Clp-dependent proteolysis of the LexA N-terminal domain in *Staphylococcus aureus*

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The SOS response is governed by the transcriptional regulator LexA and is elicited in many bacterial species in response to DNA damaging conditions. Induction of the SOS response is mediated by autocleavage of the LexA repressor resulting in a C-terminal dimerization domain (CTD) and an N-terminal DNA-binding domain (NTD) known to retain some DNA-binding activity. The proteases responsible for degrading the LexA domains have been identified in *Escherichia coli* as ClpXP and Lon. Here, we show that in the human and animal pathogen *Staphylococcus aureus*, the ClpXP and ClpCP proteases contribute to degradation of the NTD and to a lesser degree the CTD. In the absence of the proteolytic subunit, ClpP, or one or both of the Clp ATPases, ClpX and ClpC, the LexA domains were stabilized after autocleavage. Production of a stabilized variant of the NTD interfered with mitomycin-mediated induction of sosA expression while leaving lexA unaffected, and also significantly reduced SOS-induced mutagenesis. Our results show that sequential proteolysis of LexA is conserved in *S. aureus* and that the NTD may differentially regulate a subset of genes in the SOS regulon.

INTRODUCTION

Bacterial cells respond to DNA damage by changing gene expression (Butala et al., 2009). In many organisms this response is governed by the LexA repressor protein, which, upon induction, releases expression of the SOS regulon (Kelley, 2006). The SOS regulated genes carry out diverse functions in response to DNA damage such as nucleotide excision repair, translesion DNA replication, homologous recombination and cell division arrest. Of importance to bacterial pathogens, the SOS response also modulates the dissemination of mobile genetic elements expressing antibiotic resistance and virulence factors (Beaber et al., 2004; Maiques et al., 2006; Ubeda et al., 2005). In addition, it is well known that several groups of antibiotics are in fact inducers of the SOS response such as the fluoroquinolones (Cirz et al., 2005; Mesak et al., 2008), trimethoprim (Lewin & Amyes, 1991) and β-lactams (Maiques et al., 2006; Miller et al., 2004). Thus, treatment of an infection may, via the SOS response, influence both acquisition and spread of antibiotic resistance.

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Abbreviations: CTD, C-terminal dimerization domain; MMC, mitomycin C; NTD, N-terminal DNA-binding domain.
the ClpXP proteolytic complex (Neher et al., 2003). In E. coli, inactivation of clpX or overproduction of the NTD fragment results in UV sensitivity (Neher et al., 2003), suggesting that the NTD and its turnover is important in fine-tuning the DNA damage response. LexA-like regulators, such as HdiR and IvrR, which vary considerably in primary amino acid sequence but contain highly conserved domain structures, have been identified in the Gram-positive bacteria Lactococcus lactis and Streptococcus mutans, respectively. For these repressors, the importance of proteolysis appears to be conserved as the N-terminal cleavage products are also removed by Clp proteolytic systems (Boneca & Chiosis, 2003). Genes encoding antibiotic resistance and virulence are often carried by mobile genetic elements that in some instances may be mobilized in response to DNA damage. One such example is the mobile staphylococcal pathogenicity islands that occupy specific chromosomal sites but that are excised and replicated by temperate phages upon SOS induction (Novick, 2003; Ruzin et al., 2003). The staphylococcal SOS regulon may, however, be more limited as only 16 genes differed in expression between a wild-type strain (8325-4) and a mutant strain encoding a non-cleavable derivative of LexA that were exposed to the DNA damaging antibiotic ciprofloxacin (Cirz et al., 2007). One of the upregulated genes is fnbB encoding one of two fibronectin binding proteins, FnBPB, which is important for attachment of S. aureus to surfaces, a critical step in the early stage of infection. Thus, induction of the SOS response can result in virulence modulation in S. aureus through both mobilization of virulence genes and enhanced virulence factor expression (Bisignano et al., 2004; Goerke et al., 2006; Ubeda et al., 2005). In the present study, we have examined whether the S. aureus SOS response follows the E. coli paradigm with LexA autocleavage and domain degradation as important elements of SOS-induced gene expression. The biological role of the NTD has also been investigated and will be discussed.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. E. coli strains were grown in Luria–Bertani medium (LB; Oxoid) or on LB agar, while S. aureus strains were grown in brain–heart infusion broth (BHIB; Oxoid) on blood agar, while S. mutans was grown in Mitis-Salivarius Broth (MSB; Oxoid) on-blood agar. All bacteria were grown at 37°C. Bacterial strains and plasmids used in the study are listed in Table 1.

**Table 1. Bacterial strains and plasmids used in the study**

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
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<td>TOP10</td>
<td>F− mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139</td>
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<td>Δ(ara-lev) 7697 galU galK rpsL (StrR) endA1 supG λ−</td>
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</tr>
<tr>
<td>S. aureus</td>
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</tr>
<tr>
<td>8325-4</td>
<td>Wild-type strain (rsB mutant)</td>
<td>Novick (1967)</td>
</tr>
<tr>
<td>RN4220</td>
<td>Restriction-defective derivative of RN450</td>
<td>Kreiswirth et al. (1983)</td>
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<tr>
<td>RTC3002</td>
<td>8325 lexA(S130A)::SpecR</td>
<td>Cirz et al. (2007)</td>
</tr>
<tr>
<td>8325-4 ΔclpP</td>
<td>Deletion of the entire clpP gene</td>
<td>Frees et al. (2003)</td>
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<tr>
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<td>651 bp in-frame deletion in clpX</td>
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<td>1122 bp in-frame deletion in clpC</td>
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<td>Frees et al. (2004)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pCN41</td>
<td>Shuttle vector with promoterless blaZ ErmR</td>
<td>Charpentier et al. (2004)</td>
</tr>
<tr>
<td>pLexA</td>
<td>Transcriptional plexA–blaZ fusion with SOS box 1-5 in pCN41 (Fig. 1)</td>
<td>This study</td>
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<tr>
<td>pLexA_his6</td>
<td>pDEST17 derivative with inducible his−-lexA (KanR)</td>
<td>This study</td>
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* aureus* strains were grown in tryptic soy broth (TSB; Oxoid) or on tryptic soy agar (TSA; Oxoid). Ampicillin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹) or erythromycin (5 µg ml⁻¹) were added when required.

**Plasmid construction.** For construction of plasmids, PCR fragments comprising the promoter regions (see Fig. 1) were cloned into pCN41 in front of a promoterless β-lactamase gene with its own ribosome binding site using Sall and EcoRI. The constructs monitoring lexA (pLexA) and sosA (pSosA) also express the N-terminal 47 aa of sosA, which did not affect cell phenotype (data not shown). The reporter plasmids monitoring the lexA promoter activity in the presence of box 1, box 1-2 or box 1-2-3 also expressed the NTD of LexA, which does not affect lexA expression (Fig. 2a). E. coli TOP10 was used for cloning and all clones were transformed into RN4220, before being transferred by transformation to 8325-4.

**Nitrocefin assay.** The nitrocefin assay was modified from the method described by O’Callaghan et al. (1972). TSB was inoculated with an overnight culture to OD₆₀₀=0.05 and grown at 37 °C to mid-exponential phase (OD₆₀₀=0.5). An aliquot (1 ml) of culture was snap-frozen and stored at −80 °C. MMC was added (0.2 µg ml⁻¹) and the culture was allowed to continue to grow at 37 °C. At 20, 40, 60 and 80 min after MMC was added, 1 ml samples were removed and snap-frozen after OD₆₀₀ was measured. β-Lactamase enzyme activity was measured by mixing 50 µl thawed sample and diluting to OD₆₀₀=0.5, with 50 µl rehydrated nitrocefin from Oxoid (3 µg ml⁻¹) in a 96-well plate. β-Lactamase activity was measured as units (mg cell dry weight⁻¹), where one unit was defined as the hydrolysis of 1 µmol nitrocefin min⁻¹. Measurements were done by following OD₄₈₂ at 20 s intervals for 20 min at 25 °C using a PowerWave XS microplate reader from BioTek.

**Phage induction.** Phage induction, transduction and determination of titre were done as described previously (Lindsay et al., 1998; Šebda et al., 2008). *S. aureus* 8325-4 containing plasmid pLexA or pLexA-NTD89 was first made lysogenic for bacteriophage 80a. Induction of 80a from these two strains was done by adding MMC (2 µg ml⁻¹) and incubating at 32 °C for 4 h. The lysate was then filtered through a 0.2 µm filter and the phage titre was determined.

**Mutagenesis.** Mutation frequencies were measured basically as described previously (Weel-Sneve et al., 2008). Bacteria were grown with shaking at 37 °C and samples were withdrawn at mid-exponential phase (OD₆₀₀≈0.5). Cells were washed in physiological saline and a 5 ml suspension was irradiated with 50 J UV light m⁻². An aliquot (5 ml) of either irradiated or non-irradiated cells was mixed with 5 ml TSB and grown overnight with shaking at 37 °C. Appropriate dilutions of the culture were plated on TSB to determine the total number of bacteria and on rifampicin-containing TSB plates (0.1 µg ml⁻¹) to determine the number of rifampicin resistant mutants.

**Overexpression and purification of LexA protein and generation of anti-LexA antibodies.** A fragment containing the full-length lexA gene was generated and cloned into pENTR/D-TOPO (Invitrogen) and next pDEST17 (Invitrogen) according to the procedure recommended for the Gateway Technology system (Invitrogen). DNA sequence analysis was performed to verify the construct pLexA_his₆. His₆-LexA was purified from E. coli BL21-A1 (Invitrogen) carrying pLexA_his₆ as described by the manufacturer (Qiagen). The purified His₆–LexA was used for custom antibody production in rabbits (CovaLAB).

**Western blot analysis.** *S. aureus* strains were grown as specified above. At an OD₆₀₀ of 0.5 the cells were challenged with MMC (1 µg ml⁻¹) to induce the SOS response and were subsequently incubated at 37 °C for 30 min. Following induction, chloramphenicol (150 µg ml⁻¹) was added to stop translation. Cells were harvested for isolation of intracellular protein when MMC was added and at 0, 10, 20 and 40 min after chloramphenicol treatment. Cell pellets were resuspended in 50 mM Tris/HCl (pH 8.0), lysed by the addition of 50 µg lysozyme ml⁻¹ (Sigma) and incubated at 37 °C for 20 min. The protein concentration was determined by using a Bio-Rad protein assay (Bio-Rad Laboratories) and samples were separated on Novex 10% Tricine gels (Invitrogen) using Tris/glycine buffer (Invitrogen). The protein was transferred onto a polyvinylidene difluoride membrane (Invitrogen) using an XCell sure-lock mini-cell system (Invitrogen) as recommended by the supplier. LexA was probed with a 1:3000 dilution of LexA antibody and the WesternBreeze chemiluminescent anti-rabbit kit (Invitrogen).

**RESULTS**

**LexA is subjected to auto-cleavage and Clp-mediated degradation in *S. aureus***

Homologues of LexA have been identified in a number of bacteria including *S. aureus*. Commonly they are able to undergo RecA-stimulated proteolytic self-cleavage during SOS-inducing conditions (Erill et al., 2007; Kelley, 2006). To confirm if this is also the case in *S. aureus*, we followed expression of LexA by Western blot analysis using *S. aureus* LexA-specific polyclonal antibodies raised as described in Methods. When treating wild-type (strain 8325-4) cells with MMC and blocking subsequent protein synthesis by chloramphenicol, we observed that full-length LexA was rapidly processed to disappear 10 min after addition of chloramphenicol leaving a small fragment of approximately 13 kDa to react with the LexA antibody (Fig. 3a). Further, these fragments were not visible in a stain carrying a LexA derivative (RTCC002) unable to undergo auto-proteolytic cleavage (Fig. 3a; Cirz et al., 2007). When comparing *S. aureus* LexA with those of other organisms, a conserved auto-proteolytic cleavage site is present at amino...
acid sequence Ala93–Gly94 (Maiques et al., 2006) corresponding to a LexA NTD of 10.5 kDa and a CTD of 12.8 kDa. Thus, the appearance of a 13 kDa fragment after MMC treatment suggests that only the CTD is visible in wild-type cells (Fig. 3a).

Since a Clp proteolytic complex is responsible for the turnover of LexA cleavage products in *E. coli* (Neher et al., 2003), we examined the stability of the cleavage products in *S. aureus* strains lacking either the proteolytic component, ClpP, or one of the two Clp ATPases predicted to interact with ClpP, namely ClpC or ClpX (Frees et al., 2003, 2004). In the absence of clpP, clpX or clpCX, two LexA fragments were visible after MMC induction corresponding to the predicted NTD and CTD fragments (Fig. 3a). The smaller NTD was not apparent in wild-type or *clpC* mutant cells whereas in *clpP*, *clpCX* and, to a somewhat lesser extent, *clpX* mutant cells, the NTD was stabilized. Since the NTD LexA fragment accumulates to a greater extent in *clpCX* mutant cells than in the *clpX* mutant, our results indicate that while ClpXP is the main protease responsible for the turnover of NTD, ClpCP also contributes. In comparison, the LexA CTD was generally more stable than the NTD but appeared to be stabilized by the *clp* mutations, suggesting a minor role of the Clp proteins in CTD degradation.

**lexA and sosA expression is differentially induced by MMC**

With the aim of investigating whether the NTD influences the *S. aureus* SOS response, we examined two genes, namely *lexA* and *sosA*, previously shown to be controlled by LexA in *S. aureus* (Anderson et al., 2006). *lexA* and *sosA* are divergently transcribed from a common promoter region (*S. aureus* NCTC 8325, GenBank accession no. YP_499864) which shows 65% sequence identity to the *lexA–yneA* intergenic region of *B. subtilis* (http://genolist.pasteur.fr/SubtiList/). While *sosA* is predicted to encode a 77 aa product of unknown function, the relative location of *sosA* with respect to *lexA* suggests that it may encode a cell division inhibitor (Kawai et al., 2003; Ogino et al., 2008; van der Veen et al., 2010). In *B. subtilis*, LexA, also denoted DinR, binds to a consensus DNA-binding motif (the SOS box) CGAAC-N4-GTTCG (Hajjema et al., 1996). When allowing two mismatches relative to the *B. subtilis* SOS box, three putative LexA binding sites were identified in the *S. aureus lexA–sosA* intergenic region designated box 1-3 (Fig. 1) (Mesak et al., 2008). In addition, two putative SOS boxes, box 4 (CGAAC-N4-GTGC) and box 5 (GGAAC-N4-GTACG), are located within the coding region of *sosA* (Fig. 1). In *B. subtilis*, the central AA/TT of the SOS box is essential for LexA binding (Groban et al., 2005), but considering that the SOS boxes of the LexA-controlled fnbB gene in *S. aureus* all contain mismatches in one of the AA/TT pair (Bisognano et al., 2004), the *S. aureus* LexA recognition motif might deviate somewhat from the *B. subtilis* consensus.

In order to monitor *lexA* and *sosA* expression we constructed a series of *blaZ* reporter gene fusions (Fig. 1 and Table 1). Two of the constructs, designated pLexA for the *lexA* promoter and pSosA for the *sosA* promoter, encompass the *lexA–sosA* intergenic region in both orientations as well as part of the *sosA* coding region in order to include the putative box 4 and box 5. Also, reporter constructs measuring *lexA* promoter activity in the presence of box 1, box 1-2 or box 1-2-3 were created. The plasmids were introduced into 8325-4 and expression was followed after addition of MMC. Fig. 2 (dark bars) shows that MMC induced transcription of *lexA* by twofold (Fig. 2a) whereas expression of *sosA* was induced 22-fold 80 min

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**Fig. 2.** *lexA* and *sosA* promoter activity in the presence or absence of NTD89 before (0 min) and 20, 40, 60 and 80 min after addition of MMC (0.2 μg ml⁻¹). Promoter activity was measured as β-lactamase activity expressed by (a) pLexA (dark grey bars) and pLexA_ NTD89 (light grey bars) and (b) pSosA (dark grey bars) and pSosA_ NTD89 (light grey bars). The β-lactamase activity of each bar is relative to the β-lactamase activity of pLexA prior to induction with MMC [0.063 units (mg dry weight)⁻¹]; data are the mean ± SD from a minimum of three independent measurements.
after induction (Fig. 2b). Thus, expression of both \textit{lexA} and \textit{sosA} are induced by MMC, but they differ substantially with respect to the magnitude of induction. When we investigated the impact of the SOS boxes on \textit{lexA} expression, the number of SOS boxes did not influence the MMC-induced expression level of \textit{lexA} (Fig. 4). Furthermore, the construction containing only the \textit{lexA} proximal SOS box 1 was sufficient to show repression of expression.

**Fig. 3.** \textit{In vivo} stability of LexA under DNA damaging conditions (MMC) in \textit{S. aureus} 8325-4 and \textit{ΔclpP, ΔclpC, ΔclpX} and \textit{ΔclpCX} mutant derivatives and \textit{S. aureus} RTC3002. (a) Cells were treated with 1 μg MMC ml⁻¹ in mid-exponential phase (0') followed 30 min later by sample withdrawal at 0, 10, 20 and 40 min after the addition of chloramphenicol (150 μg ml⁻¹). (b) Cells were treated as in (a) and harvested in mid-exponential phase (0') and 0 and 10 min after addition of chloramphenicol. In (a) and (b), proteins were separated, transferred and hybridized as described in Methods.

**Fig. 4.** \textit{lexA} promoter activity controlled by various numbers of SOS boxes. The promoter activities of reporter constructs pLexA1\_NTD89, pLexA2\_NTD89, pLexA3\_NTD89 and pLexA\_NTD89 (with 1, 2, 3 or full promoter, respectively) were monitored either before (dark grey bars) or 80 min after (light grey bars) induction with MMC (0.2 μg ml⁻¹). Promoter activity was monitored as β-lactamase activity relative to the β-lactamase activity of pLexA\_NTD89 before MMC induction [0.063 units (mg dry weight)⁻¹]. Data are the mean ± SD of a minimum of three independent measurements.
**Accumulation of stable NTD interferes with induction of sosA**

Since the N-terminal cleavage products of LexA and a LexA-like transcriptional regulator HdiR have previously been shown to retain DNA-binding activity, we aimed to investigate the possible regulatory role of the LexA NTD in *S. aureus* (Hurstel *et al.*, 1986; Savijoki *et al.*, 2003). To this end we exploited the fact that our reporter constructs express a LexA NTD fragment (named NTD89) that lacks the C-terminal 4 aa compared with the native NTD, that are predicted to be involved in turnover of the fragment (Fig. 1). When examining NTD89, we found that it appeared stable compared with the wild-type NTD resulting from auto-cleavage (Fig. 3b). Since the *lexA* and *sosA* promoters differ in the extent to which they are induced by MMC, we monitored their respective activities at various times after SOS induction in the presence of NTD89 (Fig. 2, light grey bars). Interestingly, expression of NTD89 barely affected the induction of *lexA* expression, whereas the *sosA* promoter was repressed in the presence of NTD89 with only a 7-fold induction by MMC in the presence of NTD89 compared with 22-fold in its absence. Thus, our experiments show that a stable NTD differentially modulates expression of two genes within the SOS regulon upon induction with MMC.

**Stable NTD expression prevents UV-induced mutagenesis**

To address the biological role of the NTD we examined the accumulation of mutations in cells expressing NTD89 after exposure to UV. In cells expressing NTD89, the UV-induced mutation frequency was reduced significantly compared with cells not producing NTD89 (Table 2). In fact, the expression of NTD89 repressed mutation frequency to the same level as observed for the *S. aureus* strain RTC30002 that carries a LexA variant unable to undergo proteolytic self-cleavage and thus is unable to induce the SOS response (Cirz *et al.*, 2007). Another process depending on the SOS system is the excision of prophages. Consequently we examined whether expression of NTD89 affected the ability of the lysogenic phage 80x to excise but found that NTD89 did not affect this process (data not shown). Thus, our data demonstrate that the stable NTD89 fragment reduces UV-induced mutagenesis but not SOS-induced phage excision in *S. aureus*.

### DISCUSSION

In order to study the fate of the LexA protein in *S. aureus* following DNA damage, we raised antibodies against the protein and followed turnover after MMC addition. *S. aureus* LexA appears to undergo auto-proteolytic cleavage when exposed to DNA damaging agents, resulting in two fragments of 10.5 and 12.8 kDa. In *E. coli*, the ClpXP protease degrades the LexA NTD in a process depending on the exposure of a C-terminal degradation signal revealed upon auto-cleavage (Neher *et al.*, 2003). Our results show that in *S. aureus*, both ClpXP and ClpCP proteolytic complexes contribute to degradation of the NTD. Inspection of the C-terminal end of the NTD of *S. aureus* LexA further revealed a motif, Val 91–Thr 92–Ala 93–COOH, with similarity to the *E. coli* ClpXP degradation motif (Flynn *et al.*, 2001). Removal of the C-terminal 4 aa (aa 90–93) of NTD (resulting in NTD89) led to stabilization of the fragment, thus indicating that this motif also directs degradation in *S. aureus*. When comparing the stability of the NTD and CTD of *S. aureus* LexA, we found that in contrast with *E. coli*, the *S. aureus* CTD is more stable than the NTD and is completely stabilized in the absence of the Clp proteins. The slow turnover of the *S. aureus* LexA CTD in wild-type cells may be due to the absence of the Lon protease that is responsible for the CTD turnover in *E. coli* (Neher *et al.*, 2003).

When studying the impact of NTD accumulation we chose to focus on the divergently transcribed *lexA* and *sosA* genes for two reasons. First, it allowed us to construct reporter fusions that at the same time produce the stable NTD89 from its own promoter. Secondly, it enabled us to examine the effect of the NTD on expression of genes with different induction levels in response to MMC (Anderson *et al.*, 2006). Using the reporter constructs, we found that MMC induced *lexA* 2-fold and *sosA* 22-fold 80 min after addition. These results agree well with the 5- and 31-fold induction reported previously (Anderson *et al.*, 2006). Interestingly, expression of the stable NTD89 interfered differentially with MMC-mediated induction of *lexA* and *sosA* expression. While induction of *lexA* expression was barely affected by the presence of NTD89 following MMC addition, the induction of *sosA* expression was reduced from 22-fold to only 7-fold. Repressor activity of the *E. coli* LexA promoter activity in the absence of MMC, as has also been observed in *B. subtilis* (Hajjema *et al.*, 1996). Studies of the *sosA* promoter were hampered by the fact that the SOS box furthest from *sosA* (SOS box 1, see Fig. 1) lies between the putative −10 and −35 promoter sequences of *sosA* and thus, manipulation eliminated promoter activity (data not shown).

### Table 2. Number of rifampicin-resistant (rif<sup>R</sup>) colonies in *S. aureus* following UV treatment

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<th>Strain or plasmid</th>
<th>No. of rif&lt;sup&gt;R&lt;/sup&gt; colonies</th>
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<tr>
<td></td>
<td>No UV treatment</td>
</tr>
<tr>
<td>pLexA</td>
<td>4</td>
</tr>
<tr>
<td>pLexA, NTD89</td>
<td>5</td>
</tr>
<tr>
<td>RTC3002</td>
<td>2</td>
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</table>

Values are one representative of three independent experiments and show the mean (of three technical replicates) number of colonies per 10<sup>9</sup> viable cells. UV treatment was at 50 J m<sup>2</sup>.
NTD has been shown in several studies (Bertrand-Burggraf et al., 1987; Hurstel et al., 1986; Little & Hill, 1985). Neher et al. (2003) further proposed that the NTD may differentially affect induction of SOS genes and our data support this notion. In another study, Michel et al. (2006) reported that in the absence of SOS induction, expression of lexA is increased in a S. aureus strain lacking ClpP. This finding suggests that even in un-induced cells, Clp proteolytic complexes may influence LexA repression and it further stresses the significance of the Clp proteins in modulating the SOS response.

Lastly, we examined the effect of the stabilized NTD on several biological processes and, while it substantially reduced UV-induced mutagenesis, it did not interfere with induction of the 80x prophage. The latter result suggests that RecA activity is not affected by NTD89 since RecA is required for phage induction (Maquès et al., 2006). In conclusion, our data suggest that sequential proteolysis of LexA may allow a subset of the SOS regulon genes to be differentially controlled by the NTD following SOS induction. Future studies will be aimed at identifying conditions that stabilize NTD in the presence of ClpP and the biological processes differentially modulated by the NTD.

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REFERENCES


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