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Induction of Fibronectin Adhesins in Quinolone-Resistant *Staphylococcus aureus* by Subinhibitory Levels of Ciprofloxacin or by Sigma B Transcription Factor Activity Is Mediated by Two Separate Pathways

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We recently reported on the involvement of a RecA-LexA-dependent pathway in the ciprofloxacin-triggered upregulation of fibronectin-binding proteins (FnBPs) by fluoroquinolone-resistant *Staphylococcus aureus*. The potential additional contribution of the transcription factor sigma B (SigB) to the ciprofloxacin-triggered upregulation of FnBPs was studied in isogenic mutants of fluoroquinolone-resistant strain RA1 (a topoisomerase IV gyrase double mutant of *S. aureus* NCTC strain 8325), which exhibited widely different levels of SigB activity, as assessed by quantitative reverse transcription-PCR of their respective *sigB* and SigB-dependent *asp23* transcript levels. These mutants were Tn*551* insertion *sigB* strain TE1 and *rsbU*$^+$ complemented strain TE2, which exhibited a wild-type SigB operon. Levels of FnBP surface display and fibronectin-mediated adhesion were lower in *sigB* mutant TE1 or higher in the *rsbU*$^+$-restored strain TE2 compared to their *sigB*$^+$ but *rsbU* parent, strain RA1, exhibiting low levels of SigB activity. Steady-state *fnbA* and *fnbB* transcripts levels were similar in strains TE1 and RA1 but increased by 4- and 12-fold, respectively, in strain TE2 compared to those in strain RA1. In contrast, fibronectin-mediated adhesion of strains TE1, RA1, and TE2 was similarly enhanced by growth in the presence of one-eighth the MIC of ciprofloxacin, which led to a significantly higher increase in their *fnbB* transcript levels compared to the increase in their *fnbA* transcript levels. Increased SigB levels led to a significant reduction in *agr* RNAIII; in contrast, it led to a slight increase in *sarA* transcript levels. In conclusion, upregulation of FnBPs by increased SigB levels and ciprofloxacin exposure in fluoroquinolone-resistant *S. aureus* occurs via independent pathways whose concerted actions may significantly promote bacterial adhesion and colonization.

Staphylococcus aureus is a major pathogen that causes a variety of infections in humans and animals, ranging from minor skin and wound infections to life-threatening diseases (14). This high degree of flexibility in the expression of virulence is due to the highly regulated control and coordinated expression of numerous extracellular and cell wall-associated virulence determinants (1, 10, 13, 17, 32, 42, 46, 50). *S. aureus* displays a number of surface protein adhesins referred to as microbial surface components that recognize adhesive matrix molecules that promote the binding of several plasma or extracellular matrix host proteins (44). Fibronectin-binding proteins (FnBPs) FnBPA and FnBPB, encoded by the *fnbA* and the *fnbB* genes, respectively, play a prominent role in *S. aureus* attachment and colonization of host tissues or implanted biomaterials (18, 26, 58). FnBPs also promote the endocytic uptake of *S. aureus* by epithelial and endothelial cell lines and fibroblasts (20, 56).

Several complex regulons, notably, *agr* and *sarA*, control expression of cell wall-associated and extracellular virulence determinants in *S. aureus* in a growth-phase-dependent manner (1, 10, 13, 17, 42). The *agr* locus encodes a two-component quorum-sensing system that generates two divergent transcripts, RNAII and RNAIII, from two distinct promoters, promoters P2 and P3, respectively. RNAIII controls expression of many virulence genes during the postexponential phase by downregulating a majority of cell wall-associated proteins while upregulating the release of potent exotoxins and proteolytic enzymes (7, 13, 17, 32, 42). In contrast, the *sarA* locus encodes a single 14.5-kDa DNA-binding protein, but with three upstream promoters driving three overlapping transcripts (10). Like *agr*, the *sarA* locus was shown by transcription profiling studies to influence the expression of >100 genes of *S. aureus* (10, 17, 50). The SarA protein can either directly regulate several target genes (8, 30), e.g., *hla* (alpha-hemolysin gene), *spa* (protein A gene), *fnbA* (61, 63), *cna*, and *sspA*, or indirectly influence target gene expression by changing P2 or/and P3 *agr* promoter activities (11, 39, 63). Several additional *sar* homologues (*sarS*, *sarR*, *sarT*, *sarU*, and *rot*) that are all DNA-binding regulatory elements have recently been revealed by genomic and genetic studies (33, 35, 37, 50, 55). In addition,

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several of the 16 two-component regulatory systems identified in *S. aureus* (e.g., *arlRS*, *srrAB*, *saeRS*, *lytRS*, and *yccFG)* (16, 19, 22, 43, 45, 64) contribute to the regulation of virulence genes either directly or via the global regulators *agr* and/or *sarA* (1, 10, 42, 65).

The alternative transcription factor *sigB* operon of *S. aureus* is composed of four open reading frames (*rsbU*, *rsbV*, *rsbW*, and *sigB*) that show some structural and functional homology to those of *Bacillus subtilis* (3, 12, 25, 28, 38, 62). The SigB activity is controlled posttranslationally by a multicomponent signal transduction system involving the RsbU, RsbV, and RsbW proteins. The SigB factor, which is normally sequestered by the anti-sigma factor RsbW, may be released from the SigB-RsbW complex by transduction of external signals mediated by RsbU (a phosphatase) that dephosphorylates RsbV, thus permitting its interaction with RsbW. A number of virulence-associated target genes, such as *asp23*, *coa*, *clfA*, *sarS*, and the P3 promoter of *sarA*, are reported to be transcriptionally regulated by the level of free SigB (2, 28, 40, 41).

Subinhibitory concentrations of antibiotics may downregulate or upregulate specific adhesins (44) or secreted virulence factors (27) of *S. aureus*, such as FnBPs, collagen-binding protein, or alpha-toxin. In addition, emergence or acquisition of antibiotic resistance determinants by *S. aureus*, such as methicillin or glycopeptide resistance, can significantly alter expression of global regulators and virulence factors, such as *agr* (51, 52) and alpha-toxin (52), and can downregulate (54, 59) or upregulate (47) expression of cell wall-associated surface components, such as clumping factor A and FnBPs.

Previous studies performed in our laboratory (5, 6) revealed that subinhibitory concentrations of some antibiotics such as ciprofloxacin could raise the fibronectin-mediated attachment of fluoroquinolone-resistant *S. aureus* by selectively inducing the *fnbB* gene. Recently, a RecA-LexA-dependent pathway was shown to mediate the ciprofloxacin-induced *fnbB* upregulation in *S. aureus* (4). The aims of the present study were to (i) evaluate a potential additional contribution of SigB to the ciprofloxacin-triggered upregulation of FnBP by using isogenic derivatives of the recently described fluoroquinolone-resistant, *grlA gyrA* strain RA1 of *S. aureus* (4), which exhibit widely different levels of SigB activity, and (ii) evaluate the potential contribution of global regulons *agr* and *sarA* to either the SigB- or the ciprofloxacin-modulated effects on *fnbA or fnbB* transcription. These results provide evidence that optimal expression of a stress response factor and triggering of a drug-induced DNA repair system may independently, but in an additive manner, significantly promote FnBP-mediated *S. aureus* adhesion.

(This study was presented in part at the 13th European Congress for Clinical Microbiology and Infectious Diseases, Glasgow, Scotland.)

MATERIALS AND METHODS

Bacterial strains. Strain RA1 is a *gyrB142 grlA542 gyrAΩ105* quinolone-resistant mutant of NCTC8325 strain ISP794 (4). Strain RA1, like all members of the NCTC8325 family, has an 11-bp deletion in *rsbU* and yields weakly pigmented colonies on Muller-Hinton agar (MHA).

Strain TE1 (kindly provided by A. L. Cheung) is a Tn*551* insertion *sigB* mutant of RA1 that was constructed by transducing RA1 with a phage lysate of strain ALC1001 carrying the *sigB* mutation (12, 62). Correct insertion of Tn*551* in the *sigB* locus of strain TE1 was confirmed by PCR assays. Strain TE1 yields completely white colonies on MHA.

Strain TE2 is a derivative of RA1 whose SigB functional activity was restored by transducing RA1 with a phage lysate prepared from strain GP268 (kindly provided by M. Bischoff) *rsbU*$^+$ *rsbV*$^+$ *rsbW*$^+$ *sigB*$^+$ Tc^r (25). Strain TE2 yields strongly pigmented colonies on MHA, and its genotype was verified by a PCR assay.

Identical MICs (32 μg/ml) of ciprofloxacin, determined by a macrodilution method in Mueller-Hinton broth (MHB; Difco, Detroit, Mich.) as described by NCCLS (40a), were recorded for strains RA1, TE1, and TE2.

Assay for bacterial adhesion to fibronectin. The attachment properties of the *S. aureus* strains were measured by an adhesion assay with polymethylmethacrylate coverslips coated in vitro with three different concentrations (0.5, 1, and 2 μg/ml) of purified human fibronectin, as described previously (5, 6). Briefly, the strains were grown and metabolically radiolabeled with [^{3}H]thymidine for 5 h without shaking at 37°C in MHB in the presence or absence of one-eighth the MIC (4 μg/ml) of ciprofloxacin. This sub-MIC of ciprofloxacin was previously shown (6) to optimally promote FnBPs without significantly affecting the bacterial growth rate. Thereafter, 10^7 CFU of washed cultures of radiolabeled bacteria was incubated for 1 h at 37°C with the fibronectin-coated coverslips in human albumin-supplemented phosphate-buffered saline (PBS), as described previously (5). The coverslips were washed, and the amount of radioactivity was determined. Bacterial adhesion data for the different strains whose cell-associated radioactivity and viable counts differed slightly (<20%) were normalized as described previously (57). Relative changes in bacterial adhesion were expressed as the percent increase or decrease in attachment of strains TE1 and TE2 compared to that of strain RA1 grown in ciprofloxacin-free medium or the relative changes in bacterial adhesion between exposure and no exposure to ciprofloxacin for each strain. Each experiment was performed at least three times, and the results were expressed as the mean percent changes ± standard errors of the means (SEMs). The statistical significance of pairwise differences in bacterial adhesion of isogenic strains differing in SigB functional levels or in ciprofloxacin exposure versus no exposure was evaluated by paired *t* tests of the relative increases or decreases pooled for the three coating concentrations of fibronectin by using *P* values of <0.05 with a two-tailed significance level (49).

Quantification of FnBPs by flow cytometry. The FnBP-mediated fibronectin binding displayed by the different strains of *S. aureus* was monitored by flow cytometry, as described previously (21, 57). The specificity of the flow cytometry data was assessed by parallel analysis of control strains DU5883, a mutant of strain 8325-4 simultaneously defective in expression of both FnBPA and FnBPB, and DU5883(pFNBB4), which overexpresses FnBPB (21, 26, 57).

Relative changes in flow cytometric data were expressed as the percent increase or decrease in fluorescein isothiocyanate (FITC)-labeled fibronectin binding by strains TE1 and TE2 compared to that of strain RA1 grown in ciprofloxacin-free medium or the change in binding between exposure and no exposure to ciprofloxacin for each strain. Each experiment was performed three times, and the results were expressed as mean percent changes ± SEMs. The statistical significance of pairwise differences in FITC-labeled fibronectin binding of isogenic strains differing in SigB functional levels or in ciprofloxacin exposure versus no exposure was evaluated by paired *t* tests by using *P* values of <0.05 with a two-tailed significance level (49).

Total RNA extraction. Cultures of strains RA1, TE1, and TE2 were grown for 5 h in MHB in the absence or presence of 4 μg of ciprofloxacin per ml and were then harvested and immediately processed as described previously (27, 47). Briefly, bacteria were recovered, fixed in acetone-ethanol (1:1), and washed in *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid–sucrose buffer. Samples were treated with ice-cold lysostaphin, and RNA was purified as described previously (47).

Real-time RT-PCR. mRNA levels were determined by quantitative reverse transcription-PCR (qRT-PCR) with the one-step reverse transcriptase qPCR Master Mix kit (Eurogentec, Seraing, Belgium) and previously described primers and probes (47, 57). The reverse and forward primers and probe specific for the *sspA* gene were synthesized by Eurogentec (*sspAR*, 5′-ACTCTTTTAACAAATAAACACGTCGTAGA-3′; *sspAF*, 5′-GGTTAATTGCAGAAGGGAATGC-3′; *sspAP*, 5′-AAGCATGAGGATCACCGTGCGTAGC-3′). Conditions for reverse transcription, PCR, detection of fluorescence emission, and normalization of the levels of mRNA of the target genes extracted from the different strains on the basis of their 16S rRNA levels were described previously (47, 57). The statistical significance of strain-specific differences in normalized cycle threshold (C_T) values of each transcript was evaluated by paired *t* tests, and data were considered significant when *P* was <0.05.

Northern blotting. Total RNA (16 μg) was separated in a formaldehyde-agarose gel and blotted onto a nylon membrane, as described previously (9, 47). For *agr* RNAIII detection, a 439-bp RNAIII fragment amplified by PCR with primers RNAIIIa (5′-GTCATTATACGATTTAGTAC- 3′) and RNAIIIb (5′-G

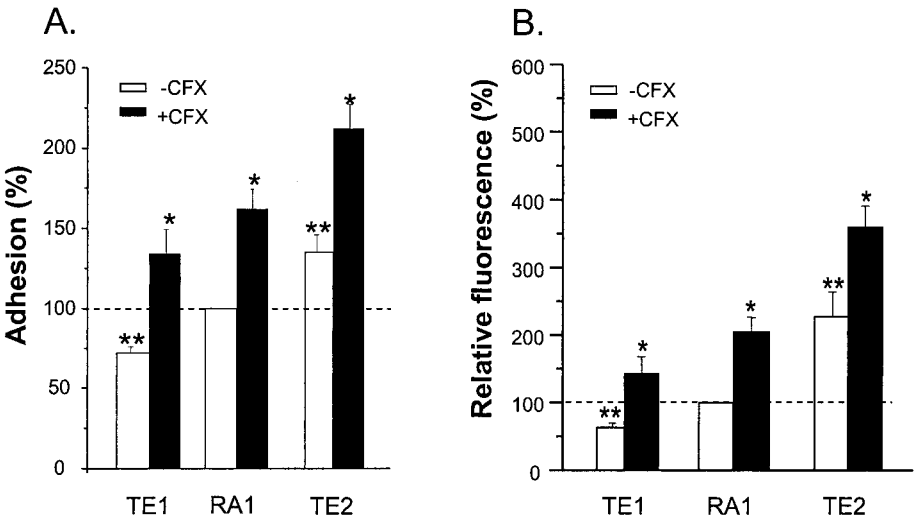

FIG. 1. Adhesion to fibronectin-coated coverslips (A) or binding of soluble FITC-labeled fibronectin (B) of *sigB* null strain TE1 and *rsbU*$^+$-restored strain TE2 grown in either the absence or the presence of 4 μg of ciprofloxacin (CFX) per ml (one-eighth the MIC) expressed as the percentages of adhesion (A) or fibronectin binding (B) for strain RA1 in ciprofloxacin-free medium. Values represent means + SEMs (error bars) of individual adhesion data accumulated over the three fibronectin coating concentrations in three experiments. *, results significantly different ($P < 0.01$) for each strain grown in ciprofloxacin-containing MHB from those for strains grown in ciprofloxacin-free MHB; **, results significantly different ($P < 0.01$) from those for strain RA1 grown in ciprofloxacin-free medium.

GTTATTAAGTTGGGATG-3′) was labeled with digoxigenin-dUTP by using DIG High Prime DNA labeling and detection starter kit II (Roche), as specified by the manufacturer. Blots were prehybridized with DIG Easy Hyb buffer for 1 h at 50°C and then hybridized with the labeled RNAIII probe at 50°C overnight. After the membrane was washed, the hybridized probes were immunodetected with anti-digoxigenin-alkaline phosphatase and then visualized with the chemiluminescence substrate disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3,3.1.1^{3,7}]decan]-4-yl) phenyl phosphate (CSPD), as specified by the manufacturer (Roche). Light emission was recorded on X-ray film.

Northern affinity blotting of the *sarA* transcripts was performed with a 290-bp ^{32}P-labeled *sarA* probe, as described previously (47).

Measurement of protease activity in culture supernatants. Proteolytic activity was assayed by determination of the increase in trichloroacetic acid-soluble azopeptides produced upon incubation of the culture supernatants with azocasein (Sigma), as described previously (60).

RESULTS

Modulation of *S. aureus* adhesion on fibronectin-coated surfaces by SigB levels and ciprofloxacin exposure. Following growth in ciprofloxacin-free medium, the level of attachment of *sigB* null mutant strain TE1, averaged over the three fibronectin coating concentrations, was significantly lower ($P < 0.01$) than that of its *rsbU sigB*$^+$ parent, strain RA1. In contrast, the average adhesion of the *rsbU*$^+$ *sigB*$^+$ strain TE2 ($P < 0.01$) increased significantly ($P < 0.01$) compared to that of its parent, strain RA1 (Fig. 1A). Thus, the adhesion values for strains TE1, RA1, and TE2 increased as a function of the predicted increase in their SigB functional activities.

To assess whether the stepwise increase in adhesion from strain TE1 to strain RA1 and from strain RA1 to strain TE2 resulted from increased levels of surface display of fibronectin adhesin molecules, FnPB-mediated fibronectin-binding sites were monitored by flow cytometry. Figure 1B shows that strains RA1 and TE2 bound two- and fivefold more FITC-labeled fibronectin ($P < 0.01$) than TE1, respectively.

Upregulation of bacterial attachment. Upregulation of bacterial attachment by the different strains by growth in the presence of one-eighth the MIC of ciprofloxacin (4 μg/ml) was not significantly influenced by modulation of their SigB functional levels. The average ciprofloxacin-promoted increase in the level of adhesion over the three fibronectin coating concentrations was significant ($P < 0.01$) and equivalent for strains TE1, RA1, and TE2 (Fig. 1). Flow cytometry (Fig. 1B) also showed a similar approximately twofold increase in the level of FnBP surface display in each strain grown in the presence of one-eighth the MIC of ciprofloxacin.

Upregulation of *fnbA* and *fnbB* transcripts by SigB levels and ciprofloxacin exposure. The steady-state *fnbA* and *fnbB* mRNA levels of strains RA1, TE1, and TE2 were also influenced by their respective functional SigB levels and growth conditions in the presence versus absence of ciprofloxacin. After growth in ciprofloxacin-free medium, *fnbA* mRNA levels were equivalent in strains RA1 and TE1 but were significantly increased fourfold in strain TE2 compared to those in strain RA1 (Fig. 2). Under similar conditions, *fnbB* mRNA levels were also equivalent in strains RA1 and TE1 but were sharply ($P < 0.01$) increased 12-fold in strain TE2 compared to those in strain RA1 (Fig. 2). Upregulation of both *fnb* transcripts in ciprofloxacin-free medium occurred not only in 5-h cultures but also during early-exponential-phase growth. Control experiments demonstrated that *fnbA* and *fnbB* mRNA levels significantly ($P < 0.01$) increased by 6- and 18-fold, respectively, in TE2 cells compared to the levels in RA1 cells grown for 2 h.

As expected from bacterial adhesion and flow cytometry data (Fig. 1), the relative increases in *fnbA* and *fnbB* transcript levels triggered in the different strains by growth in the presence of 4 μg of ciprofloxacin per ml were not correlated with their respective SigB functional levels. The ciprofloxacin-mediated increases in *fnbB* transcript levels in strains TE1, RA1, and TE2 were 5.0-, 5.8-, and 2.8-fold, respectively, being significantly ($P < 0.01$) greater than the 2.5-, 1.9-, and 1.7-fold

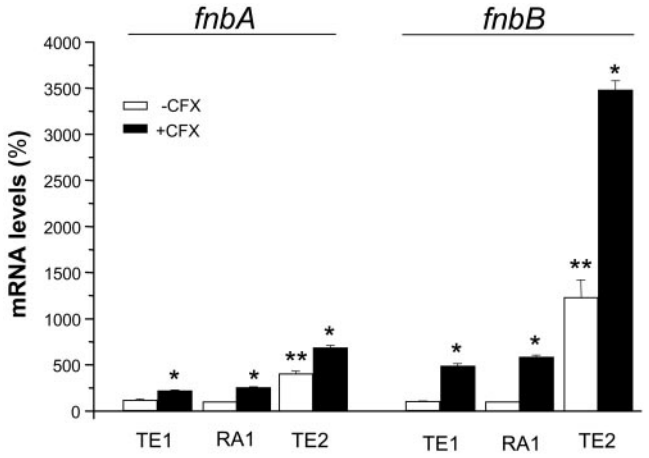

FIG. 2. Steady-state levels of the *fnbA* (left) and *fnbB* (right) transcripts of strains TE1 and TE2 grown in the absence or the presence of 4 μg of ciprofloxacin (CFX) per ml (one-eighth the MIC) expressed as the percentages of those of strain RA1 grown in ciprofloxacin-free medium. mRNA levels were determined by real-time RT-PCR and were normalized on the basis of their 16S rRNA levels. Values represent the means + SEMs of three experiments performed in triplicate. *, results significantly different ($P < 0.01$) for each strain grown in ciprofloxacin-containing MHB compared to those for the strain grown in ciprofloxacin-free MHB; **, results significantly different ($P < 0.01$) from those for strain RA1 grown in ciprofloxacin-free medium.

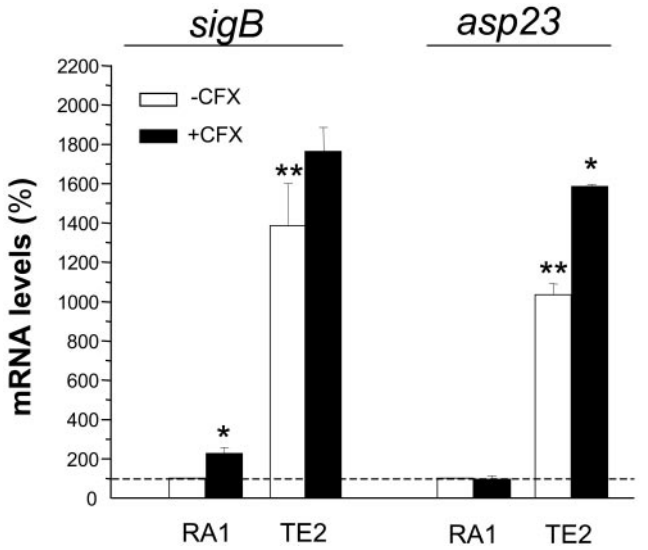

FIG. 3. Steady-state mRNA levels of the *sigB* (left) and *asp23* (right) genes of strain TE2 grown in the absence or the presence of 4 μg of ciprofloxacin (CFX) per ml (one-eighth the MIC) expressed as the percentages of those of strain RA1 grown in ciprofloxacin-free medium. mRNA levels were determined by real-time RT-PCR and were normalized on the basis of their 16S rRNA levels. Values are the means + SEMs of three experiments performed in triplicate. *, results significantly different ($P < 0.05$) for each strain grown in ciprofloxacin-containing MHB compared to those for the strain grown in ciprofloxacin-free MHB; **, results significantly different ($P < 0.01$) from those for strain RA1 grown in ciprofloxacin-free medium. The *asp23* transcripts levels of strain TE1 were too small to be visualized (see text).

increases in *fnbA* transcript levels, respectively, recorded in these strains (Fig. 2).

Assessment by qRT-PCR of SigB functional levels in the different strains. The *sigB* transcript levels were strongly (13.8-fold) elevated in the *rsbU*$^+$-restored strain TE2 compared to those in its *rsbU* parent, strain RA1 (Fig. 3). A similar trend was observed in the transcript levels of the *asp23* gene, frequently used as a marker for SigB functional activity, which showed a 10.3-fold increase in strain TE2 compared to that in strain RA1. A striking finding was the 100-fold decrease in the *asp23* transcript levels recorded in strain TE1 compared to those in RA1, which were too small to be visualized in Fig. 3. These data confirm the strong reduction of SigB functional levels in the *sigB* null mutant compared to those in its *sigB*$^+$ but *rsbU* parent.

Ciprofloxacin exposure led to a significant ($P < 0.05$) increase in the *sigB* transcript levels of strain RA1 but not those of strain TE2. On the other hand, ciprofloxacin exposure led to significantly ($P < 0.05$) increased *asp23* levels in strains TE1 (data not shown) and TE2 but not in strain RA1 (Fig. 3).

Influences of SigB levels and ciprofloxacin exposure on *agr* transcript levels. To detect any potential change in the activity of the global regulator *agr* that might contribute to either the SigB-mediated or the ciprofloxacin-triggered upregulation of *fnb* genes, we assayed the *agr* RNAII and RNAIII levels in strains TE1, RA1, and TE2. While the RNAII levels of strains grown in ciprofloxacin-free medium were not significantly different (Fig. 4A), RNAIII levels were similar in strains TE1 and RA1 but showed a significant 79% decline in strain TE2 compared to those in the other strains, as confirmed by Northern blotting analysis (Fig. 4B).

Ciprofloxacin exposure led to a significant ($P < 0.05$) but slight (less than twofold) increase in RNAII levels for strains RA1 and TE2 but not strain TE1. On the other hand, ciprofloxacin exposure led to significantly ($P < 0.01$) increased RNAIII levels only in strain TE2 and not in strains TE1 and RA1.

Impacts of SigB levels and ciprofloxacin exposure on *sarA* transcript levels. We next evaluated whether the global regulator *sarA* could contribute to either the SigB-mediated or the ciprofloxacin-triggered upregulation of *fnb* genes. While overall the steady-state *sarA* mRNA levels assayed by qRT-PCR were equivalent in strains TE1 and RA1, they showed a slight (53%) but significant ($P < 0.05$) increase in the *rsbU*$^+$-restored strain TE2 compared to those in strain RA1 (Fig. 5A). Northern blotting (data not shown) confirmed a stepwise increase in the levels of the SigB-dependent promoter P3-driven transcript from strain TE1 to RA1 and from strain RA1 to TE2, as expected from previous studies (2, 3, 15, 24, 34).

Ciprofloxacin exposure led to a significant ($P < 0.05$) approximately twofold increase in *sarA* transcript levels in all three strains, strains TE1, RA1, and TE2 (Fig. 5A).

Impacts of SigB levels and ciprofloxacin exposure on *hla* and *spa* transcript levels. Since previous reports demonstrated the strong impacts of the *agr*, *sarA*, and *sigB* regulons on the expression of hemolysin and protein A (1, 2, 10, 17, 42), we assayed the transcript levels of the target genes *hla* and *spa* in the SigB-modulated strains grown in the absence or the presence of ciprofloxacin. Striking differences in the *hla* and the *spa* transcript levels of strain TE2 compared to those of strains RA1 and TE1 were found. In the *rsbU*$^+$-restored strain, *hla*

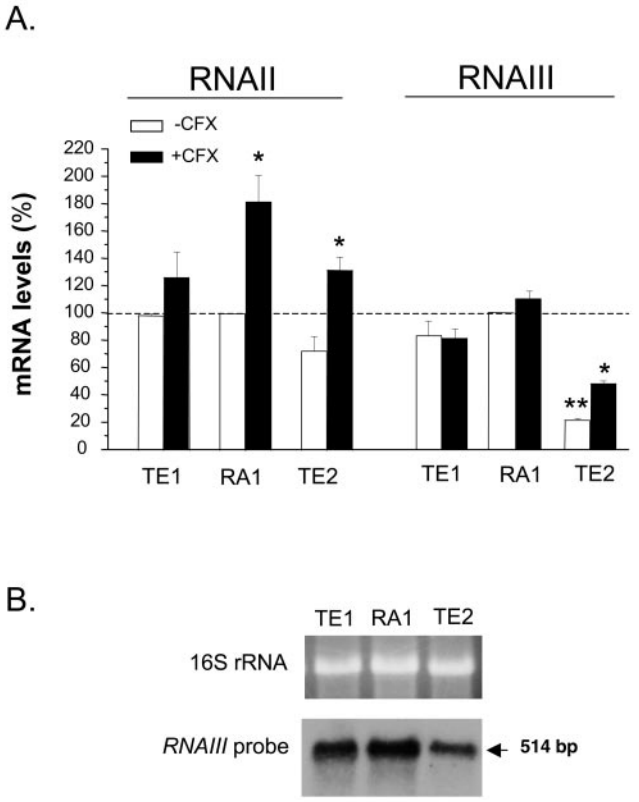

FIG. 4. (A) Steady-state levels of RNAII and RNAIII of strains TE1 and TE2 grown in the absence or the presence of 4 μg of ciprofloxacin (CFX) per ml (one-eighth the MIC) expressed as the percentages of those of strain RA1 grown in ciprofloxacin-free medium. mRNA levels were determined by real-time RT-PCR and were normalized on the basis of their 16S rRNA levels. Values represent the means + SEMs of three experiments performed in triplicate. *, results significantly different ($P < 0.05$) for each strain grown in ciprofloxacin-containing MHB compared to those for the strain grown in ciprofloxacin-free MHB; **, results significantly different ($P < 0.05$) from those for strain RA1 grown in ciprofloxacin-free medium. (B) Northern blot analysis of RNAIII in strains RA1, TE1, and TE2.

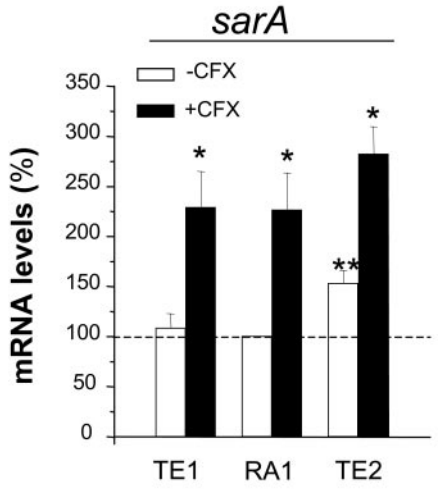

FIG. 5. Steady-state levels of the *sarA* transcripts of strains TE1 and TE2 grown in the absence or the presence of 4 μg of ciprofloxacin (CFX) per ml (one-eighth the MIC) expressed as the percentages of those of strain RA1 grown in ciprofloxacin-free medium. mRNA levels were determined by real-time RT-PCR and were normalized on the basis of their 16S rRNA levels. Values represent the means + SEMs of three experiments performed in triplicate. *, results significantly different ($P < 0.05$) for each strain grown in ciprofloxacin-containing MHB compared to those for the strain grown in ciprofloxacin-free MHB; **, results significantly different ($P < 0.05$) from those for strain RA1 grown in ciprofloxacin-free medium.

transcript levels were <10% of the levels recorded in RA1 and TE1, the levels of which in the last two strains were nearly equivalent (Fig. 6A). These strain-specific differences in *hla* mRNA levels likely accounted for the strongly reduced hemolytic zones produced on sheep blood agar by strain TE2 compared to those produced by strains RA1 and TE1 (data not shown). An inverse situation was found for *spa* transcript levels, which were increased by >25-fold in strain TE2 compared to the low levels recorded in strains RA1 and TE2, which were nearly equivalent in the last two strains (Fig. 6B).

While ciprofloxacin exposure led to a significant increase in the *hla* transcript levels of strain TE2 but not those of strains RA1 and TE1, an inverse situation was seen with *spa* transcripts, whose levels were elevated in strains RA1 and TE1 but not strain TE2.

Expression of extracellular proteases in the isogenic SigB-modulated strains. To evaluate putative strain-dependent differences in the levels of production and the extracellular release of proteases, which might account for the differential remodeling of surface-expressed FnBPs, we assayed the levels of the V8 protease gene transcripts (*sspA*) in strains RA1, TE1, and TE2 by qRT-PCR and extracellular proteolytic activity by the colorimetric detection of azopeptides in the culture supernatants incubated with azocasein (60). While the *sspA* transcript levels in 5-h cultures of strains RA1 and TE1 were equivalent, those of strain TE2 were slightly reduced (<50%) compared to those of strain RA1, thus confirming repression of this serine protease in the SigB-restored strain TE2 compared to its expression in the nonrestored isogenic derivatives (30). However, phenotypic detection of proteolytic activity in supernatants of 5-h cultures revealed only background levels of azopeptides. The levels released from 5-h cultures of the isogenic SigB-modulated strains, which represented <10% of those released from overnight (18-h) cultures of the same strains, did not allow assessment of strain-specific differences in the levels of production of extracellular proteases. In contrast, phenotypic detection of proteolytic activity of the SigB-modulated strains grown for 18 h revealed a ca. 75% reduction in extracellular proteolytic activity for strain TE2 compared to those for strains RA1 and TE1, in agreement with previous observations (30). Taken together, these data suggest that extracellular protease release from 5-h cultures of the SigB-modulated strains probably plays a minor role, if any, on FnBP surface display. However, differential remodeling of surface-expressed FnBPs is likely to play a more important role in cultures at more advanced stages of growth, as expected from previous studies (28, 30, 31).

DISCUSSION

Growing evidence suggests that expression and surface display of FnBPs in *S. aureus* are regulated by a complex network of global regulators, transcription factors, and stress response

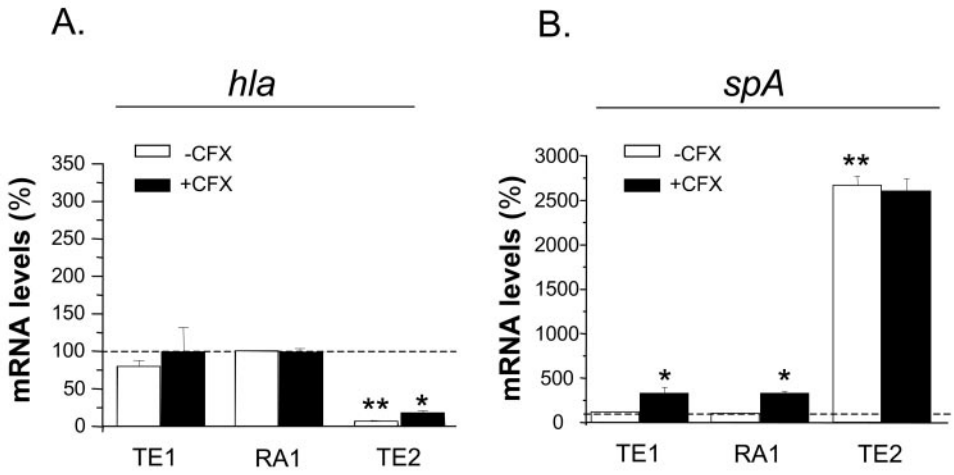

FIG. 6. Steady-state levels of the *hla* and *spa* transcripts of strains TE1 and TE2 grown in the absence or the presence of 4 μg of ciprofloxacin (CFX) per ml (one-eighth the MIC) expressed as the percentages of those of strain RA1 grown in ciprofloxacin-free medium. mRNA levels were determined by real-time RT-PCR and were normalized on the basis of their 16S rRNA levels. Values represent the means + SEMs of three experiments performed in triplicate. *, results significantly different ($P < 0.01$) for each strain grown in ciprofloxacin-containing MHB compared to those for the strain grown in ciprofloxacin-free MHB; **, results significantly different ($P < 0.05$) from those for strain RA1 grown in ciprofloxacin-free medium.

pathways (2–7, 29, 31, 53, 54, 57, 59, 61, 63). Besides the previously reported growth phase- and quorum sensing-controlled effects of *agr* and *sarA* regulons on transcription of *fnb* genes and/or FnBP surface display (7, 53, 61, 63), a variety of environmental and/or stressful conditions may also alter expression of fibronectin adhesins. These diverse situations include switching to small-colony variant phenotypes (57), acquisition of methicillin resistance (48, 54, 59), emergence of teicoplanin resistance (47), and exposure of fluoroquinolone-resistant *S. aureus* strains to subinhibitory levels of ciprofloxacin (4–6). Except for the indirect impact of the methicillin resistance element, which does not affect *fnb* transcription but which is believed to interfere with FnBP surface display via production of the *pls* surface protein (29, 54), all other conditions mentioned above were shown to regulate *fnb* transcription (4–6, 57). The upregulation of FnBP expression by specific environmental and stressful stimuli, including fluoroquinolone exposure, may play a significant role in promoting *S. aureus* attachment to and colonization of host tissues or implanted biomaterials (58). In addition, the higher levels of surface display of FnBPs involved in fibronectin-mediated bridging with the host receptor integrin $\alpha_{5\beta}1$ may also increase their endocytic uptake by nonprofessional phagocytes (20, 56), as demonstrated with *hemB* mutants of *S. aureus* displaying small-colony variant phenotypes (57).

The production of isogenic derivatives of fluoroquinolone-resistant *grlA gyrA* double-mutant strain RA1 of *S. aureus*, which displayed widely different levels of SigB activity, allowed exploration of the potential interaction of the SigB-mediated and ciprofloxacin-triggered pathways. Combination of transcriptional and phenotypic assays provided indirect, although consistent, evidence that the SigB-mediated and the ciprofloxacin-triggered responses involve separate regulatory networks whose characterization is still incomplete. We recently reported (4) on the contribution of a RecA-LexA pathway on the induction of fibronectin binding via selective upregulation of the *fnbB* gene, which did not require any functional *agr* or *sarA* activities. This study extends those previous findings by showing that the SigB functional activity does not interfere with the ciprofloxacin-triggered transcriptional and phenotypic responses. While the strong induction of the *fnbB* gene by a sub-MIC of ciprofloxacin was confirmed in the SigB-modulated derivatives of strain RA1, the *fnbA* gene was also induced, although to a much lower extent. The ciprofloxacin-triggered *fnbA* induction may possibly result from the longer exposure of each strain with ciprofloxacin in this study compared to the shorter 20-min exposure in the previous study (4), which failed to significantly induce *fnbA*. Further studies are required to elucidate the molecular basis of the differential ciprofloxacin-triggered *fnbA* versus *fnbB* upregulation.

In contrast to the ciprofloxacin-triggered responses, those promoted by genetic modulation of SigB functional activity appear to be more complex at both the transcriptional and the phenotypic levels, as supported by a recent microarray-based analysis of the *S. aureus* SigB regulon (2). Under our experimental conditions, similar transcript levels were recorded for either *fnbA* or *fnbB* when those of *sigB* null strain TE1 were compared with those of its *rsbU* parent, strain RA1. In contrast, there was a sharp, although disproportionate, increase in *fnbB* transcript levels compared with *fnbA* transcript levels in *rsbU*$^+$-restored strain TE2 compared to those in its *rsbU* parent, strain RA1. The selective increase in *fnbB* transcript levels over *fnbA* transcript levels in the *rsbU*$^+$-restored strain TE2 compared to those in its *rsbU* parent, strain RA1, is an original observation whose molecular basis is unknown at present. The *fnbA* and *fnbB* transcriptional dose-response data contrasted with the smoother increase in the levels of FnBP surface display from strain TE1 to TE2 via RA1, as recorded by bacterial adhesion and flow cytometry assays. The molecular basis of these contrasting data is not understood, and its elucidation will require improved understanding of the SigB-controlled pathway and its interactions with other regulatory networks

controlling expression of fibronectin adhesins. Since expression of extracellular proteases was shown to be downregulated by high SigB and *sarA* functional levels (30) and upregulated by *agr* (42), we assayed in our set of SigB-modulated strains the levels of extracellular proteases that could potentially alter the half-lives of surface-exposed FnBPs (31, 36). The levels of proteolytic activity recorded in supernatants from 5-h cultures were too low to allow assessment of strain-specific differences. While qRT-PCR data indicated a decreased level of expression of the V8 protease gene *sspA* in the SigB-restored strain TE2 compared to that in the nonrestored isogenic derivatives, the extracellular protease release seems to be too marginal to play a major role in 5-h cultures. Previous reports (28, 30, 31) have shown that production of proteases mainly occurs during the late exponential and postexponential phases of growth. Thus, our initial hypothesis that strain-dependent differences in the production and extracellular release of proteases may account for the lack of correlation between *fnb* transcription and fibronectin adhesion was not supported by our experimental data. This discrepancy between *fnb* transcript levels and FnBP surface display may possibly result from differences in *fnbA* and/or *fnbB* mRNA decay between strain TE2 and strain RA1 or TE2 or from the presence of saturating amounts of cell wall-anchored FnBP molecules that may be displayed on bacterial cell surfaces.

The potential contribution of the major global regulators *agr* and *sarA* to increased levels of both *fnb* transcripts in the *rsbU*$^+$-restored strain TE2 compared to those in strains RA1 and TE1 was also evaluated. It should be emphasized that transcript levels from global regulators and their putative target genes from 5-h cultures grown without shaking cannot be directly compared with those from cultures grown with rotatory shaking, whose growth rates and final biomasses are much higher. In contrast to *agr* RNAII levels, which were equivalent in all three strains, RNAIII levels were reduced by less than 1 order of magnitude in strain TE2 compared to the levels in strains RA1 and TE1, thus confirming the previously reported downregulation of the *agr* response regulator in strains displaying fully functional levels of SigB compared to its expression in their SigB-defective derivatives (3, 28). While the decreased RNAIII levels in strain TE2 compared to those in strains RA1 and TE1 may explain, at least in part (17), the changes in *hla* and *spa* transcript levels recorded for strain TE2 compared to those recorded for strains RA1 and TE1, the molecular details of the RNAIII-mediated downregulation of each *fnb* gene are still unknown. Since no selective effect of RNAIII on *fnbB* transcript levels compared to the effect on *fnbA* transcript levels has yet been reported, it is likely that other global regulatory systems or transcription factors may play a significant role in this complex regulatory process (1, 10, 42).

The impact of SigB functional levels on the activities of the *sarA* regulon is controversial. While some studies indicate a SigB-promoted upregulation of *sarA* transcription (2, 3, 23) and translation (24), other studies indicate no change in SarA protein levels (28) or even a SigB-promoted downregulation of *sarA* (12). These conflicting observations may potentially arise from either technical variables or significant differences in the genetic backgrounds of the strains examined. In our study, overall *sarA* transcript levels determined by real-time RT-PCR were increased by less than twofold in *rsbU*$^+$-restored strain TE2 compared to the levels in strains RA1 and TE1. Nevertheless, it is unlikely that increased *sarA* transcript levels may account for increased *fnbB* mRNA levels, in particular, because it was established previously (61, 63) that *sarA* upregulates *fnbA* transcription but not *fnbB* transcription.

In conclusion, the results presented here provide evidence that optimal expression of a stress response factor and triggering of a drug-induced DNA repair system may independently, but in an additive manner, lead to an impressive >30-fold increase in *fnbB* transcript levels and promote *S. aureus* attachment to fibronectin. Ongoing studies performed in our laboratory also provide preliminary evidence that increased levels of FnBP expression in the SigB-restored strain TE2 grown in the presence of ciprofloxacin can upregulate FnBP uptake by nonprofessional phagocytes (A. Renzoni, unpublished data). Further studies of SigB-regulated pathways by using combined approaches of transcription profiling, targeted mutagenesis, and functional assays are required to better understand how multiresistant clinical isolates may benefit from the highly flexible regulation of *S. aureus* colonization and virulence.

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