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A RecA-LexA-dependent Pathway Mediates Ciprofloxacin-induced Fibronectin Binding in *Staphylococcus aureus**

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Subinhibitory concentrations of ciprofloxacin (CPX) raise the fibronectin-mediated attachment of fluoroquinolone-resistant *Staphylococcus aureus* by selectively inducing *fnbB* coding for one of two fibronectin-binding proteins: FnBPB. To identify candidate regulatory pathway(s) linking drug exposure to up-regulation of *fnbB*, we disrupted the global response regulators *agr*, *sarA*, and *recA* in the highly quinolone-resistant strain RA1. Whereas *agr* and *sarA* mutants of RA1 exposed to CPX still displayed increased adhesion to fibronectin, the CPX-triggered response was abolished in the *uvs*-568 *recA* mutant, but was restored following complementation with wild type *recA*. Steady-state levels of *recA* and *fnbB*, but not *fnbA*, mRNA were co-coordinately increased >3-fold in CPX-exposed strain RA1. Electrophoretic mobility shift assays revealed specific binding of purified *S. aureus* SOS-repressor LexA to *recA* and *fnbB*, but not to *fnbA* or *rpoB* promoters. DNase I footprint analysis showed LexA binding overlapping the core promoter elements in *fnbB*. We conclude that activation of *recA* and derepression of *lexA*-regulated genes by CPX may represent a response to drug-induced damage that results in a novel induction of a virulence factor leading to increased bacterial tissue adherence.

Staphylococcus aureus is an important cause of infections that prolong the mean length of hospital stays and increase mortality significantly (1, 2). Implanted biomaterials, such as indwelling catheters and orthopedic devices, also become rapidly coated with plasma proteins, predominantly fibrinogen and fibronectin (2). Adhesion of *S. aureus* to these extracellular matrix or coated implants is a crucial step in the early stage of infection. *S. aureus* and many other bacterial species express surface adhesins collectively called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)[1] that specifically recognize particular plasma or extracellular matrix proteins (3). Of these, two distinct, but related, fibronectin-binding protein genes in *S. aureus*, *fnbA* and *fnbB*, have been cloned and analyzed. These two adjacent genes appear partly redundant because both must be inactivated to eliminate fibronectin adhesion (4).

Several complex regulons, notably *agr* and *sarA*, regulate fibronectin-binding proteins (FnBPs) and other surface proteins (5). During the exponential phase, *sarA* can up-regulate *fnb* genes, whereas in contrast, *agr*, which is activated by an octapeptide quorum-sensing signal, down-regulates *fnb* genes during the post-exponential phase by a short regulatory RNA, RNAIII (5). Studies also suggest that *sigB*, which encodes a stress σ factor, σ^B (6), is involved in the regulation of both the *sarA* and *agr* loci (7–9). Fibronectin-binding protein expression therefore remains incompletely explained and is a consequence of complex interplay of multiple regulatory elements.

Subinhibitory concentrations of various antibiotics on *S. aureus* can also affect the production of virulence factors such as FnBPs (10, 11), collagen-binding protein (12), or α-toxin (13, 14). The acquisition of drug resistance determinants, such as the methicillin resistance mec-element, may alter the expression of surface adhesins (15).

Fluoroquinolones are widely used clinical antibiotics, but shortly after their introduction, resistant strains of *S. aureus* appeared, in particular among methicillin-resistant strains (16). High level resistance to fluoroquinolones in *S. aureus* involves combined mutations in two distinct chromosomal loci: *grlA*, the gene coding for the topoisomerase IV A subunit GrlA, and in *gyrA*, the gene coding for DNA gyrase A subunit GyrA (16). Recently, we showed that the exposure of a *grlA gyrA* mutant of *S. aureus* to subminimal inhibitory concentrations (sub-MICs) of ciprofloxacin (CPX) significantly increased the surface FnBPs, and also concomitantly led to increased bacterial attachment to both *in vitro* fibronectin- and *ex vivo* coated polymethylmethacrylate coverslips (17, 18). This response to CPX was more robust in the *grlA gyrA* double mutant than in single *gyrA* or *grlA* mutants, or in their isogenic quinolone-susceptible parents. Increased adhesion resulting from growth in the presence of CPX was also observed in clinical isolates of fluoroquinolone-resistant, methicillin-resistant, and methicillin-sensitive (18) strains, which suggested a general, rather than strain-specific, response to the drug. Promoter fusions to luciferase suggested that CPX preferentially up-regulated *fnbB*, but not *fnbA in vivo*. The drug-induced effect on *fnbB* was abolished by rifampin, which further suggested that the cellular response to the drug was mediated at the transcriptional level.

Bacterial exposure to antibiotics may trigger several types of stress responses including the SOS response (19). Two major regulatory genes of the classical SOS response have been de-

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[1] The abbreviations used are: MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; CPX, ciprofloxacin; FnBP, fibronectin-binding protein; MIC, minimal inhibitory concentrations.

TABLE I
Strains and plasmids used in this study

	Relevant characteristics	MIC of CPX	Source/Ref.
		μg/ml	
Strain			
S. aureus			
ISP794	8325 *pig-131*	0.25	28
SS1	*gyrA gyrB*142	0.25	17
EN1252a	*grlA*542 *gyrA*Ω1051	32	17
ISP2272	8325–4, *uvs-568* Ω(Tn*551*)1074	ND	29
BF10	ISP794, *uvs-568* Ω(Tn*551*)1074	<0.06	30
CYL316	RN4220 pYL112Δ19 (L54a integrase)	ND	31
CYL316 $recA^{+}_{attB}$	CYL316 *geh*::pCB1	ND	This study
CYL316 $pCL84_{attB}$	CYL316 *geh*::pCL84	ND	This study
CYL316 $recA(+5)_{attB}$	CYL316 *geh*::pCB2	ND	This study
RA1	8325 *gyrB*142 *grlA542 gyrAΩ105*	32	This study
RA1*recA*	RA1, *uvs-568* Ω(Tn*551*)	2	This study
RA1*recA* pYL112Δ19	RA1, *uvs-568* Ω(Tn*551*) pYL112Δ19	2	This study
RA1*recA*$pCL84_{attB}$	RA1, *uvs-568* Ω(Tn*551*) *geh*::pCL84	2	This study
RA1*recA*/$recA^{+}_{attB}$	RA1, *uvs-568* Ω(Tn*551*) *geh*::pCB1	32	This study
RA1*recA*/$recA(+5)_{attB}$	RA1, *uvs-568* Ω(Tn*551*) *geh*::pCB2	2	This study
RA1Δ*agr*::*tetM*	*agr* null mutant, Tc^{r}	32	This study
RA1*sarA*::Tn*917*LTV1	*sarA* null mutant, Em^{r}	32	This study
RA1*sarA*::kan	*sarA* null mutant, Kan^{r}	32	This study
RA1*recA, sarA*::kan	RA1, *uvs*-568Ω(Tn*551*) *sarA*::kan	2	This study
Plasmids			
pFNBB6	Cm^{R}, Amp^{R}, *fnbB* promoter fused to *luxAB*		4
pCL84	Sp^{R}, Tc^{R}, pSC101 low copy replicon		31
pYL112Δ19	Cm^{R}, Amp^{R}, L54a Integrase plasmid		31
pBluescript II KS+	Amp^{R}, cloning vector		Stratagene
pGEM-T	Amp^{R}, cloning vector		Promega
pET20b+	Amp^{R}, T7 expression vector		Novagen
pCB1	Sp^{R}, Tc^{R}, pCL84 $recA^{+}$		This study
pCB2	Sp^{R}, Tc^{R}, pCL84 *recA*(+5)		This study
pCB3	Amp^{R}, pET20b+ *lexA*		This study
pCB4	Amp^{R}, p*recA*-pBluescript II KS+		This study
pCB5	Amp^{R}, p*fnbB*-pBluescript II KS+		This study
pCB6	Amp^{R}, p*fnbA*-pBluescript II KS+		This study
pCB7	Amp^{R}, p*rpoB*-pBluescript II KS+		This study
pCB8	Amp^{R}, p*sigB*-pGEM-T Easy		This study

scribed in detail: *lexA* (also called *dinR* in *Bacillus subtilis*) and *recA* (20–22). Classically, derepression of the SOS-response genes occurs when RecA, in response to genotoxic damage, is activated. RecA then serves as a coprotease to aid LexA repressor autocleavage thus provoking the subsequent induction of an ensemble of DNA repair and recombination genes. LexA affinity for each targeted promoter is variable and some genes may be partially induced, whereas others remain repressed until high or persistent DNA damages occur (23). Thus, the SOS response may represent a graded monitor of the inducing environmental stress rather than a simple on-off switch (22, 24).

Because fluoroquinolones are DNA-damaging agents, DNA repair mechanisms and/or homologous recombination are likely to be activated. Whereas sporadic reports provided evidence of SOS induction by fluoroquinolone exposure in various bacterial species, the predominant use of high and rapidly bactericidal antibiotic concentrations limited the physiological significance of these observations (25–27).

In this study, we sought to identify molecular pathway(s) that linked sub-MICs CPX exposure to transcriptional up-regulation of FnBP(s) expression in highly fluoroquinolone-resistant strains. Combined genetic and biochemical approaches suggest that both RecA and LexA are specifically implicated in the mechanism linking fluoroquinolone exposure and *fnbB* virulence factor up-regulation. Importantly, our results suggest that the LexA-SOS regulon in *S. aureus* comprises more than genes strictly involved in recombination and DNA repair and extends to include a novel regulation of an MSCRAMM virulence factor.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—Bacterial strains and plasmids are listed in Table I. *S. aureus* and *Escherichia coli* were propagated in Mueller-Hinton broth/agar (Difco, Detroit, MI) and LB (Luria-Bertani) broth/agar, respectively. Antibiotics used were: ampicillin (50 μg/ml), erythromycin (5 μg/ml), chloramphenicol (10 μg/ml), kanamycin (50 μg/ml), spectinomycin (25 μg/ml), tetracycline (3 μg/ml), or novobiocin (3–10 μg/ml).

Functional Binding Assay of Fibronectin Adhesins—Strains were grown for 5 h at 37 °C in the presence, or absence, of ⅛ of the MIC of CPX, except where indicated, as described (17). Results were evaluated using the Kruskal-Wallis test and the Dunn procedure for comparison of specific groups (32). Comparisons were considered significant when all increases or decreases accumulated for three coating concentrations of fibronectin yielded p values <0.05 with two-tailed significance.

Quantitative Steady-state mRNA Analysis—Total RNA was prepared using the RNeasy™ midi-Kit (Qiagen), or the FastRNA kit Blue (Bio 101, Inc.) in conditions minimizing RNA degradation (33). RNA was quantified using the Platinum™ quantitative reverse transcriptase-PCR kit (Invitrogen). PrimerExpress (Applied Biosystems) was used to design primers and probes, which were used at 0.2 and 0.1 μM, respectively. The primer sets were: 5′-caccgaaaactgtgcaagca and 5′-ttcctgtagtttccttatcagcaactt for *fnbB*; 5′-acaagttgaagtggcacagcc and 5′-ccgctacatctgctgatcttgtc for *fnbA*; 5′-agactcagttgctgctttaacacct and 5′-tacgtaacgcttgtgacattaaacg for *recA*; and 5′-ggcaagcgttatccggaatt and 5′-gtttccaatgaccctccacg for the 16 S rRNA of *S. aureus*. 5′-FAM and a 3′-TAMRA derivatized probes were: 5′-tagaaacttcgcgagttgatttgccatcg for *fnbB*; 5′-agaacggcatcagaaagtaagccacgtg for *fnbA*; 5′-aaggagaaatgggagacactcacgttggt for *recA*; and 5′-cctacgcgcgctttacgccca for 16 S rRNA. Data were acquired on an ABI Prism 7700 and analyzed with Sequence Detector (Applied Biosystems). Total RNA in each sample was normalized to 16 S rRNA.

Genetic Manipulations—Transduction was performed using bacteriophage $\phi 85$ or $\phi 80\alpha$. Transformation of genomic DNA and electropora-

tion were performed as described (28, 34). RA1 was constructed using high molecular weight DNA prepared from SS1 to transform EN1252a. Transformants were screened for loss of erythromycin resistance but retention of novobiocin resistance. The removal of the erythromycin marker exerted no significant influence on *fnbB* gene activation and promotion of fibronectin-mediated adhesion by ⅛ of the MIC of CPX. The *recA uvs-568* allele (29) was transduced from strain BF10 into RA1 by a tight association (95%) with Tn*551.* Strains ALC355, ALC637, and ALC2057 were used to transduce Δ*agr*::*tetM*, or *sarA*::Tn*917*LTV1 and *sarA*::kan, respectively, to RA1. Genotypes were verified using a PCR assay.

For chromosomal insertions, plasmids pCL84, pCB1, or pCB2 were first transferred into CYL316 (31). Plasmid *geh* integrates were then transferred by generalized transduction with phage ϕ85 to strain RA1, followed by a second step transfer of *uvs*-568. Transductants were screened for failure to express lipase activity with Bacto Spirit Blue Agar (Difco). UV sensitivity was evaluated with increasing UV (254 nm) from 0 to 5 s using an XL1000 Spectrolinker (Spectronics Corporation) at 15 cm and 7200 μJ cm^{-2}.

Recombinant DNA Methods—Genomic DNA from RA1 was purified as described (18). The *recA*$^+$ gene was PCR amplified using primers: 5′-cgggatcccgaagattattaaattggcttagaaca-3′ and 5′-cggaattccgctactatttttctaaagttttgaagc-3′ corresponding to nucleotides −156 to −131 and +1261 to +1286, respectively, of contig 8104 in the TIGR *S. aureus* COL data base.[2] EcoRI and BamHI (underlined) sites were incorporated in the primers and the PCR product was cloned into EcoRI-BamHI-cleaved pCL84 to yield plasmid pCB1. The *recA* sequence was verified from two independent isolates and was identical to the composite *recA* sequence published previously (29) except for one silent mutation in codon 260. Plasmid pCB2 was constructed using the QuikChange™ method (Stratagene). Complementary primers that inserted 5 bp, including an EcoRI restriction site resulting in a frame shift from RecA-codon 34, were: (5′-gtgacaatataggtgaattcgccgagtttcaac-3′ and 5′-gttgaaactcggcgaattcacctatattgtcac-3′.

The *S. aureus lexA* gene was PCR-amplified using a 5′ primer that introduced the NdeI restriction site (underlined) at the initial ATG codon 5′-cgggaattccatATGagagaattaacaaaacgac-3′. The downstream primer contained a XhoI site (underlined), 5′-ccgctcgagcggttacatttcgcggtacaaaccaattac-3′. The product was cleaved with NdeI and XhoI and ligated with pET20b+ (Novagen). The expression clone was sequence verified.

The promoters of *recA*, *rpoB*, *fnbA*, and *fnbB* were PCR-amplified using primers that incorporated upstream EcoRI and downstream SmaI, or *Bam*HI, sites (underlined). Amplified product sizes are indicated: 5′-ggaattccttggcttagaacaacaaattaattg-3′ and 5′-tccccgggggataaagctttttgacgatcgttatcc-3′ for p*recA* (230 bp); 5′-cgggatccgagcttgaaatgaaatggatattctg-3′ and 5′-cggaattccagattcacccctcaaaaattatgt-3′ for p*rpoB* (238 bp); primers for FnbA and FnbB were as described (4) and yielded 425- and 465-bp products, respectively. PCR products were digested and were cloned in pBluescript II KS+ (Stratagene). All promoter clones were sequence verified.

Luciferase Assays—Luciferase activity was measured as described (4, 18). The specific light units were determined by normalization of the relative light units by the $A_{540\ nm}$ of the culture at the time of sampling.

Purification of S. aureus LexA—BL21 (λDE3)/pLysE (Novagen) carrying pCB3 was diluted 1:100 into LB broth supplemented with ampicillin (50 μg/ml) and grown at 37 °C until A_{600} = 0.8. Cells were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, grown for an additional 3 h, harvested, and then resuspended in ice-cold Buffer A (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 10% sucrose). Lysozyme was added to 0.2 mg/ml and the cells were incubated on ice for 30 min and then quick frozen in liquid nitrogen. Cells were thawed on ice, and phenylmethylsulfonyl fluoride and β-mercaptoethanol were added to 1 and 2 mM, respectively. The lysate was sonicated to reduce viscosity, then centrifuged at 100,000 × *g* in a Beckman 60 Ti rotor for 30 min. Polyethyleneimine was added to a final concentration of 1% (v/v) and then centrifuged at 15,000 × *g* (Sorvall HB6) for 10 min. The supernatant was recovered and solid ammonium sulfate (0.331 g/ml, 55% w/v) was added. The extract was centrifuged at 15,000 × *g* (Sorvall HB6) for 15 min and the pellet was resuspended in a minimal volume of ice-cold Buffer B (20 mM Tris-Cl, pH 7.5, 0.2 M NaCl, 10% (v/v) glycerol, 5 mM β-mercaptoethanol (Fraction I). Fraction I was loaded onto a 15-ml heparin-Sepharose column (Amersham Biosciences) equilibrated in Buffer B. The flow-through was collected and dialyzed against 1 liter of Buffer C (20 mM potassium phosphate, pH 7.2, 0.5 M NaCl, 0.1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol). One and a half volumes of Buffer C without NaCl was added to the dialysate and the solution was loaded immediately onto an 80-ml P11-phosphocellulose (Whatman) column equilibrated in Buffer D (20 mM potassium phosphate, pH 7.2, 0.2 M NaCl, 0.1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol). The column was washed with 5 bed volumes of Buffer D, then eluted with a linear gradient 0.2 M to 1 M NaCl in Buffer D. The majority of LexA eluted at 0.4 M NaCl (Fraction II). Peak fractions were pooled, diluted with 2 volumes of Buffer D, and immediately applied to a hydroxyapatite column (20 ml) equilibrated in Buffer D. The column was washed with 5 bed volumes of Buffer B, then LexA was eluted with a linear gradient of 20 mM to 0.4 M potassium phosphate, pH 7.2, in Buffer D. The majority of LexA eluted at 0.2 M potassium phosphate (Fraction III). Peak fractions were pooled, dialyzed overnight against 2 liters of Buffer E (10 mM HEPES-NaOH, pH 7.5, 0.2 M NaCl, 0.1 mM EDTA, 10% glycerol), and concentrated using Centriprep-10 columns (Millipore). The dialysate was adjusted to 50% glycerol and stored at −80 °C. Protein concentrations were determined using Bradford assay and bovine serum albumin standards. The Fraction III protein was greater than 95% pure as judged by Coomassie Blue-stained SDS-PAGE gels. The specific activity (3000 units/mg) was defined as the amount of LexA required to gel shift 5 fmol of radiolabeled p*recA* promoter fragment.

Electrophoretic Mobility Shift Assays—DNA fragments were generated by digesting plasmids pCB4, pCB5, pCB6, pCB7, and pCB8 with HpaII, or with HpaII and SapI for pCB5. The fragments were 3′-radiolabeled with [^{32}P]dCTP. Labeled fragments were incubated with the indicated amounts of purified LexA in binding buffer (40 mM Tris acetate, pH 7.5, 4 mM magnesium acetate, 50 mM potassium glutamate, 2 mM β-mercaptoethanol, 0.1 mM EDTA) containing 1 μg of poly (dI-dC)·poly(dI-dC) (Amersham Biosciences) in a final volume of 50 μl. Binding reactions were incubated for 10 min at 37 °C, then analyzed on 6% polyacrylamide gels and autoradiographed. For quantitative gel shifts 10 pM of each promoter fragment prepared by PCR was 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase to a specific activity of 10^7 cpm/μg. LexA was serially diluted in binding buffer supplemented with 100 μg/ml gelatin.

DNase I Footprinting—The promoters of *recA* and *fnbB* were re-PCR amplified from pCB4 and pCB5. Fragments were 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and divided into two aliquots. pCB4 was digested with ClaI, or HindIII; pCB5 was digested with HincII, or DdeI. Fragments were gel purified and desalted. LexA binding reactions were assembled on ice as described for gel shift analysis. DNase I (Amersham Biosciences) conditions were titrated in pilot cleavage reactions. Reactions were terminated by adding 12.5 mM EDTA, pH 8.0, 0.5% (w/v) SDS, and Proteinase K at 0.4 mg/ml, extracted with phenol-chloroform, and ethanol precipitated using 0.4 mg/ml glycogen carrier. DNA pellets were resuspended in loading buffer (98% deionized formamide, 10 mM EDTA, pH 8.0, 0.025% (w/v) xylene cyanol FF, and 0.025% (w/v) bromphenol blue), applied on 6% sequencing gels, dried, and autoradiographed. Dideoxy sequencing marker ladders for each fragment were run in parallel and prepared using the same primers as for PCR.

RESULTS

Inactivation of recA, but Not agr or sarA, Abolishes the CPX-promoted Fibronectin Adhesion of Fluoroquinolone-resistant S. aureus—To evaluate the role of global regulators *agr* and *sarA* on drug-induced enhanced attachment to fibronectin, null mutants in strain RA1 were tested in the absence or presence of subinhibitory levels of CPX. We observed that promotion of fibronectin-mediated adhesion by growth in the presence of ⅛ of the MIC of CPX (4 μg/ml) was significant ($p < 0.05$) and equivalent when comparing strains RA1 and RA1 Δ*agr*::*tetM* (hereafter RA1*agr*) after growth in the presence of antibiotic (Fig. 1).

Whereas adhesion of RA1 *sarA*::Tn*917*LTV1 (hereafter RA1*sarA*) after growth in CPX-free medium was markedly lower than adhesion of either RA1 or RA1*agr*, we observed that the CPX-promoted increase in fibronectin-mediated attachment was higher with strain RA1*sarA* (about 7-fold) than with strains RA1 or RA1*agr* (about 2-fold) (Fig. 1). The lower relative adhesion of RA1*sarA* when grown in CPX-free medium compared with strains RA1 and RA1*agr* can be explained, in

[2] www.tigr.org.

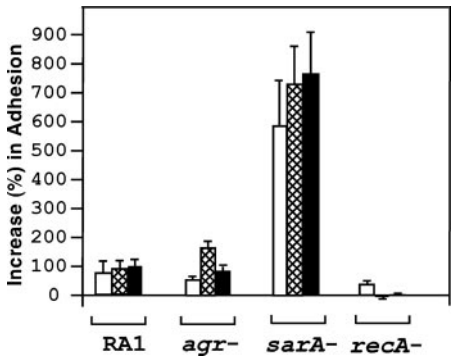

FIG. 1. **Increased adhesion (%) on fibronectin-coated coverslips of strains RA1, RA1*agr*, RA1*sarA*, and RA1*recA* after growth in the presence of 1/8 of the MIC of CPX.** Amount of fibronectin on coverslips: 96 (*white*), 199 (*hatched*), and 301 ng (*black*). Results are the mean ± S.E. of at least three independent experiments.

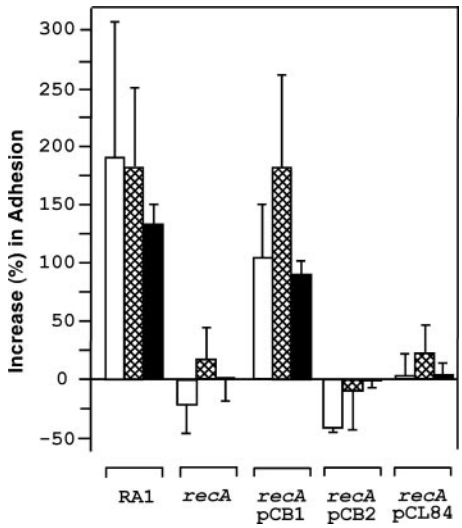

FIG. 2. **Increased adhesion (%) on fibronectin-coated coverslips of strains RA1, RA1*recA*, RA1*recA*/pCB1*attB*, RA1*recA*/pCB2*attB*, and RA1*recA*/pCL84*attB* after growth in the presence of ⅛ of the MIC of CPX.** 96, *white*; 199, *hatched*; and 301 ng, *black*. Results are the mean ± S.E. of three independent experiments.

part, by (*a*) the previously reported down-regulation of *fnbA* transcription in a *sarA* mutant of *S. aureus* (35), and also by (*b*) the derepression of *sar*A-regulated extracellular proteases known to accelerate degradation of cell surface proteins such as FnBP (36). We also observed a stimulation of the *fnbB* promoter in RA1*sarA* strain with ⅛ of the MIC of CPX using an independent p*fnbB*-luxAB reporter assay (data not shown). Collectively, we conclude that insertional inactivation of either *agr* or *sarA* does not abolish CPX-induced *fnbB* expression.

The contribution of *recA* to the CPX-induced up-regulation of FnBP was analyzed next. The introduction of the *uvs*-568 *recA* mutation in strain RA1 (hereafter RA1*recA*) led to increased UV sensitivity (data not shown) and decreased fluoroquinolone resistance, as assessed by a 16-fold decrease in CPX MIC in RA1*recA* compared with RA1, RA1*agr*, and RA1*sarA*. Inactivation of *recA* did not significantly alter its adhesion profile compared with its parent RA1 in the absence of drug treatment (data not shown). However, when RA1*recA* was grown in the presence of either ¼ (not shown) or ⅛ of the MIC of CPX, drug-dependent promotion of fibronectin-mediated adhesion was abolished (Fig. 1). This result suggested that *recA* was involved in the mechanism linking CPX exposure to induction of a virulence adhesion. We also tested a RA1*sarA*/*recA* double mutant constructed using an alternative *sarA*::kan null allele that was phenotypically comparable in RA1 to the *sarA*::Tn*917*LTV1 mutant (not shown). Our results showed that the double mutant, RA1 *sarA*/*recA*, behaved similarly to the RA1*recA* single mutant alone. In the presence of ⅛ of the MIC of CPX, drug-dependent promotion of fibronectin-mediated adhesion was abolished with the double mutant (data not shown). Importantly, this result indicated that the *recA* mutation could abolish the pronounced increase in CPX-promoted fibronectin adhesion brought about by the *sarA* mutation alone and pointed to *recA* as a predominant mediator of CPX-induced changes in adhesion.

The specific role of *recA* was further examined using RA1*recA* complemented with cloned *recA* reinserted in the chromosome (Fig. 2). Previous attempts to stably clone the entire *recA* gene from *S. aureus* were unsuccessful (29). To minimize potential toxicity resulting from *recA* carried on high copy plasmid, we cloned *recA* using a very low-copy plasmid, pCL84, which is also used as a suicide vector for directing integration into the lipase gene (*geh*) of *S. aureus* by bacteriophage L54a integrase (31). The plasmid pCB1 carrying the entire *recA* gene, including 200 bp of upstream promoter sequence, could be stably maintained in *E. coli* DH5α. Subsequent chromosomal integration of pCB1 into the *geh* gene *att* site of RA1*recA* resulted in strain RA1*recA*/pCB1$_{attB}$, which showed an equivalent UV resistance phenotype (data not shown) and CPX MIC (32 μg/ml) to that of parental *recA*$^+$ strain RA1.

When we examined the fibronectin adhesion phenotypes, we observed that complemented expression of RecA in strain RA1*recA*/pCB1$_{attB}$ led to restoration of the CPX-induced adhesion response to the level of parental strain RA1 (Fig. 2). To further substantiate the role of RecA and to eliminate the trivial possibility of vector sequences contributing to the observed complementation, we also tested two RA1*recA* derivatives carrying either the empty vector pCL84, or pCB2 that contained a frame-shifted copy of *recA*. We observed that neither RA1*recA*/pCL84$_{attB}$ nor RA1*recA*/pCB2$_{attB}$ showed any CPX-induced adhesion response (Fig. 2). We conclude that a functional copy of *recA* is essential for CPX-induced adhesion to fibronectin.

Subinhibitory Concentrations of CPX Coordinately Increase Transcription of Both recA and fnbB Genes in Strain RA1—In previous work, we reported that CPX-induced fibronectin adhesion was correlated with up-regulation of *fnbB*, but not *fnbA*, as judged *in vivo* by luciferase-linked promoter fusion assay (18). Because the CPX effect on FnbB was sensitive to rifampin, we reasoned that CPX induction was regulated, in part, at the transcriptional level. To examine coordinate CPX induction of promoters in greater detail, we next quantified the impact of CPX on the steady-state transcription levels of *recA* and *fnb* genes in exponential growth phase of RA1 by quantitative real time reverse transcriptase-PCR. These experiments showed that *recA* and *fnbB*, but not *fnbA* mRNA, steady-state levels increased by 5.8 ± 0.9 and 3.1 ± 1.3-fold, respectively, in cells exposed for 20 min to ⅛ of the MIC of CPX compared with control cells (Fig. 3). These data strongly suggested that RecA was a key component of the regulatory pathway(s) linking drug exposure to transcriptional up-regulation of *fnbB*. Furthermore, the coordinate up-regulation of both *recA* and *fnbB* by drug exposure is most easily explained by a *recA*-dependent SOS response and subsequent derepression of *lexA*-regulated genes. To test this hypothesis, we purified LexA to examine its specific binding to a series of *S. aureus* promoter fragments.

Purification of S. aureus LexA—The LexA repressor and the LexA regulon have not been previously described in *S. aureus*. A search of the *S. aureus* N315 nucleotide sequence data base

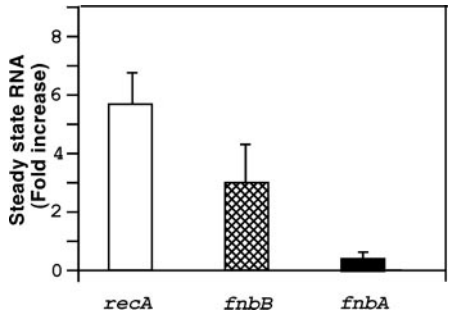

FIG. 3. **Steady-state mRNA levels of *recA* (*white*), *fnbB* (*hatched*), and *fnbA* (*black*) genes of strain RA1 measured by reverse transcriptase-PCR.** Results are expressed as -fold mRNA increase after 20 min of CPX treatment. The mean ± S.E. of at least three independent experiments are shown.

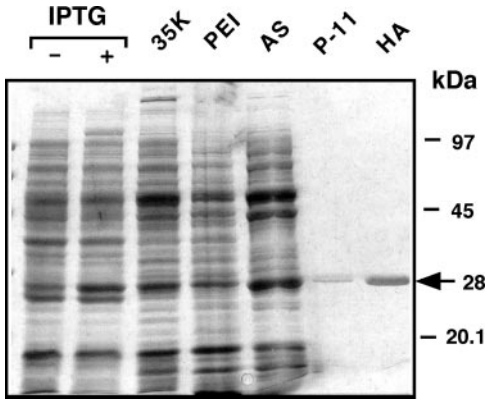

FIG. 4. **Coomassie Brilliant Blue-stained SDS-polyacrylamide gel (12%) showing the steps of the purification of recombinant *S. aureus* LexA in *E. coli*.** The apparent M_r of LexA is indicated. *35K*, high speed supernatant. *PEI*, polyethylenimine supernatant. *AS*, 55% ammonium sulfate pellet. *P-11*, phosphocellulose pooled fractions. *HA*, hydroxyapatite pooled fractions. Molecular weight marker mobilities are indicated. *IPTG*, isopropyl-1-thio-β-D-galactopyranoside.

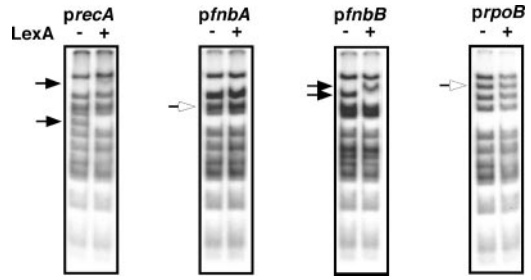

FIG. 5. **Electrophoretic mobility shift assay showing specific *S. aureus* LexA binding to p*recA* and p*fnbB* promoter fragments (*filled arrowheads*).** Promoter fragments that do not shift in the presence of LexA are indicated with *open arrowheads*.

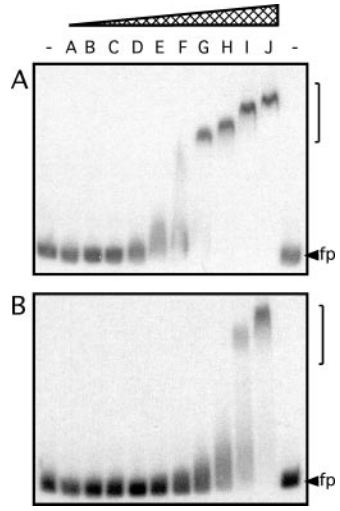

FIG. 6. **Electrophoretic mobility shift assay showing relative affinities of *S. aureus* LexA binding to p*recA* and p*fnbB* promoter fragments.** *A*, *precA*. *B*, p*fnbB*. *Fp* marks the position of free probe. A *dash* (–) indicates no protein control. *Lanes A–J* contain 5.4, 10.9, 21.8, 42.8, 87.5, 175, 350, 700, 1400, and 2800 ng of LexA, respectively.

revealed an open reading frame (SA1174) with strong predicted similarity (65.4%) to the DinR(LexA) repressor of *B. subtilis* (37). Conceptual translation of the putative *S. aureus* LexA opening reading frame predicted an acidic protein of 207 amino acids and M_r = 23,300 and pI = 5.05. The DNA coding sequence of SA1174 was PCR amplified, cloned, and sequence verified.

S. aureus LexA was overexpressed in *E. coli* and purified to homogeneity. The purified protein migrated with an apparent M_r = 28,000 on 12% SDS-PAGE gels (Fig. 4). The slightly retarded mobility of LexA relative to its predicted size has also been reported for *B. subtilis* DinR(LexA), but not for *E. coli* LexA (38). An aliquot of the purified protein was subjected to electrospray matrix-assisted laser desorption ionization time-of-flight mass spectroscopic analysis and showed the N-terminal tryptic peptide sequence: MRELTKR. This confirmed its identity as *S. aureus* LexA as well as its predicted start site. During the course of purification, we also observed that *S. aureus* LexA displayed a propensity to form insoluble aggregates if kept in low salt buffers (<200 mM NaCl). This aggregation tendency has also been described for *E. coli* LexA (38).

LexA Binding to recA and fnbB Promoters—The binding of purified LexA to the cloned promoter regions of five different *S. aureus* genes was examined *in vitro* using electrophoretic mobility shift assay. Plasmids carrying cloned *S. aureus recA*, *fnbA*, *fnbB*, or *rpoB* promoter fragments were first digested with HpaII, or HpaII and *Sap*I for pCB5, yielding a collection of multiple fragments from each plasmid ranging from 26 to 762 bp. The ensemble of fragments from each plasmid was 3′-end-labeled with [^{32}P]dCTP, titrated with increasing concentrations of purified LexA, and subjected to gel shift analysis. The results are shown in Fig. 5. We observed that LexA was able to bind specifically only to those DNA fragments corresponding to both *recA* and *fnbB* promoters. It is important to note that at this representative level of added protein (300 ng of LexA), and in the presence of excess nonspecific poly(dI-dC)·poly(dI-dC) carrier DNA, that no other radiolabeled vector fragments specifically bound to LexA. In contrast to p*recA* and p*fnbB*, no shift was observed when LexA was incubated with radiolabeled plasmid digest carrying the *fnbA*, or *rpoB* promoter that is not known to be under *lexA* control in either *E. coli* or the *B. subtilis* SOS response (21, 39). In all bacterial systems studied to date, RecA itself is known to be under LexA repression. We conclude from our analysis that both *recA* and *fnbB* promoters specifically bind LexA *in vitro*. Because we observed coordinate transcriptional up-regulation of both promoters *in vivo* following transient drug exposure, we also conclude that both genes comprise a portion of the *lexA* regulon in *S. aureus*. Importantly, the specific binding of LexA to the *fnbB*, but not the *fnbA*, promoter could account for the observed change in fibronectin binding by selective transcriptional up-regulation of only the *fnbB* promoter upon subinhibitory CPX administration.

We next examined the relative affinities of LexA for the *recA* and *fnbB* promoters by gel shift assay using DNA fragments radiolabeled to the same specific activity. The results are shown in Fig. 6. When we titrated LexA through successive 2-fold dilutions, we observed that the *fnbB* promoter fragment

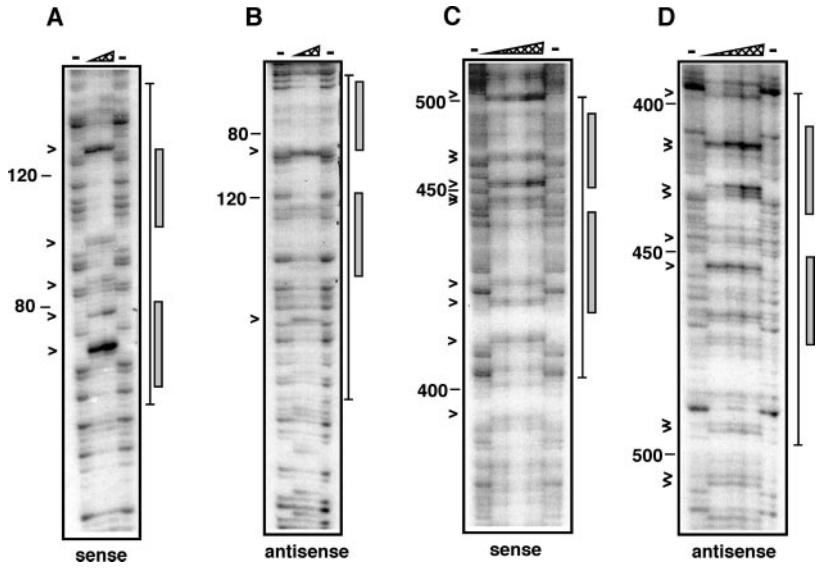

FIG. 7. **DNase I footprint assay of LexA with p*recA* and p*fnbB* promoter fragments.** *A*, p*recA* *Hind*III fragment (215 nucleotide); *B*, p*recA* ClaI fragment (194 nucleotide); *first* and *fourth lanes*, control DNase I digest without LexA; *second* and *third lanes*, 0.2 and 0.8 μg of LexA, respectively; *C*, p*fnbB* DdeI fragment (154 nucleotide); *D*, *pfnbB* HincII fragment (200 nucleotide). *First* and *fifth lanes 1*, control DNase I digest without LexA; *second*, *third*, and *fourth lanes*, 0.3, 0.5, and 0.7 μg of LexA, respectively. Dideoxy sequencing marker reactions were run in parallel (not shown). *Numbers* on the *left* correspond to the GenBank™ published sequence of *recA* and *fnbB* (respectively, AF317802 and X62992). Hypersensitive sites are shown with *arrows*, protected regions are delimited by *lines*, and LexA boxes are indicated by *gray rectangles*.

displayed an ~8-fold lower relative affinity for LexA compared with *recA* (compare Fig. 6, *A*, *lane G*, and *B*, *lane J*). In addition, we noted that the formation of a specific LexA-DNA complex was preceded by a detectable shift of the free probe, but with indistinct complex formation. This observation likely reflects the initial LexA monomer binding followed by the stable dimerization of LexA on DNA that has been described in other systems (21, 40). As LexA concentrations were increased, we observed an incremental increase in complex retardation in gels that probably reflects either the nonspecific aggregation tendency of LexA or binding to additional weak sites.

FnbB Promoter Mapping and the CPX Effect—The *fnbB* mRNA start site has been mapped by primer extension to position −50 upstream of the gtg start codon of FnbB in strain 8325-4 (4). We have identified the same mRNA start site in strain EN1252a (the parent strain of RA1 used in this study) using 5′ rapid amplification of cDNA ends (data not shown). Because our p*fnbB* reporter fusion containing 387 bp of upstream sequence fused to luciferase responds to CPX, we asked whether engineered mutants in the promoter region would abolish basal transcription and the CPX-induced effect, or whether the administration of CPX prompted the activation of a cryptic upstream promoter(s) on the same fragment. When we engineered separate mutations resulting in HindIII or EcoRI sites in the plasmid pFnBB6 at positions −9 and −21 upstream of the mRNA +1 without changing the phasing of −10 or −35 promoter elements, or the relative G/C content, we observed loss of all basal luciferase activity, indicating that the mutations had disrupted crucial elements of the p*fnbB* promoter (data not shown). Importantly, we also observed no increased luciferase reporter activity with these mutant promoters upon treatment with CPX (data not shown). We conclude from these results that CPX-induced transcriptional up-regulation of *fnbB*, and likely LexA-mediated repression, must act on the one mapped promoter contained within this fragment.

Footprinting LexA on precA and pfnbB—To examine the LexA binding precisely, we analyzed the LexA-DNA complexes *in vitro* by DNase I footprint assay on both the *recA* and *fnbB* promoters. Representative footprints on both coding and template strands of p*recA* and p*fnbB* are shown in Fig. 7. Composite data are summarized in Fig. 8. The strongly protected region on p*recA* (Fig. 7, *A* and *B*) corresponded to coordinates 67 to 146 of the *recA* upstream sequence. This region, encompassing >80 nucleotides, overlaps one predicted LexA binding site spanning coordinates 112–123, 5′-CGAACAAATATTCG-3′ on the p*recA* promoter. This predicted site was based on the *B. subtilis* DinR (LexA) consensus sequence, 5′-CGAACRNRYGTTYC-3′ and shows a 2-nucleotide mismatch (21). A second strong adjacent region of DNase I protection was also observed on the p*recA* promoter at coordinates 67–81, together with the appearance of DNase I-hypersensitive sites. This second protected region overlaps with a second consensus on the template strand, 5′-CGAACAAACGTGCT-3′, with a 2-nucleotide mismatch from the *B. subtilis* consensus. The precise start site of the *recA* message has not been mapped and coordinates are given relative to the position of the start codon.

When LexA binding to p*fnbB* was examined, we also observed an extended region of DNase I protection by LexA on *pfnbB* that corresponded to a region spanning nucleotides 411 to 474 (Figs. 7, *B* and *C*, and 8). Other protections were also observed that extended to coordinate 502. We observed that the major clear region of DNase I protection was positioned over predicted −10 and −35 elements of the *fnbB* promoter and included two sites, 5′-CGAACAATATAGAA-3′ and TGAAAAAAAGCGAGT-3, which were similar to, but contained multiple mismatches when compared with the *B. subtilis* DinR (LexA) consensus site (Table II). An additional LexA binding site more distantly related to the consensus site is positioned upstream of the ribosome binding site and may account for the observed weak protection on one strand extending to nucleotide 502 (Fig. 8). The existence of at least two observed LexA binding sites is consistent with mutational analysis. An engineered XhoI restriction site that disrupts and replaces the major contact point residues, GAAC (38), of the promoter proximal site (CGAACAATATAGAA) weakens, but does not entirely abolish LexA binding to p*fnbB* in gel shift assays (data not shown). We conclude from these analyses that the native promoters of p*recA* and p*fnbB* both bind LexA *in vitro* are both are coordinately responding to CPX-induced LexA derepression *in vivo* in strain RA1. We also conclude from this analysis that the LexA repressor most likely mechanistically interferes with the *fnbB* promoter by steric occlusion of RNA polymerase. The weak LexA binding to this promoter shown by gel shift analysis explains the strong basal expression of FnbB. CPX-induced full derepression of LexA results in significant detectable transcription up-regulation with the concomitant physiological consequence of measurably altered fibronectin-adhesion.

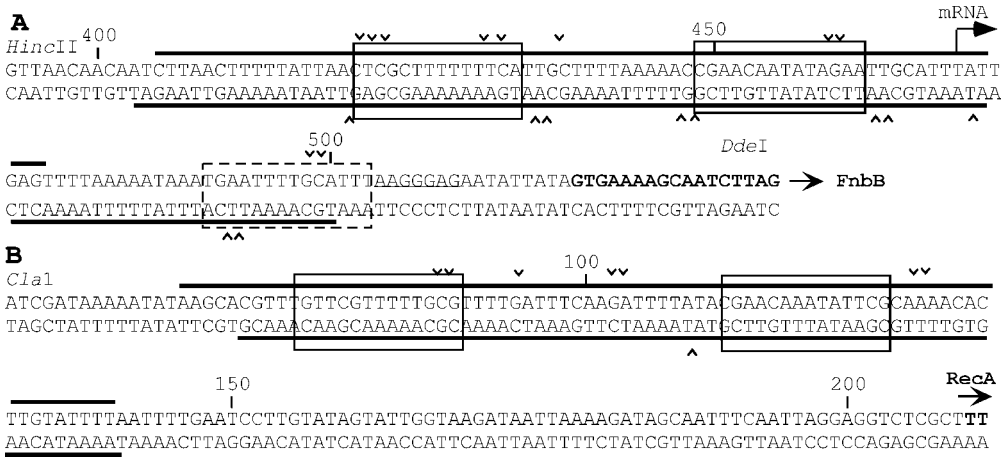

FIG. 8. **Composite summary of footprinting data on the *fnbB* (*A*) and *recA* (*B*) promoters.** DNase I protected regions are marked by *solid bars above* and *below* the sequence. DNase I-hypersensitive sites are marked by *circonflex*. Coordinates are indicated *above* each sequence and correspond to the legend of Fig. 7. The position of the mapped *fnbB* mRNA start site is indicated. LexA sites are indicated by *solid boxes*. The beginning of each protein coding region is indicated in *bold*.

TABLE II

Comparison of LexA binding sites in B. subtilis and S. aureus

The *B. subtilis* experimentally determined sites and consensus have been published (21). Our experimentally determined *S. aureus* LexA binding sites are indicated for *recA* and *fnbB* and are closely related to the published *B. subtilis* consensus. Additional putative LexA boxes are indicated that are positioned in promoter elements of likely orthologs of SOS-regulated genes in *S. aureus.*

B. subtilis lexA (−39)	CGAACCTATGTTTG
B. subtilis lexA (−67)	CGAACAAACGTTTC
B. subtilis lexA (−104)	GGAATGTTTGTTCG
B. subtilis recA	CGAATATGCGTTCG
B. subtilis LexA consensus	CGAACRNRYGTTYC
S. aureus recA-1	CGAACAAATATTCG
S. aureus recA-2	CGAACAAACGTGCT
S. aureus fnbB-1	CGAACAATATAGAA
S. aureus fnbB-2	TGAAAAAAAGCGAG
S. aureus lexA	CGAACAAATGTTTG
S. aureus recQ	CGAACGATTAAAAT
S. aureus recN	CGAAGCAAAGAGGC
S. aureus uvrA	CGAAAGATTTAGAT
S. aureus uvrB	CGAACAAACGTTTG
S. aureus uvrC	CGAAGATGTTGATT

DISCUSSION

This study reveals a novel link between a fluoroquinolone-triggered SOS response and the up-regulation of a specific fibronectin binding adhesin virulence factor in *S. aureus*. The inclusion of *fnbB* in the LexA regulon explains increased selective transcription of *fnbB* that leads to increased fibronectin adhesion. Our study also showed that several global gene regulators *agr* and *sarA* are not involved in CPX-induced FnbB. Recent work from our laboratory also indicates that *sigB*, an alternative σ factor involved in stress response, is not involved in CPX-induced FnbB (41).

The SOS response and the ensemble of LexA-regulated genes have not been previously studied in *S. aureus*. Nearly all known genes in the SOS response in *E. coli* and *B. subtilis* under LexA control are involved in DNA repair, recombination, and cell division arrest (22–24, 39). None have been described as virulence factors affecting extracellular matrix adhesion. Sequence similarity searches performed with *S. aureus* (37) revealed many putative SOS-response genes compared with their counterparts in *E. coli* or *B. subtilis* (23, 24). For example, and based upon our experimentally derived consensus, putative *S. aureus* LexA boxes are found in the promoter regions of genes *lexA*, *recQ*, *recN*, *uvrA*, *uvrB*, and *uvrC*. It is likely that most elements of the SOS response are conserved in *S. aureus*.

Nalidixic acid, the chemical backbone of derivative fluoroquinolones, has been shown to induce *recA* in *B. subtilis* and thus the ability of fluoroquinolones *per se* to trigger an SOS response in *S. aureus* is not unexpected (42). The exact drug-dependent triggering mechanism of RecA and the SOS response in our system remains unknown and it is unclear why the specific up-regulation of *fnbB* was observed in double *grlA gyrA* mutants of both clinical and laboratory isolates of *S. aureus* rather than in single *gyrA*, *grlA*, or fluoroquinolone-susceptible strains. One possible triggering mechanism is that particular tertiary drug-enyzme complexes on DNA that predispose, or sensitize, to double strand breaks (30). Preliminary data indicate that the threshold of a stress response to CPX in *S. aureus* may be significantly higher than that tolerated by fluoroquinolone-susceptible strains.[3] A number of fluoroquinolones can also trigger FnbB up-regulation, which indicates that the effect is not specifically restricted to CPX (data not shown).

What advantage would *S. aureus* obtain by placing FnbB under LexA control? Because fluoroquinolones are DNA damaging agents, a plausible hypothesis is that rapid acquisition of fluoroquinolone resistance could result in a subpopulation of survivors that possess new virulence traits such as enhanced extracellular matrix attachment, invasion kinetics, and perhaps globally increased mutation frequency as a consequence of SOS error-prone repair (27). *E. coli* is now known to engage in stress-induced mutagenesis in aging cultures that may aid in adaptive evolution (43, 44). Investigation of drug-induced stress mechanisms will be an important subject to pursue.

MSCRAMM virulence factor regulation, and fibronectin-binding proteins in particular, are subjects of intensive study (5, 45). In most infection routes, professional phagocytes engulf and destroy *S. aureus*. *S. aureus* may occasionally evade immune clearance by invading cells and remaining in an intracellular niche. A key role for fibronectin-binding proteins in invasion has recently been elucidated (46–49). Fibronectin-binding proteins promote the attachment of *S. aureus* to $\alpha_5\beta_1$ integrins on the host cell surface using surface, or soluble, fibronectin as a bridging molecule (46). Fibronectin attachment is crucial for invasion that does not otherwise require active bacterial processes, and indeed, heterologous expression of cloned *S. aureus fnbB* expressed in non-invasive *Staphylococcus carnosus*, *Lactococcus lactis cremoris* spp., or clinical iso-

[3] D. Li, unpublished data.

lates of non-invasive *S. aureus* rendered them invasive in a variety of cell types (46, 47). Of particular importance is the recent discovery that *S. aureus* small colony variants have significantly increased surface expression of fibronectin-binding proteins that are correlated with enhanced invasion kinetics (32).

In this context, our study now suggests that drug exposure may not only select for highly resistant strains, but can subsequently provoke the enhanced expression of a key colonizing factor that could promote persistent infection among drug-resistant survivors. It is noteworthy that a recent signature tag mutagenesis study also uncovered a transposon insertion in *S. aureus recA* in a screen for attenuated virulence in a murine model of bacteremia (50). These authors reported that the less virulent phenotype of *recA* mutants might be, in part, caused by an effect on the expression of colonizing factors. Identification of gene components of the fluoroquinolone-triggered response pathways may help to elucidate their contribution to survival and virulence of *S. aureus* and may reveal additional targets for effective antimicrobial chemotherapy.

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