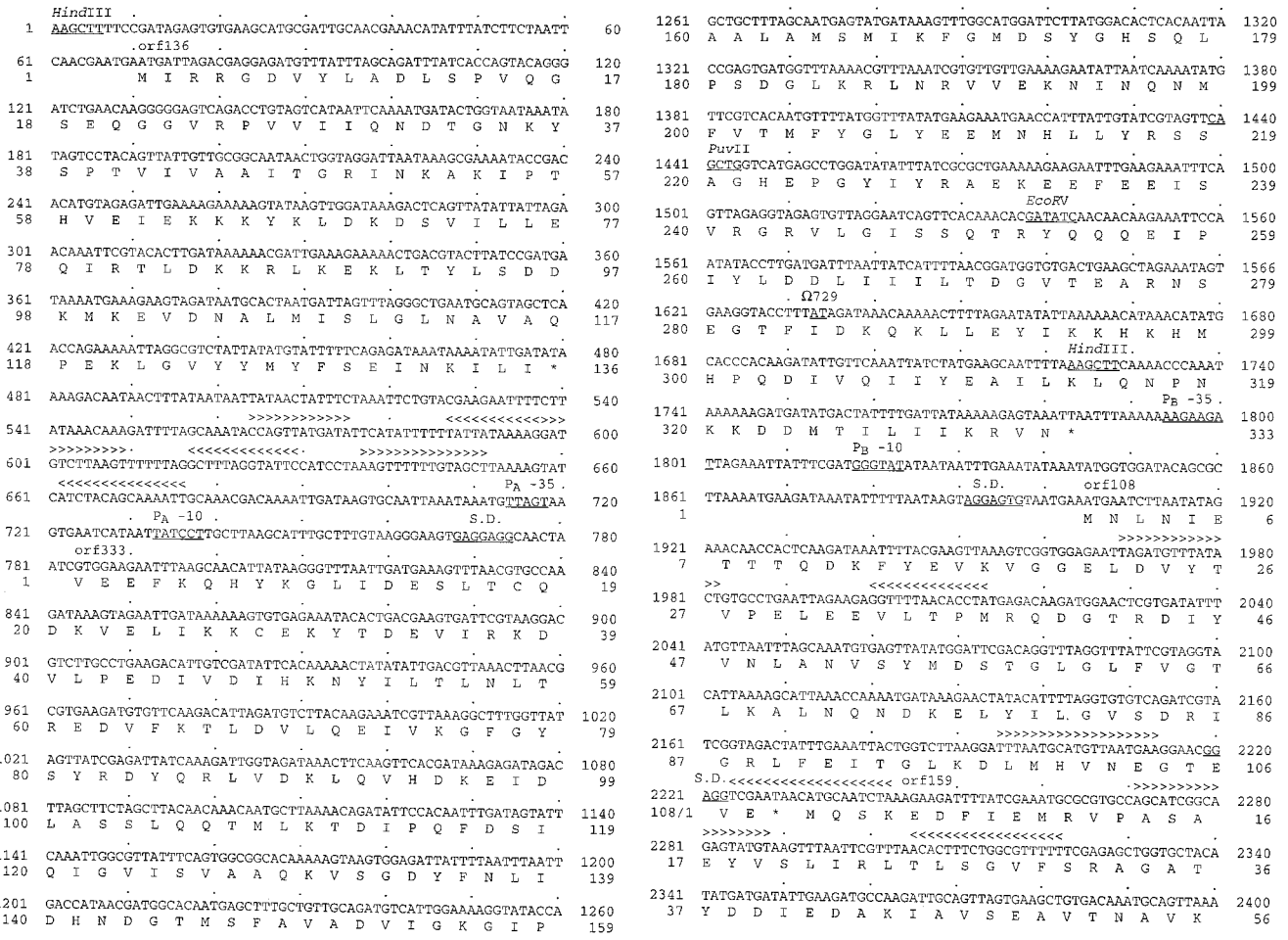

FIG. 2. Nucleotide sequence of the 4,607-bp *Hin*dIII-*Eco*RI fragment containing the Ω727/729 region. Numbering starts at the *Hin*dIII site and ends at the *Eco*RI site (position 4607). The putative start codon is indicated below the gene designation, and the stop codon is designated with an asterisk. A putative Shine-Dalgarno (S.D.) sequence is underlined. The possible candidates for promoter sequences (−35 and −10 regions) are shown, and the pairs of −10 and −35 regions belonging to one possible consensus are indicated with same letter designation, e.g., −35a and −10a. Inverted repeat sequences are indicated by arrows. The insertion sites of Tn*551* are shown by underlining of the two spanning nucleotides and labeled Ω727 and Ω729. Amino acids deduced from the nucleotide sequence are specified by standard one-letter abbreviations.

located on the same 9.8-kb *Eco*RI fragment but different *Hin*dIII (4 kb for Ω727 and 1.9 kb for Ω729) and *Eco*RV (about 0.6 kb for Ω727 [the Tn*551* insertional site Ω728 of RUSA150 was determined to be on the same *Eco*RV fragment as Ω727] and 1.4 kb for Ω729) fragments. The *Pst*I, *Pvu*II, *Spe*I, and *Bgl*I recognition sites on the flanking sequence of Tn*551* were also mapped with respect to the unique *Sal*I restriction site of Tn*551* (Fig. 1a and e).

The 4.1-kb *Ava*I-*Pst*I fragment of λDII/R122, which includes a 7-bp Tn*551* left junction and 4.1-kb Ω729 left flanking region, was subcloned into *Ava*I-*Pst*I-digested pGEM-3Z to form the recombinant plasmid pSW-7 (Fig. 1c). pSW-7 was subsequently digested with *Hin*dIII, the 4.3-kb fragment was separated from the 2.0-kb fragment, and plasmid pSW-7A was generated by self-ligation of the 4.3-kb fragment (Fig. 1d). Plasmid pSW-2 was constructed by ligating the 5.9-kb *Sal*I fragment of λDII/R122 with *Sal*I-digested pGEM-3Z vector. The insert DNA in pSW-2 included a 2.9-kb Tn*551* right junction and 3.0-kb Ω729 right flanking region (Fig. 1b); actually this insert had spanned the insertion site Ω727, according to the physical map (Fig. 1a and e). In addition, the 3.3-kb *Sal*I-*Eco*RV fragment of λDII/R168 was subcloned into pGEM-3Z vector to create plasmid pSW-11 (Fig. 1f); thereby the right flanking region of Ω727 with a 2.9-kb Tn*551* right junction was also isolated.

DNA sequencing of the Ω727/729 region. The DNA sequence of the Ω727/729 region was determined by separately sequencing the DNA inserts of pSW-7A and pSW-2 through both strands, using the strategy of primer walking. In the 4,607-bp DNA sequence depicted in Fig. 2, the sequence of the first 1,633 bp of the 5′ portion was obtained from sequencing of the pSW-7A insert DNA which covered the area between the *Hin*dIII cloning site and the insertion site Ω729 (Fig. 1d); the 2,974-bp region of the 3′ portion from bp 1634 to 4607 was the region from the insertion site Ω729 to the *Eco*RI cloning site of λDII/R122 (Fig. 1b), and this was a part of the insert DNA in pSW-2.

The 4,607-bp region was analyzed for open reading frames (ORFs). As shown in Fig. 2 and 3, this region contained six ORFs (one truncated by an *Eco*RI cloning site in the C terminus), which were tentatively designated according to the number of deduced amino acid residues in the order orf136-orf333-orf108-orf159-orf256-CTorf239 (CT, C-terminally truncated). orf136 was initiated with a typical ATG codon, but no multiple guanine sequence which could be considered a ribo-

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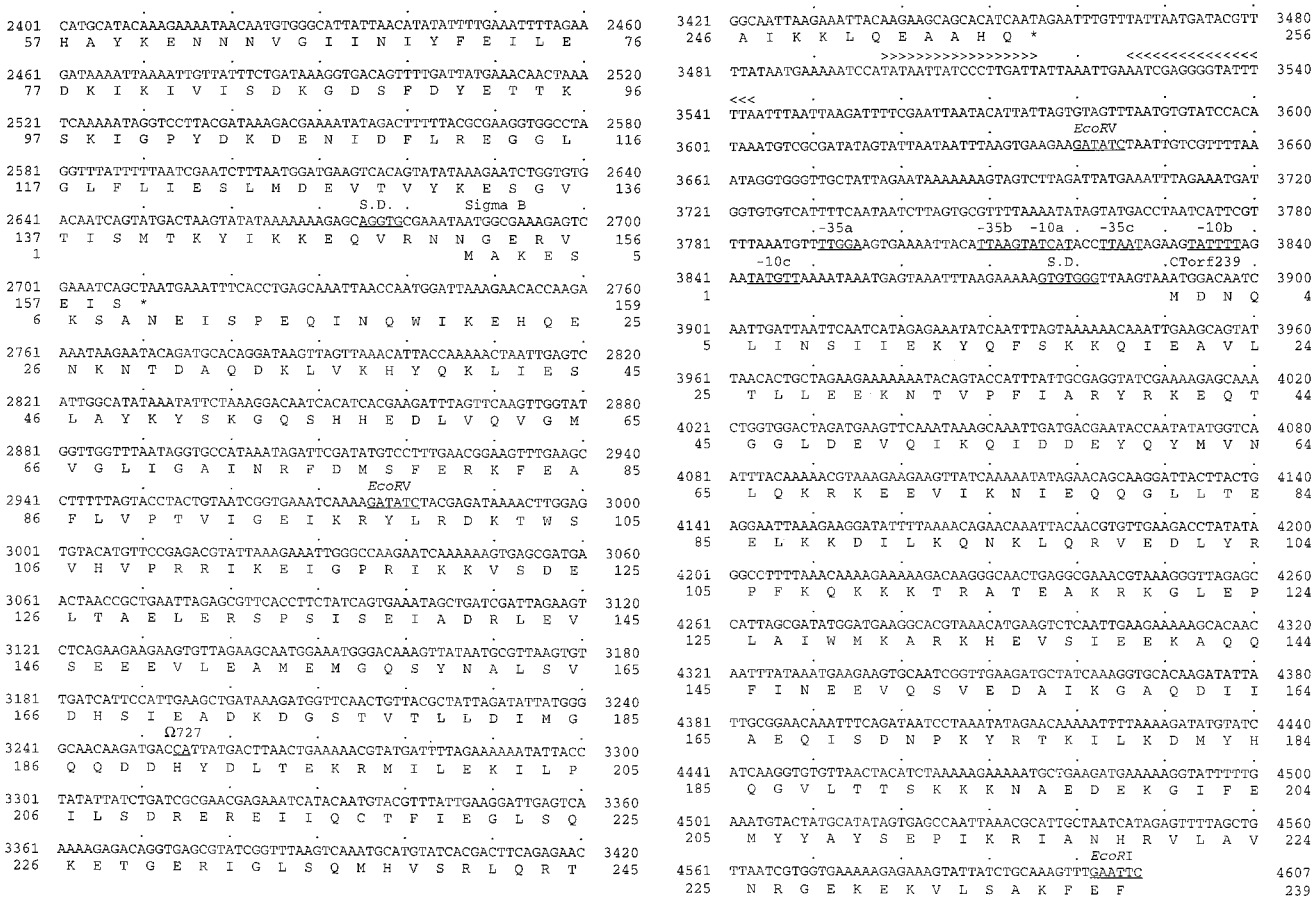

FIG. 2—*Continued.*

some binding site was observed prior to the initiation codon (Fig. 2). orf333 was 303 nucleotides downstream of orf136 (Fig. 2), began with an uncommon GTG initiation codon, and was preceded by a putative promoter sequence (20), with the sequences TTAGT and TATCCT for the −35 and −10 regions, respectively, and a Shine-Dalgarno sequence of GAGGAGG (23). A region of multiple dyad symmetry sequences, which contained at least three stem-loop structures, was identified within an area of 112 bp (bp 564 to 676), and this area was 36 bp upstream from the −35 region of orf333. With respect to the known Tn*551* sequence, the insertion site Ω729 in RUSA122 was located in orf333; it was 283 amino acid residues from the initiation codon and 50 amino acid residues from the termination codon (Fig. 2). Following orf333 with an interval of 120 nucleotides, three ORFs, orf108, orf159, and orf256, were closely connected (orf159 followed orf108 by a single nucleotide, and orf159 and orf256 overlapped by 26 nucleotides). Each of them was preceded by a ribosome binding consensus sequence: AGGAGTG for orf108, GGAGG for orf159, and AGGTG for orf256 (Fig. 2). However, only one putative promoter sequence, AAGAAGAT for the −35 region and GGGTAT for the −10 region, was found upstream of orf108. The palindromic sequences possibly associated with the orf108-orf159-orf256 cluster were found in the start and end portions of orf108 (bp 1969 to 2013 and 2195 to 2242), the part close to the N terminus of orf159 (bp 2271 to 2322), and 40 nucleotides downstream of orf256 (bp 3589 to 3543). By sequencing the DNA insert of pSW-11, the insertion site Ω727 in RUSA168 was found to be located in orf256, 189 amino acid residues from the initiation codon and 67 amino acid residues from the termination codon (Fig. 2). CTorf239 was 434 bp downstream of orf256 and was preceded by a ribosome binding consensus sequence of GTGTGGG (Fig. 3). Within an area 100 nucleotides upstream from the putative ribosome binding site of CTorf239, at least three pairs of DNA segments possessed the characteristics of a promoter sequence; TTGGA and TATCAT for −35a and −10a, TTAAG and TATTTT for −35b and −10b, TTAAT and TATGTT for −35c and −10c (Fig. 2). Despite the relatively short spacing between its −35 and −10 regions, we believe that P_A may function as a promoter. No ORF of significant length was found on the reverse complement of the sequence.

Comparison of the amino acid sequences of the Ω727/729 region and known proteins. The deduced amino acid sequences from the ORFs in the sequenced Ω727/729 region were compared with the sequences of known polypeptides in both Tblastn and Blastp databanks. The most significant homologies were observed between the ORFs in the Ω727/729 region and two operons, *sigB* and *spoIIA*, in *B. subtilis*; the gene cluster orf333-orf108-orf159-orf256 was not only highly homologous with the *sigB* and the *spoIIA* operons in terms of primary sequences of the gene products (Fig. 4) but also strikingly similar to the two operons in the overall organization (Fig. 3). The *sigB* operon of *B. subtilis* comprised eight genes in the order orfR-orfS-orfT-*rsbU-rsbV-rsbW-sigB-rsbX* (*rsb* stands for regulator of sigma-B) (3, 5, 14, 28). The cluster orf333-orf108-

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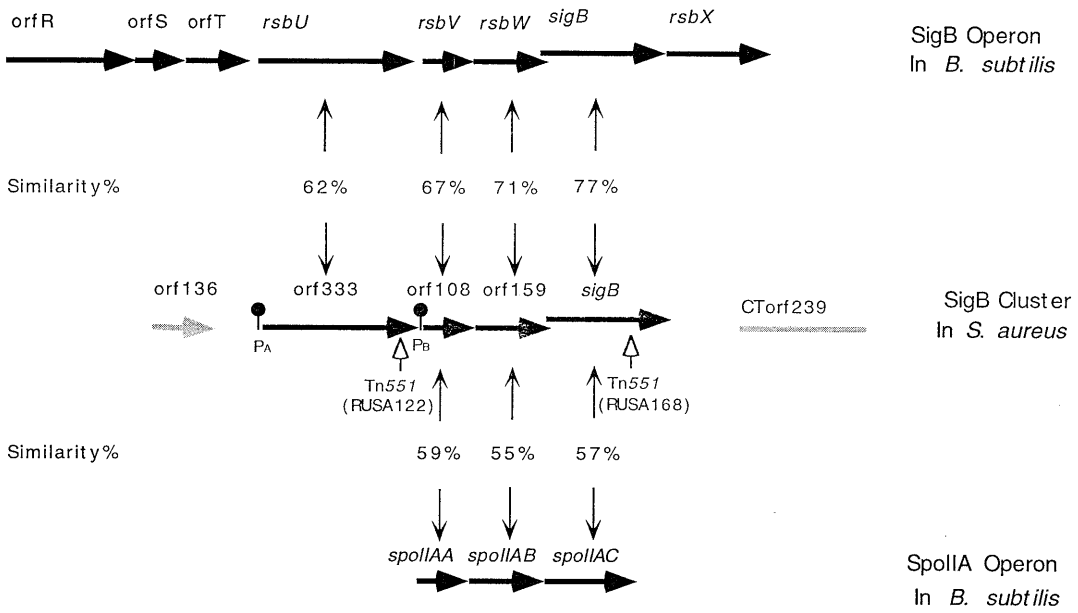

FIG. 3. Similarity in organization of the *S. aureus sigB* cluster and the *B. subtilis sigB* and *spoIIA* operons. In the physical maps of the *sigB*, *sigB*, and *spoIIA* operons, ORFs are indicated by arrows. *sigB* encodes the stationary-phase alternative sigma factor in *B. subtilis*, and *spoIIA* encodes the sporulation-essential σ^F in *B. subtilis*. Similarities between the predicted products of the corresponding genes are shown with percentages. The two Tn*551* insertion sites (Ω727 and Ω729) in the *S. aureus sigB* cluster are indicated with arrows.

orf159-orf256 showed a high degree of similarity to the *rsbU-rsbV-rsbW-sigB* cluster in the *B. subtilis sigB* operon (Fig. 3); the polypeptides encoded by orf333 (38 kDa), orf108 (12 kDa), orf159 (18 kDa), and orf256 (29 kDa) were correspondingly homologous with the products of the 334-residue RsbU, 109-residue RsbV, 160-residue RsbW, and 264-residue SigB (62, 67, 71, and 77% similarities) (Fig. 3 and 4). The *B. subtilis spoIIA* operon consists of three genes, *spoIIAA*, *spoIIAB*, and *spoIIAC*, with *spoIIAC* encoding the sporulation-essential σ^F (9, 19, 24, 25). The similarity in organization of the *spoIIA* operon and the ORFs in the Ω727/729 region was evidenced by the fact that the products of *spoIIAA*, *spoIIAB*, and *spoIIAC* had similarities of 59, 55, and 57% to the products of orf108, orf159, and orf256 (Fig. 3).

Furthermore, the glycine residue (Gly-93) important for RsbV function (4) was conserved in orf108 product (Fig. 4b). Also conserved in the orf159 product was Ala-14 in RsbW (Fig. 4c). The orf136 and CTorf239 products had no significant similarity with any protein in the Tblastn and Blastp databanks.

DISCUSSION

Comparison of amino acid sequences has shown that the gene cluster in the *S. aureus* Ω727/729 region is highly homologous with the *sigB* and *spoIIA* operons of *B. subtilis* (Fig. 3 and 4) and that there is a high degree of similarity between the products of *S. aureus* orf333, orf108, orf159, and orf256 and their counterparts *rsbU*, *rsbV*, *rsbW*, and *sigB* in the *sigB* operon of *B. subtilis* (Fig. 3 and 4). *B. subtilis rsbU* is also known to have high degree of similarity to *spoIIE* (7). Therefore, there may also be a functional similarity between *spoIIE* and orf333. The similarities in both product sequence and overall genetic organization suggest that the gene cluster in the Ω727/729 region may be regulated in a manner similar to that of the *sigB* operon of *B. subtilis*. We infer that orf256, which encodes a polypeptide of about 29 kDa (29,443 Da) and corresponds to the *sigB* structural gene, is the corresponding structural gene of the staphylococcal orf333-orf108-orf159-orf256 cluster. We propose to name this cluster the sigma-B operon, to name orf256 the *sigmaB* gene, and to name the product of orf256 the σ^B factor.

Several observations suggest that the staphylococcal *sigB* and *B. subtilis sigB* operons may perform similar physiological roles. (i) There are close similarities between the four genes of the *S. aureus sigB* cluster and their counterparts in the *B. subtilis sigB* operon. (ii) The gene *rsbU* is believed to encode a *trans*-acting, positive regulatory factor that controls σ^B (28). We speculate that orf333 and *sigB* correspond to an analogous pair of positive regulator and structural genes in *S. aureus*. This conclusion is consistent with the less severe reduction in antibiotic resistance level in mutant RUSA122 (Tn*551* insert of orf333) compared with the substantially lower methicillin MIC (25 μg ml^{-1}) in mutant RUSA168 (Tn*551* insert in *sigB*). (iii) The staphylococcal *sigB* operon may also be preceded by two promoters: the putative upstream promoter of orf333 may have a function similar to that of the *B. subtilis sigB* P_A; the putative downstream promoter of orf333 is identical to most σ^B-dependent promoters (26) in the −10 region (GGGTAT) and might be an internally σ^B-dependent promoter detected in the *sigB* operon. (iv) Like the *rsbV-rsbW-sigB* cluster in *B. subtilis*, the staphylococcal orf108, orf159, and *sigB* are closely linked genes, suggesting that expression of the gene products may be translationally coupled, presumably in order to ensure equimolar synthesis of proteins which must act in concert in the cell.

Numerous observations and speculations have been described concerning the functioning of *B. subtilis sigB*, such as induction of σ^B activity upon entry into the stationary phase (14) or by environmental stress during logarithmic growth (4), control of σ^B activity through stationary-phase signals (2, 4), and possible roles in the induction of general stress genes (27). However, null mutants in the σ^B structural gene (*sigB*) did not show any obvious growth or sporulation phenotype (3, 8, 13, 14), and therefore the physiological function of σ^B in *B. subtilis*

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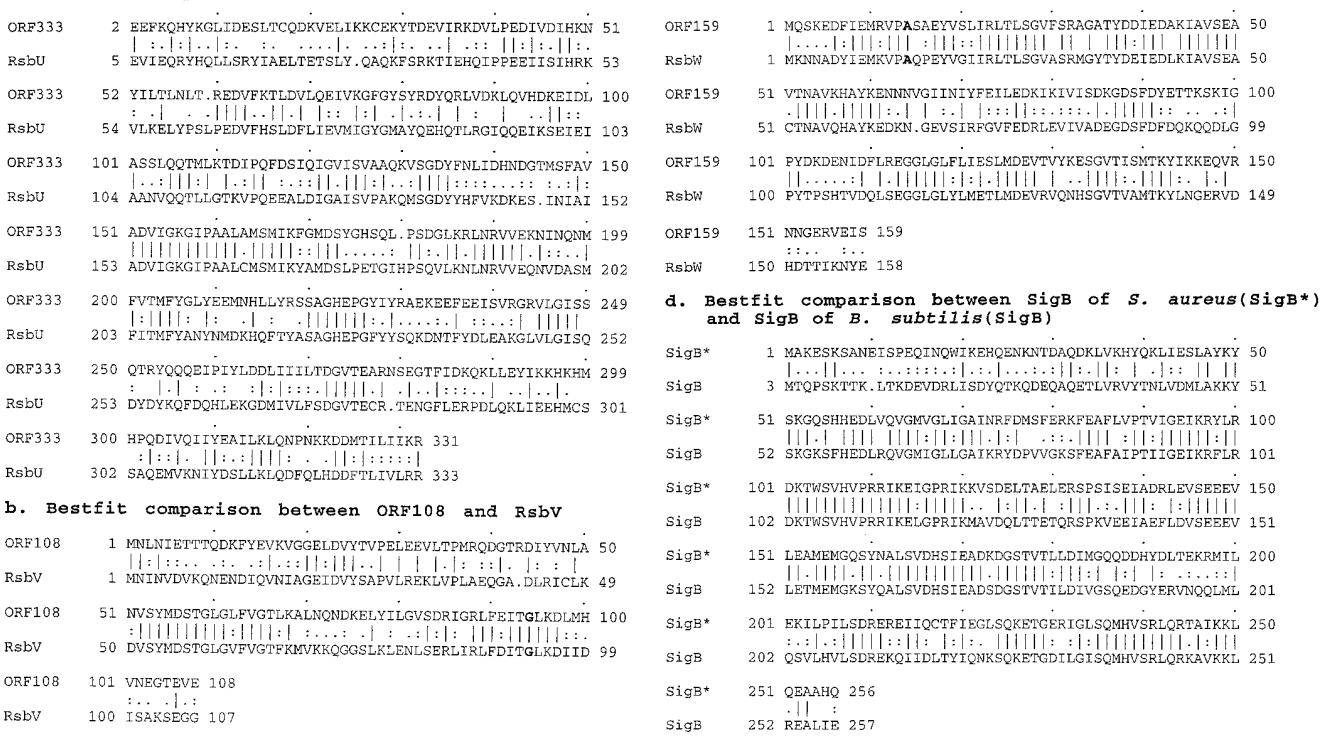

FIG. 4. Genetics Computer Group Bestfit amino acid sequence comparisons. (a) orf333 and RsbU (39% identity and 62% similarity); (b) orf108 and RsbV (42% identity and 67% similarity); (c) orf159 and RsbW (54% identity and 71% similarity); SigB of *S. aureus* and SigB of *B. subtilis* (59% identity and 77% similarity). The residues critical for protein function, Gly-93 in RsbV and Ala-14 in RsbW, are in boldface. Vertical bars indicate identical amino acids; colons and periods indicate two degrees of similarity of amino acids.

remains to be identified. A *sigB*-like gene, apparently involved with the stationary phase, has recently also been identified in *Mycobacterium tuberculosis* (6).

The most striking difference between the *sigB* mutants RUSA122, -150, and -168 and the parental strain COL is the drastic reduction of antibiotic resistance in the mutants: the MIC of the parental strain was 1600 $\mu g\ ml^{-1}$, whereas MICs were 25 $\mu g\ ml^{-1}$ for RUSA168 and 50 $\mu g\ ml^{-1}$ for RUSA122 (5). The decreased resistance to methicillin in RUSA168 and RUSA122 was not related to a reduction in the cellular amounts of PBP2A (the gene product of *mecA*). Moreover, in contrast to several other auxiliary or *fem* mutants, no alteration in the muropeptide composition of the peptidoglycans of mutant RUSA168, -122, or -150 could be detected (unpublished observations).

The mechanism through which inactivation of the *sigB* cluster results in the suppression of the methicillin resistance phenotype is currently under investigation in our laboratory. Our observation that inactivation of a cluster of staphylococcal stress response genes also inactivates antibiotic resistance introduces the possibility that for optimal expression of antibiotic resistance, staphylococci require an uninterrupted functioning of stress response genes which, presumably, are activated by the metabolic disturbances induced in the cells during exposure to the antibacterial agent.

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