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NOTES

Effects of Subinhibitory Concentrations of Antibiotics on SOS and DNA Repair Gene Expression in *Staphylococcus aureus*▽†

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Reporter clones of *Staphylococcus aureus* with different SOS response- and DNA repair-associated promoter-*lux* gene fusion constructs were constructed to study the effects of sub-MICs of antibiotics on the transcription of the SOS and methyl mismatch repair (MMR) genes. Fluoroquinolones (FQs) upmodulated both the SOS and the MMR genes. The patterns of antibiotic-induced transcriptional modulation were altered in FQ-resistant mutants.

Subinhibitory concentrations (sub-MICs) of antibiotics are known to provoke extensive transcriptional changes in bacteria (28, 29). The expression of virulence functions such as toxins, adhesins, and biofilm formation in the human pathogen *Staphylococcus aureus* is affected by exposure to sub-MICs of antibiotics (2, 6, 7, 11, 12, 14, 19). Sub-MICs of certain antibiotics, in particular, compounds whose primary mode of action is DNA damage, are known to enhance mutation rates in bacteria (15). This is usually the result of transcriptional changes in the genes responsible for DNA repair and preservation of the integrity of the genome, such as the SOS and methyl mismatch repair (MMR) pathways (10, 24, 25). DNA polymerases of the SOS system lack intrinsic proofreading activity, which leads to mutations when DNA replication bypasses lesions or errors (24). The MMR system maintains the fidelity of DNA replication by postreplicative correction of base mismatches, small insertions, or deletions (8); a strong mutator phenotype is associated with genetic defects in the MMR system (21).

We have studied the effects of sub-MICs of antibiotics of different chemical classes and with different modes of action on the principal mediators of the SOS response in *S. aureus*, *lexA* and *recA* (16) (Fig. 1A). We also examined other known or presumptive SOS response genes, *umuC*, *sosA* (SAOUHSC_01334), *dinB*, and *recF* (9), and known or presumptive MMR genes, *mutSL* (25, 27), *mutS2* (SAOUHSC_01099), *mutS3* (SAOUHSC_02276), and *mutT* (SAOUHSC_00429) (see Table SA in the supplemental material). The respective promoter regions (234 to 661 bp) were amplified by PCR and inserted into pAmiLux (L. R. Mesak et al., unpublished data), a promoter cloning vector, at a BamHI site upstream of a modified *Photobacterium luminescens luxABCDE* (*lux*) operon encoding luciferase (*luxAB*) and fatty acid reductase (*luxCDE*) from pAL2 (4). The constructs were introduced into *S. aureus* RN4220 (18), and the effects of the antibiotics on transcription in cells grown in NYE medium (26) were monitored by obtaining luminescence measurements. A single colony of *S. aureus* from NYE agar was resuspended in 200 μl water, mixed with 0.7% agar (1:1,000), and poured as an overlay on NYE agar. Paper disks containing selected antibiotics were placed on the overlay, and the culture was incubated at 37°C. After 20 h, luminescence was detected with a luminograph LB980 photon camera (Berthold). Liquid assays were performed at room temperature in a clear-bottom 96-well plate by using starting cultures with an optical density at 595 nm of 0.150. Luminescence was recorded hourly for 20 h in a Wallac 1420 Victor multilabel counter (Perkin-Elmer). Each determination was replicated two to four times (for experiments in broth) or six times (for experiments on solid medium).

The overall level of *lux* expression in the absence of antibiotics was low for all the constructs tested, whether expression was tested on agar or in broth, although the basal levels of expression of *lexA*, *recA*, and *recF* were higher than those of the other SOS genes (Fig. 1B). When antibiotic disks were placed on lawns seeded with reporter strains, promoter activation, as indicated by luminescence at the border between the inhibition zone around the disks (concentrations greater than the MIC) and the growing cells beyond (concentrations less than the MICs), was observed after 20 h at 37°C. Expression of the *lexA*, *recA*, *sosA*, *recF*, and *umuC* genes was upmodulated by all nine fluoroquinolones (FQs) tested to levels higher than those in the presence of mitomycin C, a DNA-damaging agent known to activate *lexA*-regulated genes (3) (Fig. 2A). Although the function of *sosA*, a gene that appears to be unique to *Staphylococcus* spp., remains to be identified, the upmodulation of *sosA* transcription by FQs was similar to that of the SOS genes, suggesting that the SOS boxes associated with *lexA* may influence *sosA* (Fig. 1A). In recent studies, *sosA* has been shown to be regulated by *lexA* (3, 9). SOS boxes were not found in the *dinB* promoter region, and the expression of *dinB* was not affected by FQs. MMR gene transcription was upmodulated to a lesser extent than SOS gene transcription by FQs after 20 h of incubation at 37°C (Fig. 2A).

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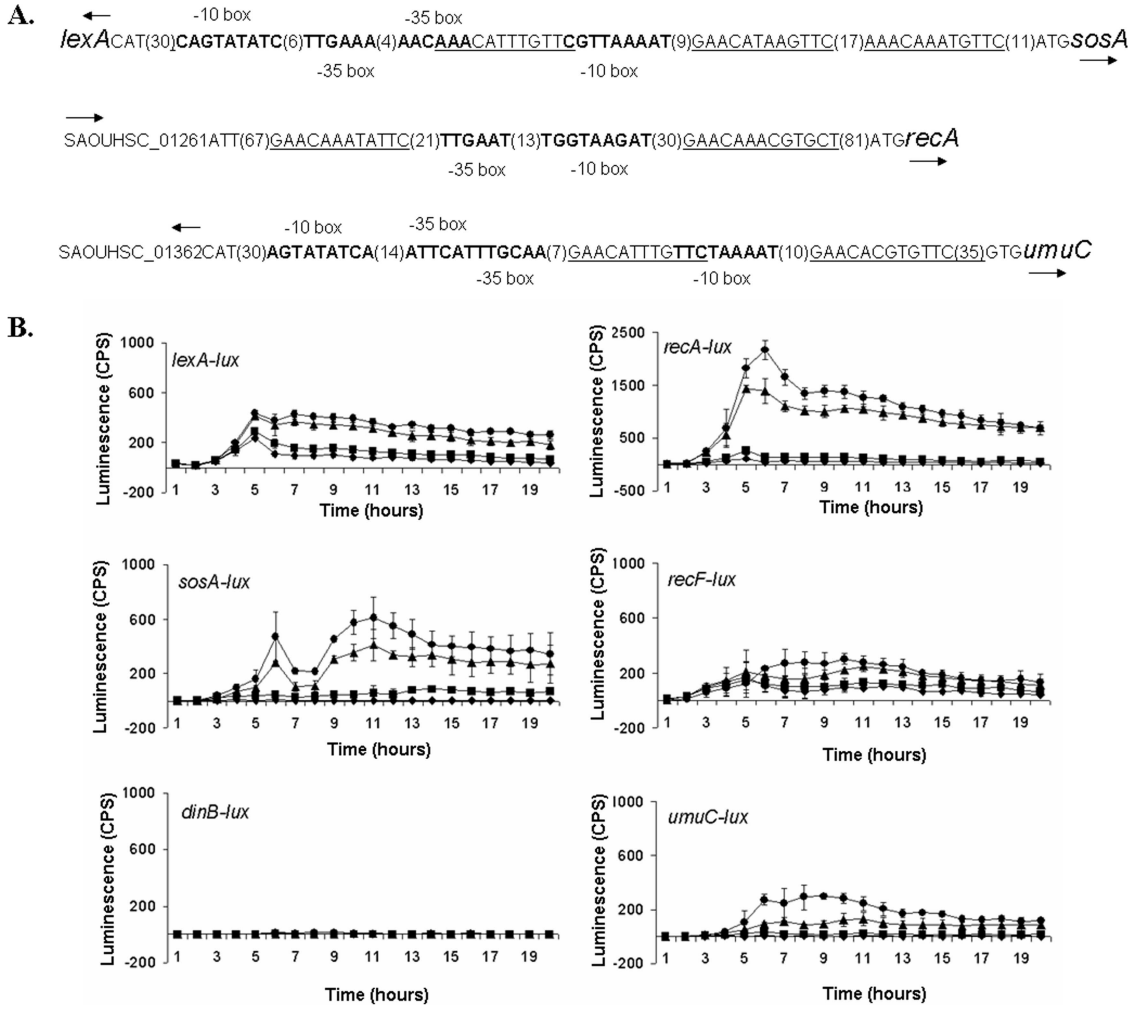

FIG. 1. (A) Promoter regions of SOS response genes. The SOS boxes (underlined) are conserved sequences upstream of many genes in the SOS regulon that repress transcription when they are bound by the LexA protein (5, 9). The putative −10 and −35 boxes (boldface), the direction of transcription (arrow), and intervening nucleotides not shown (parenthetical numbers) are indicated. (B). Expression of SOS genes. The luminescence in the presence of 0 μg/ml (diamonds), 0.2 μg/ml (squares), 0.5 μg/ml (triangles), and 1 μg/ml (circles) CIP in broth was measured with a Wallac 1420 Victor multilabel counter (Perkin-Elmer).

The results from the studies with solid medium were confirmed by monitoring the luminescence during the growth of the reporter strains in liquid culture with ciprofloxacin (CIP); *recA* and *umuC* expression was strongly activated by 1 μg/ml CIP (Fig. 1B). The level of induction of the *lexA*, *recF*, and MMR genes was lower than that of the *recA*, *umuC*, and *sosA* genes (Fig. 1B). The SOS response was dependent on the antibiotic concentration, but *mutSL* expression was not. Interestingly, the behavior of *mutS2* and *mutT*, proposed here to be MMR genes on the basis of their deduced similarity to proteins of the MutS and MutT families, respectively, was similar to that of *mutSL*. Further studies are necessary to determine if they have a role in the MMR pathway or other pathways.

With the exceptions of trimethoprim, phleomycin, and ethidium bromide, none of the other compounds tested (Table 1), regardless of their modes of action, upmodulated the *S. aureus* RN4220 SOS and MMR promoter-*lux* fusions (Fig. 2A). Trimethoprim had a strong upmodulating effect on *recA* (Fig. 2A); this compound inhibits nucleoside biosynthesis and results in the accumulation of damaged DNA (1, 17). Phleomycin is a member of the glycopeptide antibiotic family, generates free radicals, and produces DNA breaks. It is considerably more cytotoxic than bleomycin (22). Ethidium bromide is a well-known intercalating agent. Sub-MICs of the β-lactams, e.g., penicillin G, had no effect on the transcription of the SOS genes. This is surprising, because β-lactams have been reported to induce *lexA* transcription and *recA*-dependent prophage induction in *S. aureus* lysogens (20). The reason for the difference is not clear.

The effects of sub-MICs of antibiotics on *recA* expression in *S. aureus* Newman and 458, both of which are clinical strains, was largely similar to the effect in RN4220 (Fig. 2A); however, the level of induction of *recA* expression by netropsin in *S. aureus* Newman was increased compared to that in RN4220 and 458 (Fig. 2A).

We also investigated the effects of FQs on the expression of *recA* in Cip^r mutants of RN4220 (Table 1). Spontaneous CIP-resistant mutants of *S. aureus* were selected by plating 2.5 ×

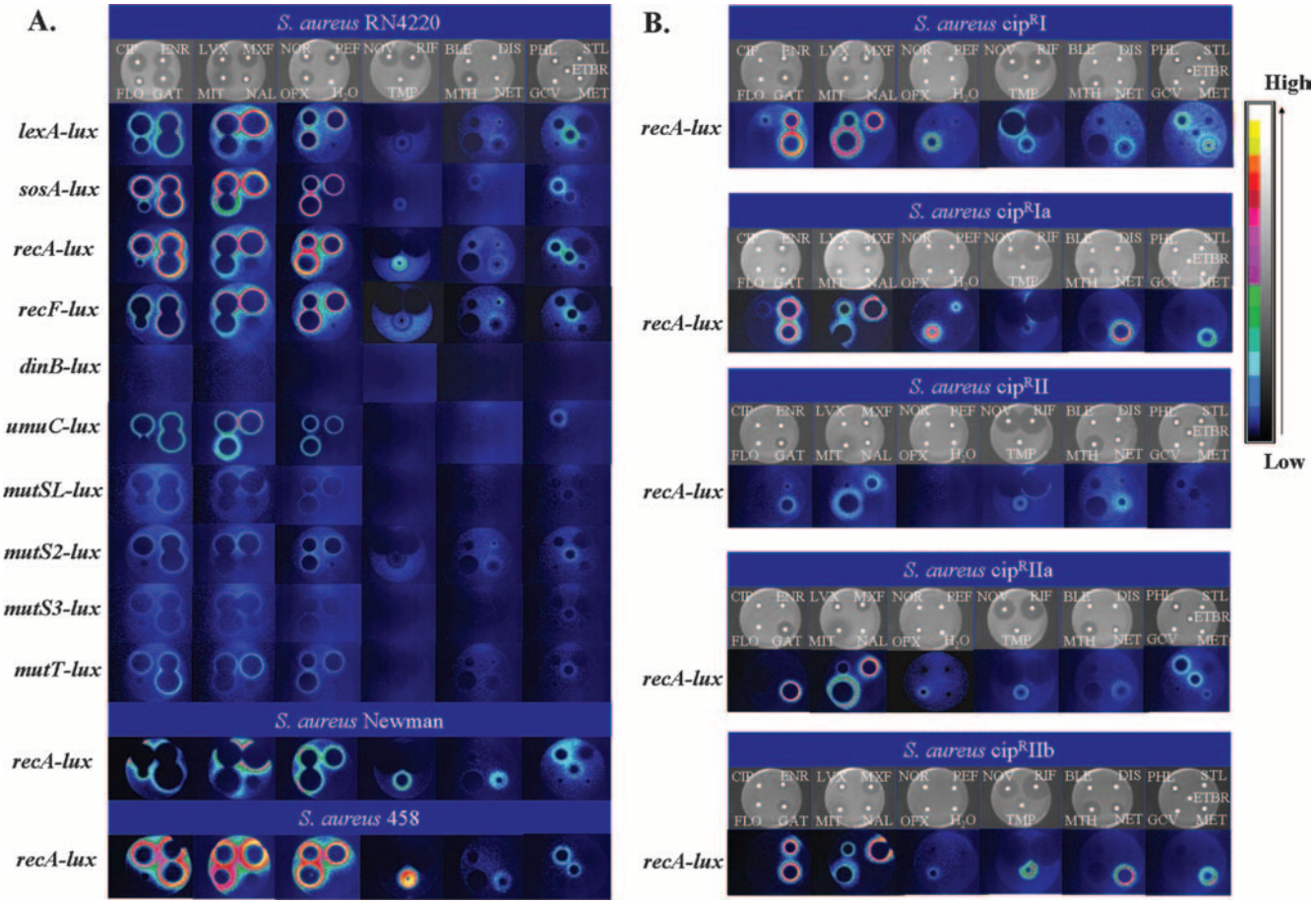

FIG. 2. Effects of antibiotics on SOS and MMR gene expression in the indicated wild-type *S. aureus* strains (RN4220, Newman, and 458) (A) and Cip^r mutants (B). The first row in each set shows the inhibition zones in representative disk diffusion assays after 20 h; the other rows show the effects of the antibiotics on the indicated reporter strains, as seen with a luminograph LB980 photon camera (Berthold) and as converted to the color scale on the right. FQs and mitomycin C did not affect the promoterless-*lux* constructs. The antibiotic disks are as follows: CIP, CIP at 5 μg; ENR; enrofloxacin at 5 μg; FLO, florofloxacin at 5 μg; GAT, gatifloxacin at 5 μg; LVX, levofloxacin at 5 μg; MXF, moxifloxacin at 5 μg; MIT, mitomycin C at 5 μg; NAL, nalidixic acid at 30 μg; NOR, norfloxacin at 5 μg; PEF, pefloxacin at 5 μg; OFX, ofloxacin at 5 μg; H_2O, water; NOV, novobiocin at 5 μg; RIF, rifampin at 5 μg; TMP, trimethoprim at 5 μg; BLE, bleomycin A at 10 μg; DIS, distamycin at 10 μg; MTH, mithramycin A at 10 μg; NET, netropsin at 10 μg; PHL, phleomycin at 10 μg; STL, streptolydigin at 10 μg; GCV, ganciclovir at 10 μg; MET, methotrexate at 50 μg; ETBR, ethidium bromide at 10 μg.

10^9 cells on NYE medium containing 2 μg/ml of CIP. Five colonies that appeared after 3 days were picked and purified on medium containing 2, 4, or 8 μg/ml CIP to identify those mutants that were more resistant to CIP. One derivative of each mutant (mutants Cip^{r}I, Cip^{r}Ia, Cip^{r}II, Cip^{r}IIa, and Cip^{r}IIb; Table 1) was selected from medium containing 8 μg/ml CIP and characterized by sequencing of the *norA* promoter and the *gyrA*, *gyrB*, *grlA*, and *grlB* genes (23). The strains were cross tolerant to most FQs, including extended-spectrum FQs (moxifloxacin and gatifloxacin), with mutant Cip^{r}II being generally more resistant than the other mutants. The mutants with an altered GrlA (S80F), mutants Cip^{r}I and Cip^{r}IIa, were more resistant to nalidixic acid. The plasmid carrying the *recA-lux* construct was then introduced into the different Cip^r strains and the strains were tested for their responses to antibiotics (Fig. 2B). CIP did not stimulate *recA* transcription in the mutants; but enrofloxacin, levofloxacin, and ofloxacin upmodulated *recA* expression in mutants Cip^{r}I, Cip^{r}Ia, and Cip^{r}IIb. Gatifloxacin and moxifloxacin, which both have GrlA and GyrA as targets (23), induced *recA* expression in all mutants. Unexpectedly, certain inhibitors that had no effect on the transcription of the SOS and MMR genes in RN4220 influenced *recA* transcription in the Cip^r strains (Fig. 2B). Notably, novobiocin induced *recA* expression in Cip^{r}I, while rifampin induced *recA* expression in Cip^{r}II. Phleomycin and ethidium bromide did not induce *recA* expression in Cip^{r}II or the *norA* promoter mutants (mutants Cip^{r}Ia and Cip^{r}IIb). In these two mutants, *recA* expression was also strongly induced by the DNA-binding agent netropsin. The atypical responses of spontaneous Cip^r mutants indicate that mutations to resistance may lead to a variety of phenotypes affecting bacterial responses to completely different classes of antibiotics. However, the latter possibility could be due to the presence of additional unidentified mutations. There is no simple explanation for this phenomenon, but the fact that resistance to one class of antibiotic may influence bacterial responses to other inhibitors could be of therapeutic importance.

Using a modified *lux* operon expression system, we have

TABLE 1. *S. aureus* strains used in this study[a]

Strain	Relevant characteristic(s)	CIP MIC[b] (μg/ml)	Reference or source
RN4220	Restriction-deficient derivative of 8325-4 r_K^- m_K^+	0.65–1.3	18
Newman	Clinical isolate	0.3–0.65	13
458	Clinical isolate	0.3–0.65	L. Friedman
Cip^{r}I	*grlA* (GrlA [S80F])	42	This study
Cip^{r}Ia	Insertion in *norA* promoter[c]	21	This study
Cip^{r}II	*grlA* (GrlA [S80Y]), *gyrA* (GyrA [E88K])	42	This study
Cip^{r}IIa	*grlA* (GrlA [S80F]), *gyrA* (GyrA [E88Q])	10.5	This study
Cip^{r}IIb	*grlA* (GrlA [S80Y]), insertion in *norA* promoter[d]	42	This study

[a] The promoter-*lux* fusion constructs were transformed into *S. aureus* strains and then tested with FQs, other DNA-damaging agents (bleomycin A, phleomycin, distamycin, netropsin, nalidixic acid, mithramycin A, ethidium bromide), and a variety of antibiotics with other modes of action: cell membrane-damaging agents (daptomycin, polymyxin B), cell wall biosynthesis inhibitors (bacitracin, fosfomycin, imipenem, penicillin G, vancomycin), protein synthesis inhibitors (clindamycin, erythromycin, gentamicin, kanamycin, neomycin, pristinamycins I and II, spectinomycin, streptomycin, tetracycline, tobramycin), the nucleoside analog ganciclavir, a DNA replication inhibitor (novobiocin), the metabolic inhibitor trimethoprim, RNA polymerase inhibitors (rifampin, streptolydigin), and a nucleotide biosynthesis inhibitor (methotrexate). Antibiotic disks were obtained from Becton Dickinson or Difco or were made by using the laboratory collection.

[b] The MICs of CIP were determined in NYE broth: an aliquot of a culture grown overnight from a single colony at 37°C was diluted in water (1:100), and 10 μl was inoculated into 1.5 ml NYE broth in tubes containing CIP representing twofold serial dilutions starting from 170 μg/ml.

[c] A direct tandem duplication (ATATGTAGCAATGTTGTAATACAAT) of native sequences was observed at position 379 within the promoter region of *norA* (23), adjacent to the −10 box.

[d] A direct tandem duplication (TGTTGTAATACAAT) of native sequences was observed at position 379 within the promoter region of *norA* (23), adjacent to the −10 box.

demonstrated that a number of antibiotics at their sub-MICs have specific transcriptional effects on the SOS and MMR systems of *S. aureus*. One consequence of increased expression might be that the therapeutic use of one antibiotic could increase the numbers of mutations to resistance to other classes of antibiotics. The effects of sub-MICs of antibiotics on the mutation rate in wild-type and resistant mutants of *S. aureus* are under investigation. Given the specificities of the responses, the promoter-*lux* constructs generated in this study have utility in screening for different antibiotic classes or for use in promoter-*lux* reporter panels for screening of antibiotics for their modes of action.

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