

**SOS Regulation of the Type III Secretion System
of Enteropathogenic *Escherichia coli***

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ABSTRACT

Genomes of bacterial pathogens encode, and coordinately regulate virulence-associated genes in order to cause disease. Enteropathogenic *Escherichia coli* (EPEC), a major cause of watery diarrhea in infants and a model Gram-negative pathogen, expresses a type III secretion system (TTSS) that is encoded by the locus of enterocyte effacement (LEE) and is necessary for causing attaching and effacing intestinal lesions. Effector proteins encoded by the LEE and in cryptic prophage are injected into the host cell cytoplasm by the TTS apparatus, ultimately leading to diarrhea. The LEE is comprised of multiple polycistronic operons, most of which are controlled by the global, positive regulator Ler. Here we demonstrated that the *LEE2* and *LEE3* operons also respond to SOS signaling and this regulation was LexA-dependent. By DNase I protection assay, purified LexA protein bound *in vitro* to a predicted SOS box located in the divergent, overlapping *LEE2/LEE3* promoters. Expression of the *lexA1* allele, encoding an uncleavable LexA protein in EPEC, resulted in reduced secretion, particularly in the absence of the Ler regulator. Finally, we present evidence that the cryptic phage-located *nleA* gene encoding an effector molecule is SOS regulated. Thus we demonstrate, for the first time to our knowledge, that genes encoding components of a TTSS are regulated by the SOS response, and these data might explain how a subset of EPEC effector proteins, encoded within cryptic prophage, are coordinately regulated with the LEE-encoded TTSS necessary for their translocation into host cells.

INTRODUCTION

Coordinate regulation of virulence determinants within a host is critical to the bacterial pathogen's ability to cause disease. Enteric pathogens perceive a number of environmental cues within the human intestinal tract, signaling the bacterium that it resides in the correct niche and allowing pathogenesis to commence.

Enteropathogenic *E. coli* (EPEC), the causative agent of watery diarrhea primarily of infants living in the developing world (42), also responds to a requisite set of environmental cues, permitting colonization of the small intestine. EPEC forms attaching and effacing (AE) intestinal lesions, mediated by a type III secretion system (TTSS), whereby more than 20 protein effector molecules are injected into the host cell cytoplasm (16), leading to cytoskeletal rearrangement, intimate attachment to the host cell membrane, and pedestal formation. In the LEE-containing, related *E. coli* O157:H7 Sakai strain, Tobe et al. recently demonstrated that 39 effector proteins are translocated into host cells, and many of these effectors are encoded within cryptic prophages (55). Similarly, the SopE effector protein of a few *Salmonella typhimurium* strains is encoded within a temperate P2-like phage (37).

EPEC diarrhea results from loss of absorptive tissue from destruction of the microvilli in the formation of AE lesions, alteration of host cell signaling events causing active ion secretion, increased intestinal permeability, loosening of tight junctions and inflammation at the site of infection (for review see (7, 23)). Effector molecules translocated into the host cell by the TTS apparatus include the locus of enterocyte effacement (LEE)-encoded Tir, EspF, EspG, EspH, EspZ, and Map proteins, as well as the proteins NleA (also termed EspI), EspD, EspJ, EspG2 and Cif that are encoded outside the LEE pathogenicity

island (PAI) (16). EspG2 is encoded within the EspC PAI (34), and interestingly, the NleA, NleD, EspJ and Cif proteins are encoded within cryptic prophage (10, 19, 40).

Maximal secretion of EPEC effector molecules occurs during early exponential phase of growth at 37° C, at pH7, and is influenced by media conditions (24, 25, 48, 58). To date, however, there is not an understanding as to how the prophage-encoded effector molecules are coordinately regulated with the components of the LEE-encoded TTSS.

A number of regulatory proteins have been demonstrated to control expression of the EPEC LEE (for review see <http://www.ecosal.org/ecosal/toc/index.jsp>), including the master regulator Ler, which is encoded in the *LEE1* operon (13, 33). PerC, encoded on the *E. coli* attachment factor (EAF) virulence plasmid and integration host factor (IHF) directly affect the expression of *LEE1* and thus expression of Ler (3, 15, 33, 45). Fis and BipA have also been demonstrated to regulate *LEE1* transcription, and thus indirectly control the AE phenotype (17, 18). H-NS is an important regulator of LEE transcription at temperatures below that of the human host, directly silencing the *LEE1*, *LEE2* and *LEE3* operons (58) encoding the membrane-spanning components of the TTSS, and *LEE5* operon (20) encoding Tir, its chaperone CesT, and the outer membrane protein intimin. The LEE is subject to quorum sensing signaling (51, 53), and lastly the GrlRA (for global regulator of LEE-repressor and activator, respectively) proteins have been described as part of a complex regulatory loop controlling LEE gene expression, acting upstream of Ler (11).

Recent reports have implicated the SOS regulon in the control of virulence-associated phenotypes in both Gram-negative and Gram-positive pathogens. In *E. coli* β -lactam antibiotics induce the SOS response and mitigate the lethal effects of drugs on these

bacteria (35). Additionally, resistance to the DNA gyrase inhibitor ciprofloxacin in *E. coli* requires a specific mutation that is mediated by cleavage of the SOS repressor LexA, inducing associated expression of error-prone polymerases (5). Ciprofloxacin induces *Staphylococcus aureus* fibronectin binding in a RecA-LexA-dependent manner (1), and in *E. coli* serotype O157:H7, prophage-encoded Stx₂ production is enhanced by the presence of DNA-damaging antibiotics (54, 62). In *Vibrio cholerae*, LexA and the phage-encoded protein RstR act together to control expression of the CTX Φ phage promoter P_{rstA} (32, 46). Here we demonstrate that expression of the TTSS of EPEC is regulated by the SOS response in a LexA-dependent manner, and discuss the implications of this finding for EPEC pathogenesis, the evolution of pathogenic *E. coli* bacteria, and the clinical treatment of bacterial infections.

MATERIALS AND METHODS

Bacterial strains, plasmids and phage. Bacterial strains, plasmids and bacteriophage used in this study are listed in Table 1. Strains were grown aerobically at 37°C in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics to the following final concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; chloramphenicol, 30 µg/ml, or tetracycline, 15 µg/ml.

Generation of regulatory fragments. Promoter fragments for transcriptional studies were generated by PCR according to standard protocols (21) utilizing Taq polymerase (Sigma), oligonucleotide primers listed in Table 3 (Invitrogen) and genomic DNA isolated from *E. coli* strain MG1655 using a Qiagen Genomic DNA Kit (Qiagen).

The *recA* gene was amplified by PCR using the 5'-*recA* and 3'-*recA* primers listed in Table 2. Amplified DNA fragments were gel isolated using the Qiaquick Gel-Extraction Kit (Qiagen) and subsequently cloned with a TOPO TA Cloning kit (Invitrogen) according to the manufacturers instructions. Regulatory fragments were verified by restriction mapping and DNA sequence analysis.

Construction of a chromosomally encoded, single-copy *recA* transcriptional fusion. The *recA* transcriptional reporter gene fusion was constructed by excising the *recA* regulatory fragment (-97 to +827) from the TOPO TA plasmid vector with the restriction endonuclease *EcoRI*, and cloned upstream of the promoter-less *lacZ* gene in pRS551 previously cleaved with *EcoRI* and dephosphorylated with shrimp alkaline phosphatase (Roche), creating plasmid pKH281. Electrocompetent DH5α was transformed, using a BIO-RAD Micropulser electroporation apparatus, and selecting for ampicillin resistance. Plasmid DNA was isolated using the UltraClean Mini Plasmid Prep

Kit (Mo Bio) according the manufacturers instructions and subjected to restriction enzyme digests and DNA sequence analysis for verification of the specific and *recA-lacZ* transcriptional fusion.

In order to isolate the chromosomally encoded, single-copy *recA-lacZ* transcriptional fusion, the resultant plasmid was allowed to recombine with λ RS45 (50) in *E. coli* strain MC4100. Specialized transducing lysates were used to transduce MC4100 to kanamycin resistance. The single-copy, chromosomally encoded *recA* fusion was isolated by screening kanamycin resistant transductants for ampicillin sensitivity according to established protocols (33, 50).

Enzymatic assays. β -Galactosidase assays were performed according to Miller (1972), except that, saturated overnight cultures were diluted 50-fold and allowed to grow to exponential phase (OD_{600} of ~ 0.5). Individual cultures were subsequently diluted to an OD_{600} of ~ 0.1 to begin the time course. These cultures were then split in two, to which mitomycin C was added to one of the cultures to a final concentration of 1 μ g/ml at time zero. Aliquots were removed at 30 and 60 minutes of growth and kept on ice until β -galactosidase assays could be performed. The data presented are expressed in Miller units and represent the mean of at least two independent experiments performed in triplicate.

Generalized P1*vir* transduction. Strains encoding *recA1* or *lexA1* alleles were constructed by P1*vir* transduction (36). P1*vir* lysates were generated from donor strains that contained Tn10 insertions, which encode tetracycline resistance, linked to mutant *recA1* or *lexA1* alleles, which confer UV sensitivity (6, 39). To isolate a Tn10 insertion linked to the *lexA1* allele we transduced strain DM803 to tetracycline resistance by using

a P1*vir* lysate from strain TL229 that encodes a Tn10 linked to *lexA*, *zjb-729::Tn10*. By selecting tetracycline resistance and screening for UV sensitivity strain JLM803 was isolated. (Strains were determined to be UV sensitive if, when cross-streaked on LB agar, they exhibited death when exposed to 30 seconds of UV light from a Germicidal STERILAMP (Westinghouse)). Once strains containing Tn10 insertions linked to *lexA1* (JLM803) or *recA1* (USL10) were isolated, we then infected the appropriate recipient strains with P1*vir* lysates generated from strains JLM803 and USL10, selecting for tetracycline resistance. Transductants that had inherited the Tn10 and the mutant *lexA1* or *recA1* alleles exhibited tetracycline resistance and UV sensitivity.

RNA dot blot procedure. Total RNA was isolated from bacterial cultures grown in LB with and without exposure to mitomycin C for 60 minutes during exponential growth. Strains E2348/69 and MC4100 were inoculated in 2 mls of LB, grown overnight at 37° C with aeration and subsequently diluted 50-fold in LB the next morning. Cultures were grown at 37° C with shaking until the absorbance, A₆₀₀ was between 0.5 and 1.0. These cultures were diluted to an absorbance, A₆₀₀ ~ 0.1 in LB with and without 1 µg/ml of mitomycin C. After 60 minutes of growth cells were harvested by centrifugation and stored at -80° C. Total RNA was isolated from each culture by Trizol (Invitrogen) according to the manufacturers instructions.

Total RNA was then treated with 10 units of DNase I (Sigma) at 37° C for 30 minutes in buffer containing: 10 mM Tris pH 8.0, 2.5 mM MgCl₂, 0.5 mM CaCl₂. This reaction was quenched by the addition of EDTA to a final concentration of 5 mM and heating to 75° C for 10 minutes. RNA integrity and absence of contaminating DNA was analyzed by agarose gel electrophoresis followed by staining with ethidium bromide.

Clear, sharp 23S and 16S RNA species were observed and there was no detectable genomic DNA present. The concentration of RNA was determined spectrophotometrically by absorbance at 260 nm.

Total RNA (10 µg/dot) in Transfer Buffer (2% formaldehyde, 2.5X SSC) was heated at 75° C for 15 minutes and vacuum blotted onto Hybond-N membrane (GE Healthcare) and dried completely at 80° C for 2 hours. The blots were then rinsed with 2X SSC and incubated with pre-hybridization solution, (5X SSC, 5X Denhardt's reagent, 0.5% SDS and 100 µg/ml salmon sperm DNA (Invitrogen)) at 65° C with agitation for greater than 2 hours. The blots were then probed individually with ~100 ng of radioactively labeled DNA fragments.

Probe fragments were generated by PCR utilizing the oligonucleotide primers listed in Table 2, and subsequently cloned by Topo-TA cloning (Invitrogen) according to the manufacturers instructions. The *LEE2*, *LEE3*, *LEE5* and *nleA* probe fragments were generated from E2348/69 genomic DNA, and the *recA* fragment was generated from MG1655 genomic DNA. All plasmids used for probes were subjected to DNA sequence analysis for verification of nucleotide sequence. Each probe fragment of DNA was excised from the respective plasmid, gel isolated (Qiaquick gel extraction kit) and radioactively labeled with α -³²P-dATP (NEBlot Kit) according to manufacturers instructions. Unincorporated nucleotides were removed by spin column (Probe Quant G-50 Micorcolumn, GE Healthcare) and the DNA was denatured at 100° C for 5 minutes and chilled on ice. The probes were then added individually to each pre-hybridization solution/membrane and incubated at 65° C with agitation overnight to allow hybridization. The hybridization solution was removed and the membranes were washed

twice in 2XSSC, 0.1% SDS at 65° C for 15 minutes, twice in 1X SSC, 0.1% SDS at 65° C for 15 minutes and three times in 0.1X SSC, 0.1% SDS at 65° C for 30 minutes. The membranes were then wrapped in Saran Wrap and mounted on Kodak X-OMAT LS X-ray film and exposed for varying amounts of time at room temperature. After satisfactory images were obtained the membranes were cut into 1 cm squares containing each RNA dot and subjected to liquid scintillation analysis to quantify specific RNA levels (Packard TRICARB 2900 TR Liquid Scintillation Analyzer). Counts per minute (cpm) for each RNA dot under each condition were normalized to the 16S rRNA (cpm) and the relative increase (%) in transcript levels was calculated for each operon or gene, comparing signal in the presence versus the absence of the DNA damaging agent mitomycin C. Results for two independent RNA isolations, performed in triplicate were subjected to statistical analysis (student's paired t-test), where $P < 0.05$ was considered significant.

Cloning and expression of the *lexAI* allele. The *lexAI* allele encoding the uncleavable LexA1 protein was amplified by PCR using the *lexA* specific primers, 5'-*lexA* new and 3'-*lexA* new. These primers amplify a minimal fragment encoding a promoterless ORF corresponding to the *lexA* gene from *E. coli* strain MG1655, including the ribosome binding sequence. Genomic DNA from the strain JLM281A (Table 1), which encodes the mutant uncleavable *lexAI* allele that has been verified to confer an SOS uninducible phenotype in this work, was used as template DNA for PCR amplification. A single fragment was observed, gel-isolated and cloned directly into pBAD33 using the *Xba*I/*Hind*III sites engineered into the primer sequences, creating plasmid pKH296. The pBAD33 plasmid contains the arabinose-inducible P_{BAD} promoter, which drives transcription of the cloned ORF in the presence of arabinose. By DNA

sequence analysis this plasmid was verified to contain the G to A transition mutation that results in a G80D amino acid substitution that renders the protein encoded by the *lexA1* allele uncleavable (28).

Analysis of TTSS-dependent protein secretion. EPEC strains were inoculated into 2 mls of LB containing chloramphenicol for pBAD plasmid selection and 0.2% glucose to inhibit expression of the *lexA1* allele cloned into pKH296. Incubation proceeded overnight at 37° C with shaking. Cultures were then diluted 50-fold into Dulbecco's Modified Eagle Medium (DMEM) containing 50 mM HEPES buffer, pH 7.4, chloramphenicol and 0.1% arabinose, to induce expression of the *lexA1* allele. Cultures were incubated at 37° C with shaking until the optical density at 600 nm was between 0.8 and 1.0. Bacteria were removed from the media by centrifugation at 12,000 X g for 30 minutes. The equivalent of 4.5 OD units of media were removed by serological pipette and proteins were precipitated as described previously (24). Protein pellets were resuspended in Laemmli SDS-PAGE loading buffer and resolved on a 15% SDS-polyacrylamide gel. Proteins were visualized by overnight staining with 0.25% Coomassie R-250 in 40% methanol and 10% acetic acid. Destaining was achieved by repeated washing in 45% methanol and 10% acetic acid.

DNase I protection assays. Fragments suitable for DNase I protection assays were PCR amplified from genomic DNA. The *recA* fragment (-97 to +62) was amplified using the 5' *rec*-foot, 3' *rec*-foot primers and genomic DNA isolated from strain MG1655. The 159 base pair fragment was gel isolated, cleaved with *Eco*RI and *Bam*HI and ligated into pBluescript-II KS cut with the same restriction endonucleases, creating pKH286. The *LEE2* (-83 to +75)/*LEE3* (-94 to +64) and *LEE5* (-75 to +88) DNA fragments were PCR

amplified from genomic DNA isolated from EPEC strain E2348/69. Primers 5'LEE3 and 3'LEE3 were used to amplify the 158 base pair fragment that encodes the overlapping *LEE2/LEE3* promoters including the putative LexA binding site. Primers Tir5 and TIR+88 were used to amplify the 163 base pair fragment encoding the *LEE5* promoter and putative LexA binding site. These fragments were gel isolated and cloned using the TOPO TA Cloning Kit (Invitrogen). The *LEE2/LEE3* and *LEE5* promoter fragments were then excised using *EcoRI* and *BamHI* and cloned into pBluescript-II KS creating pKH284 and pDG588 respectively.

Specific binding of regulatory sequences by purified LexA protein *in vitro* was demonstrated by DNase I protection assays. DNA fragments encoding the *LEE2/3*, *LEE5* and *recA* promoters were asymmetrically labeled by cleavage of pKH284, pDG588 or pKH286 with *NotI*. The resulting 5' overhangs were filled in using the Klenow fragment of DNA Polymerase I (NEB), [α -³²P]dCTP (General Electric Healthcare) and dGTP (NEB) at room temperature for five minutes. The polymerase was then heat inactivated at 70°C for 15 minutes. The labeled fragment was then released from the vector by cleavage with *EcoRI* and gel isolated. LexA binding reactions were achieved by incubating 70,000 cpm of probe DNA, 40 µg/ml non-specific DNA (pBluescript-II KS) and varying amounts of purified LexA protein in binding buffer (20 mM TrisHCl at pH 7.4, 10 % sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, 1.5 mM CaCl₂, 2.5 mM MgCl₂, 0.1% bovine serum albumin and 50 mM NaCl), (30) at room temperature for ten minutes. After LexA binding, 40 mU of DNase I (Sigma) was added and incubation proceeded at room temperature for 2 minutes. The reactions were terminated by the addition of Stop Solution (200 mM NaCl, 2 mM EDTA, 1 % SDS). The reactions were then extracted

once with phenol, once with chloroform followed by ethanol precipitation (2 volumes 100% ethanol, 1/10 volume 7.5 M ammonium acetate, pH 7.5 and 1 µl glycogen (Invitrogen)). The pellets were resuspended in 2.5 µl water and 2.5 µl Sequenase Stop Solution (USB), denatured at 75° C for 2 minutes immediately prior to loading and separated in a denaturing 6% polyacrylamide TBE gel. The gel was then transferred to Whatman paper and dried at 80° C for one hour and exposed to Kodak X-Omat LS film. Sequencing reactions with each plasmid and the BluescriptNotI primer were loaded adjacent to the DNase I reactions to determine the exact positions of protected DNA sequences.

RESULTS

Putative LexA binding sites identified at the *LEE2*, *LEE3* and *LEE5* promoters.

We identified a putative LexA binding site within the overlapping EPEC *LEE2/LEE3* promoters extending from positions –4 to –23 and from –13 to +7 in relation to the start of transcription for the *LEE2* and *LEE3* promoters (33), respectively (Table 3; Fig. 1A). Similarly, we identified a putative LexA binding site in the *LEE5* promoter, extending from positions –20 to –1 in relation to the start of transcription (12) (Table 3; Fig. 1B). These sequences contained the nearly invariant CTG motif of the canonical LexA recognition sequence (9, 61) and were positioned directly within the *LEE2*, *LEE3*, and *LEE5* promoters. In comparison, the canonical LexA binding sites at *recA* spans from positions –30 to –11 in relation to the *recA* start of transcription ((30); Fig. 1). Therefore, based on the position of the putative LexA binding sites in relation to the *LEE2/LEE3* and *LEE5* promoters, we predicted that these loci would be responsive to DNA damage-associated signaling, in a RecA-LexA-dependent manner.

LEE promoters responsive to DNA damage associated signaling in a RecA-dependent manner. To test our hypothesis we assayed β -galactosidase activity derived from single-copy *LEE-lacZ* fusions constructed in the *E. coli* K-12 laboratory strain MC4100 in the presence and absence of the DNA damaging agent mitomycin C. Mitomycin C induces interstrand DNA crosslinks (56, 57), causing double strand breaks, inducing the SOS response (47, 61). Because genes of the SOS regulon are induced at varying time points after DNA damage occurs, we monitored β -galactosidase activities at 30 and 60 minutes after the addition of mitomycin C. At the 60-minute time point, mitomycin C caused ~four-fold increased expression from the *LEE2-lacZ* fusion, from 50

to 213 Miller units (Fig. 2A). Similarly, addition of mitomycin C caused ~three-fold and ~four-fold increased expression from the *LEE3-lacZ* and *LEE5-lacZ* fusions, respectively, from 66 to 181, and from 45 to 181 Miller units (Figs. 2B and 2C). We did not observe mitomycin C-dependent increased expression from the *LEE2-lacZ*, *LEE3-lacZ* or *LEE5-lacZ* fusion strains encoding the mutant *recA1* allele (Figs. 2A, 2B, 2C), as mutations in *recA* prevent the bacterium from inducing the SOS response (61).

As a positive control, we constructed a *recA-lacZ* single-copy fusion in strain MC4100, showing that mitomycin C caused expression to increase from 288 to 1435, and from 275 to 2740 Miller units at the 30-minute and 60-minute time points, respectively (Fig. 2D). The ~10-fold increase in activity derived from the *recA* transcriptional fusion at the 60 minute time point was consistent with previously reported results (9, 44). As a negative control, we showed that transcriptional activity derived from a *lac-lacZ* single-copy fusion, constructed identically to all other fusions in strain MC4100, increased less than two-fold, in the presence of mitomycin C. This fusion contained the *lacZYA* promoter, as well as the *lacI* gene encoding the Lac repressor, and showed over 100-fold induction in the presence of the inducer IPTG (data not shown). We also observed that expression from single-copy *LEE1-lacZ* and *LEE4-lacZ* fusions increased less than two-fold in the presence of mitomycin C, and thus concluded that, unlike *LEE2*, *LEE3* and *LEE5*, the *lacZYA*, *LEE1* and *LEE4* operons did not respond to DNA-damage-associated signaling in the K-12-derived strains (data not shown).

SOS regulation of LEE operons is dependent upon functional LexA protein.

Based on our observation that increased LEE transcription in response to DNA damage was dependent upon functional RecA, we predicted that this signaling would also be

dependent upon functional LexA protein. We therefore transduced the *lexA1* allele, which encodes a mutant LexA protein unable to undergo auto-catalytic cleavage in the presence of the co-protease RecA and thus renders the cell unable to induce the SOS response (39), into the *LEE-lacZ* fusion strains and monitored β -galactosidase activity in response to DNA damage. Reporter gene assays in the presence of the *recA1* and *lexA1* alleles were performed in derivatives of the K-12 strain MC4100 because the wt EPEC pathogenic strain E2348/69 is recalcitrant to such genetic manipulations, *i.e.*, generalized transduction to move the *recA1* or *lexA1* allele is not possible using wt EPEC. As predicted, the addition of mitomycin C did not cause increased activity from the *LEE2-lacZ*, *LEE3-lacZ* or the *LEE5-lacZ* fusions in the presence of the *lexA1* allele (Figs. 2A, 2B, 2C). Transduction of the *lexA1* allele into the *recA-lacZ* strain also eliminated the ability of this fusion to respond to the DNA damage signaling induced by the addition of mitomycin C (Fig. 2D). Therefore, we concluded that the EPEC *LEE2*, *LEE3* and *LEE5* operons were regulated by the SOS response in K-12-derived strains in a RecA-LexA-dependent manner.

SOS regulation of the LEE in EPEC. To demonstrate SOS regulation of the LEE operons in wt EPEC we performed RNA dot blot analysis. Briefly, whole-cell RNA was isolated from strain E2348/69 growing in exponential phase in the absence and presence of mitomycin C at the 60 minute time point, the identical conditions for monitoring β -galactosidase activities derived from the *LEE-lacZ* single-copy fusions in the K-12-derived strains. Ten μ g aliquots of RNA were dotted onto a nylon membrane, and hybridized with radiolabeled DNA probes generated by PCR using the oligonucleotide primers presented in Table 2. Dots were cut from the membrane and subjected to

scintillation counting to quantify changes in transcription in response to DNA damage. Consistent with results in the K-12-derived strains, transcription of *LEE2* and *LEE3* increased 60 minutes after the addition of mitomycin C (127%, $P < 0.001$; 136%, $P = 0.038$, respectively) (Table 4). Surprisingly we did not observe a significant increase in *LEE5* transcription in EPEC as a response to DNA damage (108%, $P = 0.128$). However, predictably we observed an increase in transcription of the cryptic prophage-located, effector-encoding *nleA* gene (164%, $P = 0.016$) (Table 4). Transcription of the positive control, *recA*, also increased in the presence of mitomycin C (503%, $P < 0.001$). The negative control *lacZ*, the *LEE1*, *LEE4* operons, and the cryptic prophage-located effector molecule-encoding genes *nleD* and *espJ* did not show significantly increased transcription in response to DNA damage (data not shown).

To further demonstrate SOS regulation of the EPEC LEE we monitored protein secretion in the presence of the *lexA1* allele, which exhibits a dominant negative phenotype in *E. coli* (39). Because EPEC is intractable to genetic manipulation by transducing phage, we amplified the *lexA1* allele from strain DM803 and cloned this gene under the control of the arabinose-inducible P_{BAD} promoter in the plasmid pBAD33 (the resulting plasmid was termed pKH296), and transformed the plasmid into EPEC strains E2348/69, CVD452 deleted for *escN*, the gene encoding the ATPase of the TTSS (14), and SE796 deleted for the global regulator *ler* (33). EPEC strains containing the pKH296 plasmid and the pBAD33 empty vector control were grown in HEPES, pH 7.4, buffered DMEM, a tissue culture medium that maximizes secretion, harvested at late exponential phase, and secreted proteins were precipitated, then separated by PAGE. As expected, we observed no secretion of proteins from strain CVD452 deleted for *escN* in the presence of

either pBAD33 or pKH296 (Fig. 3, Lanes 1 and 2). In the wt EPEC strain E2348/69 we observed a modest decrease in quantity of some secreted proteins, most notably EspB/D in the presence of the *lexA1* allele (Fig. 3, Lanes 3 and 4). Because Ler is a key regulator of the EPEC virulence, we also monitored protein secretion in the strain SE796 deleted for *ler*, dampening expression of the LEE and enabling a better understanding of the contribution of the SOS response control over TTSS function. In strain SE796, expression of the uncleavable LexA protein eliminated secretion of all proteins, save one co-migrating with the 66 kDa MW marker, when compared to the pBAD33 plasmid control (Fig. 3, Lanes 5 and 6). These results were consistent with alterations in transcriptional activities of LEE operons observed in both K-12 and wt EPEC strains. We therefore concluded that the LexA protein and the SOS response regulate LEE transcription and secretion of proteins by the EPEC TTSS.

Purified LexA protein binds *in vitro* to the predicted binding site located in the EPEC *LEE2/LEE3* promoter region. Based on the genetic, and phenotypic evidence presented above, we predicted that purified LexA protein would bind to EPEC LEE regulatory DNA *in vitro*. We therefore performed DNase I protection assays using purified LexA protein, which is identical in the K-12 MG1655 and EPEC E2348/69 strains ((2); http://www.sanger.ac.uk/Projects/Escherichia_Shigella/), and *LEE2/3*, and *LEE5* regulatory fragments containing the putative LexA binding sites (Fig. 1, Table 3). As a positive control, we also performed the DNase I protection assay using a DNA fragment containing the demonstrated LexA binding site of the *recA* promoter (30). For the *recA* promoter-containing DNA fragment purified LexA protein protected positions – 2 to –30 from DNase I digestion, corresponding nearly identically to results previously

published (Fig. 4C; (30)). We observed a band corresponding to hypersensitivity to DNase I digestion at positions –32 and –5 in relation to the *recA* start of transcription, near the 5' and 3' points of LexA binding, respectively, on the coding DNA strand (Figs. 1C, 4C).

Purified LexA protein bound the *LEE2/LEE3* regulatory fragment from positions –2 to –25 in relation to the *LEE2* start of transcription (Figs. 1A, 4A). This site corresponds to positions –15 to +9 in relation to the overlapping *LEE3* promoter (Figs. 1A, 4A). We observed regions of DNA that were hypersensitive to DNase I cleavage at positions –28 +1, +2, and +3 of the *LEE2* promoter, which we interpreted as indicating the extent of LexA binding, closely corresponding to the predicted LexA binding site (Figs. 1A, 4A, Table 3). LexA protein bound discreetly over the –10 hexamers of both *LEE2* and *LEE3* promoters, even at relatively high protein concentration, 2 μ M (Fig. 4A). Though the *LEE5* operon was regulated by the SOS response in a RecA-LexA-dependent manner in the K-12-derived *lacZ* fusion strain *in vivo* (Fig. 2C), multiple DNase I protection assays demonstrated that purified LexA protein did not bind specifically to the *LEE5* promoter region *in vitro* (Fig. 4B). This result, however, was consistent with the lack of SOS regulation of the *LEE5* operon in EPEC by dot blot analysis (Table 4). Because purified LexA protein bound specifically to the predicted *LEE2/LEE3* binding site *in vitro*, we concluded that the LexA protein directly represses EPEC *LEE2/LEE3* transcription.

DISCUSSION

In this report we present genetic, biochemical and phenotypic evidence that the SOS response regulates expression of LEE genes of EPEC, encoding components of a TTSS. The DNA-damaging agent mitomycin C caused increased transcription of single-copy *LEE2-lacZ*, *LEE3-lacZ*, and *LEE5-lacZ* fusions in a K-12-derived strain and this activity was eliminated in the presence of the *recA1* allele, which prevents the bacterium from inducing the SOS response (Fig. 2) (6, 61). Additionally, increased transcriptional activity of these operons was abrogated in the presence of the *lexA1* allele, encoding an uncleavable LexA protein (39, 52). In the wt EPEC strain E2348/69 we demonstrated increased transcriptional activity of the *LEE2* and *LEE3* operons by RNA dot blot analysis (Table 4), consistent with results observed in the K-12-derived strains.

By DNase I protection assay we found that purified LexA protein specifically bound a *LEE2/LEE3* regulatory fragment. We observed LexA binding protected the *LEE2/LEE3*, overlapping, divergent promoter fragment under identical conditions whereby LexA protected a *recA* promoter fragment from DNase I digestion (Fig. 4). We therefore concluded LexA directly regulates expression of the *LEE2/LEE3* operons in EPEC. Binding of LexA at the *LEE2/LEE3* site is predicted to prevent transcription by occlusion of RNA polymerase binding, consistent with the demonstrated mechanism of LexA repression. Single operator sites have also been observed at the divergent *uvrA/ssb*, *ybiA/dinG* and the *umuCD/hylE* promoters and LexA is predicted to inhibit transcription of both operons in each case (9).

Consistent with the absence of increased *LEE5* transcription in response to DNA damage in EPEC by RNA dot blot analysis (Table 4), we did not observe binding of

LexA protein to a *LEE5* regulatory fragment *in vitro* (Fig. 4). The most likely explanation for observing a *LEE5* response to DNA damage in the K-12 strain but not in the wt pathogen is that an EPEC-specific repressor dampens transcription at *LEE5*, disallowing response to the DNA damage signal under the conditions tested. We hypothesize that without an EPEC-specific repressor in the K-12 strain, SOS regulation of the *LEE5* operon is thus demonstrable. Evidence for a non-H-NS, negative regulator acting at the *LEE5* operon in EPEC has been reported previously (49, 58). In addition, if the *LEE5* operon of EPEC were subject to SOS regulation, we would predict it to be indirect, because the purified LexA protein did not bind to the *LEE5* regulatory DNA *in vitro* (Fig. 4).

SOS induction of the LEE operons was slower than that of the *recA* gene in response to DNA damage. The *recA* gene was induced five-fold and 10-fold 30 and 60 minutes after the addition of mitomycin C, respectively, while the *LEE2* and *LEE3* operons required 60 minutes of exposure to the DNA damaging agent in order to observe induction (Fig. 2). We attributed this difference in induction kinetics to the relative strength of induction- the *LEE2* and *LEE3* operons were induced three- to four-fold while the *recA* gene was induced 10-fold at the 60-minute time point.

DNA damage signals that induce the SOS response, and ultimately phage gene expression play an important role in bacterial pathogenesis. Prophages are central to pathogenesis in *E. coli* serotype O157:H7, a pathogen closely related to EPEC, and *V. cholerae*, among others. The Stx₂ toxin of serotype O157:H7 is encoded in the 933W λ -like prophage and is transcribed from the P_{R'} promoter (43, 60). Agents that cause DNA damage, and thus phage induction increase Stx₂ biosynthesis and release in this pathogen

(54, 62). The P_{rstA} promoter of the cholera toxin-encoding CTX Φ prophage is regulated by the SOS response, and is dependent upon LexA cleavage for induction (46). We demonstrate here that expression of the TTSS of EPEC is regulated by the same mechanism, the SOS response, finding that TTSS-dependent protein secretion was reduced in the presence of the *lexA1* allele encoding an uncleavable LexA protein (Fig. 3).

Several effector molecules secreted by the TTSS of EPEC strains are encoded within defective prophage, including Cif, NleA, NleD and EspJ (16), and the genes encoding these proteins are likely to be coordinately regulated with the TTSS, necessary for their secretion in order for them to play a role in pathogenesis. The CP-933P prophage encoded NleA protein co-localizes with the Golgi apparatus in the host cell, is not required for AE lesion formation, but does play an important, though not fully understood role in the *Citrobacter rodentium*/mouse infection model (19, 40). We provide evidence in this report that transcription of the prophage-encoded *nleA* gene is regulated by the SOS response (Table 4). For bacteriophage λ , activation of the co-protease RecA leads to autocatalytic cleavage and release of the λ CI repressor (29, 41), expression of phage genes and thus phage induction. Some phage, in fact use LexA as the repressor to prevent induction (46). Activation of the SOS response upon DNA damage, leading to autocatalytic cleavage of the LexA protein and/or phage repressors by the RecA co-protease bound to single-stranded DNA might explain how a subset of the cryptic prophage-encoded EPEC effector molecules and associated phenotypes are coordinately regulated with the LEE-encoded TTSS within a host.

Multiple mechanisms most likely induce the SOS response of bacterial pathogens within the human gastrointestinal tract, and during infection the organisms are subjected to tremendous selective pressure. For example, membrane-membrane interactions cause DNA damage and activation of repair mechanisms in *Neisseria meningitidis* (38). Neutrophils of the human innate immune system produce agents, such as H₂O₂, that can cause DNA damage to the invading bacterium (59). Prescribing antibiotics, such as β -lactams that damage the bacterial cell wall also induce the SOS response (35). EPEC inhibits expression of inducible nitric oxide synthase (iNOS), which produces the antibacterial agent nitric oxide (NO), from human intestinal epithelial cells in culture (31). This activity was dependent upon attachment to, and delivery of effector molecules into the host cell, and provides further evidence that EPEC perceives and responds to DNA damage signals within the human host. We now know that the SOS response regulates expression of at least one of the phage-encoded effector molecules and its cognate TTSS, a structure necessary for EPEC, and many other Gram-negative bacteria to cause disease. This regulation links functions fundamental to bacterial survival within the host- SOS control of stalled DNA replication forks and expression of error-prone polymerases that lead to increased mutation frequencies- with expression of a structure fundamental to EPEC pathogenesis, the TTSS. These data underscore the need to diversify our therapeutic arsenal directed against EPEC and related pathogens.

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TABLE 1. Bacterial strains, plasmids and phage used in this study.

Strains	Genotype/description	Reference
E2348/69	Wild type EPEC1 pathogen	(27)
CVD452	E2348/69 $\Delta escN::aphT$	(22)
SE796	E2348/69 $\Delta ler::aphA3$	(13)
MG1655	Wild type laboratory K-12 strain	(2)
DH5 α	<i>supE44</i> $\Delta(argF-lac)U169$ <i>($\phi 80dlac\Delta(Z)M15$) deoR hsdR17 recA1</i> <i>endA1 gyrA96 thi-1 relA1</i>	Laboratory stock
MC4100	<i>araD139</i> $\Delta(argF-lac)U169$ <i>rpsL150</i> <i>relA1 flbB5301 deoC1 ptsF25 rbsR</i>	(4)
USL10	MC4100 <i>recA1 srl::Tn10</i>	(8)
DM803	<i>F- metA28 lacY1 or Z4 $\lambda+$ thi-1 xyl-5</i> <i>or -7 galK2 tsx-6 lexA1</i>	(39)
TL229	MC4100 <i>MalT^c-1ΔmalE44 zjb-</i> <i>729::Tn10</i>	(26)
JLM166	MC4100 Φ <i>LEE2-lacZ</i> (-682 to +206)	(33)
JLM172	MC4100 Φ <i>LEE3-lacZ</i> (-434 to +262)	(33)
KMTIR3	MC4100 Φ <i>LEE5-lacZ</i> (-303 to +172)	(20)
JLM281	MC4100 Φ <i>recA-lacZ</i> (-97 to +827)	This study
JLM803	<i>F- metA28 lacY1 or Z4 $\lambda+$ thi-1 xyl-5</i> <i>or -7 galK2 tsx-6 lexA1 zjb-729::Tn10</i>	This study

KH4151	JLM281 <i>recA1 srl::Tn10</i>	This study
KH4155	KMTIR3 <i>recA1 srl::Tn10</i>	This study
KH4166	JLM166 <i>recA1 srl::Tn10</i>	This study
KH4172	JLM172 <i>recA1 srl::Tn10</i>	This study
JLM166A	JLM166 <i>lexA1 zjb-729::Tn10</i>	This study
JLM172A	JLM172 <i>lexA1 zjb-729::Tn10</i>	This study
JLMTIR3A	KMTIR3 <i>lexA1 zjb-729::Tn10</i>	This study
JLM281A	JLM281 <i>lexA1 zjb-729::Tn10</i>	This study
Plasmids		
pBluescript-II KS	Cloning vector, <i>bla</i>	Stratagene
pRS551	(promoter-less) <i>lacZ</i> reporter fusion vector, <i>bla</i>	(50)
pKH281	<i>recA-lacZ</i> (-97 to +827) in pRS551	This study
pKH284	<i>LEE2</i> (-83 to +75)/ <i>LEE3</i> (-94 to +64) promoter in pBluescript	This study
pKH286	<i>recA</i> (-97 to +62) promoter in pBluescript	This study
pKH16S	300 bp of <i>rrnH</i> 16S rRNA gene	This study
pKH <i>recA</i>	245 bp of <i>recA</i> coding sequence starting from first ATG in pCR2.1- TOPO	This study

pKHLEE2	297 bp of <i>sepZ</i> coding sequence starting from first ATG in pCR2.1- TOPO	This study
pKHLEE3	300 bp of <i>escV</i> coding sequence 400 bp downstream from the first ATG in pCR2.1-TOPO	This study
pKHLEE5	301 bp of <i>tir</i> coding sequence starting from first ATG in pCR2.1-Topo	This study
pKHnleA	300 bp of <i>nleA</i> coding sequence starting from first ATG in pCR2.1-Topo	This study
pDG588	<i>LEE5</i> (-75 to +88) promoter in pBluescript	This study
Phage		
P1 <i>vir</i>	Generalized transducing phage	Laboratory stock
λRS45	Specialized transducing phage	(50)

TABLE 2. Oligonucleotides used in this study

Primer name	Sequence (5' to 3') ^a
Tir5	CCGGAATTCGTTGGAAATACAGACATGCA
TIR+88	CGCGGATCCATACATATATCCTTTTATTAGAA
5' <i>LEE3</i>	CCGGAATTCACCTGGTTATAAATAACCA

3' <i>LEE3</i>	CCG <u>GGATCC</u> GATTCATCTGCAGGCTCTGA
5'- <i>recA</i>	GTGCGTCGTCAGGCTACTG
3'- <i>recA</i>	GGATGTTGATTCTGTCATGGC
5' <i>rec</i> -foot	CCG <u>GAAATC</u> GTGCGTCGTCAGGCTACTG
3' <i>rec</i> -foot	CCG <u>GGATCC</u> CGTCGATAGCCATTTTAC
Slot 16S rRNA 5'For	AAATTGAAGAGTTTGATCATGGCTC
Slot 16S rRNA 3'Rev	TCTCAGACCAGCTAGGGATCGTCGC
Slot <i>LEE2</i> 5'For	ATGGAAGCAGCAAATTTAAG
Slot <i>LEE2</i> 3'Rev	TTAGGCATATTTTCATCGCTA
<i>LEE3</i> #2 For	GGGCCGAGCGTGTTGCTGAA
<i>LEE3</i> #2 Rev	CAGAAAACAGGCTAAGTGCC
Slot <i>LEE5</i> 5'For	ATGCCTATTGGTAACCTTGG
Slot <i>LEE5</i> 3'Rev	GAATATCAAGTGGCCCCTTA
Slot <i>nleA</i> 5'For	ATGAACATTCAACCGATCGTAACAT
Slot <i>nleA</i> 3'Rev	GAATAAACCAACATATCCAATGCT
Slot <i>recA</i> 5'For	ATGACAGGAGTAAAAATGGC
Slot <i>recA</i> 3'Rev	GTCAGCGTGGTTTTACCGGA
BluescriptNotI	GGCCGCTCTAGAACTAGTG
M13For	CCCAGTCACGACGTTGTAAAACG

^a Restriction sites used in cloning are underlined.

TABLE 3. LexA binding sites

Gene/Operon	LexA Binding Sites ^a
LexA consensus site ^b	TACTGTATATATACAGTA
<i>recA</i>	TACTGTAT GCT CATACAGTA
<i>LEE2/LEE3</i>	TACTGTATATAT TAT CCAAT
<i>LEE5</i>	TACTGT GAT T TAT T TGGT T

^a The LexA binding site at *recA* was identified previously (9, 30). The putative LexA binding sites at the *LEE2/LEE3* and *LEE5* operons were identified in this study. DNA sequences with identity to the consensus LexA binding site (9, 61) are noted by bold text.

^b The LexA consensus binding site is TACTG(TA)₅CAGTA (9, 61).

TABLE 4. Mitomycin C increased transcription of *LEE2*, *LEE3* and *nleA* in EPEC

Operon/Gene	Signal ^a	SD	<i>P</i>
<i>LEE2</i>	127%	12%	0.001
<i>LEE3</i>	136%	39%	0.038
<i>LEE5</i>	108%	12%	0.128
<i>nleA</i>	164%	51%	0.016
<i>recA</i>	503%	132%	0.0002

^a Values are for increased transcription at the listed operons or genes comparing signal in the presence versus absence of mitomycin C 60 minutes after addition. EPEC strain E2348/69 was grown in LB at 37° C to mid-exponential phase prior to treatment. Data includes two independent RNA isolations probed in triplicate.

FIGURE LEGENDS

FIG. 1. Putative and established LexA binding sites at the *LEE2/LEE3*, *LEE5* and *recA* promoters. (A) The putative LexA binding site (61) at the *LEE2* promoter spans from positions –4 to –23, corresponding to positions –13 to +7 for the *LEE3* promoter. (B) The putative LexA binding site at the *LEE5* promoter spans from positions –20 to +1. (C) The LexA binding site (SOS box) (61) at the *recA* promoter spans from positions –30 to –11, was determined previously (30), and is indicated by a dashed line. The –35 and –10 canonical hexamers for E σ^{70} RNA polymerase and start points of transcription (+1) appear in bold text, while the direction of transcription for each promoter is indicated by an arrow. Observed binding of purified LexA protein to the *LEE2/LEE3* promoter region (A) is indicated by a dashed line.

FIG. 2. DNA damage-associated response of single-copy *LEE-lacZ* fusions occurs in a RecA-LexA-dependent manner in *E. coli* K-12-derived strains. β -Galactosidase activities derived from (A) *LEE2-lacZ*, (B) *LEE3-lacZ*, (C) *LEE5-lacZ*, and from (D) *recA-lacZ* fusions were monitored 30 and 60 minutes after the addition of the DNA damaging agent mitomycin C. Assays were also performed in *recA1* and *lexA1* genetic backgrounds to establish SOS induction of *LEE-lacZ* transcriptional fusions. Error bars represent one standard deviation. Data presented is the mean of at least two experiments performed in triplicate.

FIG. 3. LexA-dependent secretion by the EPEC TTSS. Strains were grown to an A₆₀₀ of ~0.8 to 1.0 in DMEM buffered with 50 mM HEPES, pH 7.4, in the presence of 0.1%

arabinose to induce *lexA* expression. Bacterial cultures were centrifuged, supernatants removed, secreted proteins precipitated, washed and separated on a 15% polyacrylamide SDS-containing gel. Secretion was monitored from the following strains: Lane 1, CVD452 (pBAD33); Lane 2, CVD452 (pKH296); Lane 3, E2348/69 (pBAD33); Lane 4, E2348/69 (pKH296); Lane 5, SE796 (pBAD33); and Lane 6, SE796 (pKH296); relevant genotypes are indicated. Black dots appear adjacent to proteins, including the predominantly observed EspB/D, whose secretion is partially reduced by the *lexA* allele encoding the uncleavable LexA in wt EPEC strain E2348/69. An asterisk appears adjacent to proteins whereby secretion is reduced in the Δler strain SE796 in the presence of the uncleavable LexA. A dagger appears adjacent to a protein of ~66 kDa that requires EscN, but neither Ler nor a functional LexA transcriptional regulator for secretion. Molecular standards appear adjacent to their corresponding masses in kDa.

FIG. 4. DNase I protection assays to establish purified LexA protein binding at *LEE* and *recA* regulatory DNA fragments *in vitro*. (A) The region of protection from DNase I cleavage, demonstrating LexA binding at the *LEE2/LEE3* promoters, is indicated by vertical bar. (B) LexA binding at the *LEE5* promoter was not observed *in vitro* under the conditions tested. (C) The region of protection from DNase I cleavage, demonstrating LexA binding at the *recA* promoter was performed as a positive control, as LexA binding *in vitro* at this locus was previously established (30). The region of protection is indicated by vertical bar. Bands showing hypersensitivity to DNase I cleavage are indicated by arrowheads. The canonical -35 and -10 hexamers for $E\sigma^{70}$ RNA polymerase for the *LEE2*, *LEE5* and *recA* promoters are indicated by double lines, while the canonical

hexamers for the *LEE3* promoter is indicated by a dashed line. DNA sequencing ladders appear adjacent to DNase I protection assays with varying amounts of purified LexA protein as indicated.

A.

LEE3 →

-35 -10 +1

TTGCAATGTACATAAAGTACATATACTGTATATATTATCCAATACGCTTT

AACGTTACATGTATTTTCATGTATATGACATATATAATAGGTTATGCGAAA

+1 -10

← *LEE2*

TTCAAGCAATAATATTCACACGTATACTTACTTCAGAGCCTGCAGATGAA

AAGTTCGTTATTATAAGTGTGCATATGAATGAAGTCTCGGACGTCTACTT

-35

B.

LEE5 →

-35 -10 +1

TTGCATCAAAAATTAACTGTGATTTATTGGTTTATGCTCAGTTGTTTT

AACGTAGTTTTTTAATATGACACTAAATAAACCAAATACGAGTCAACAAAA

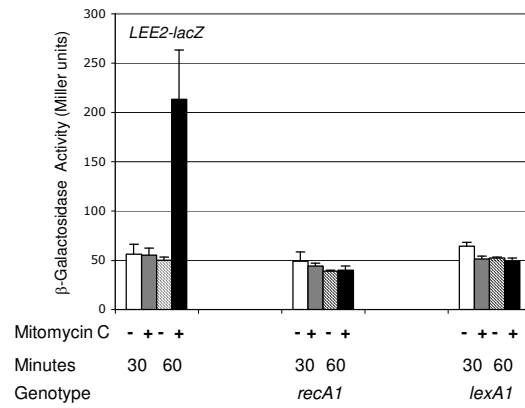
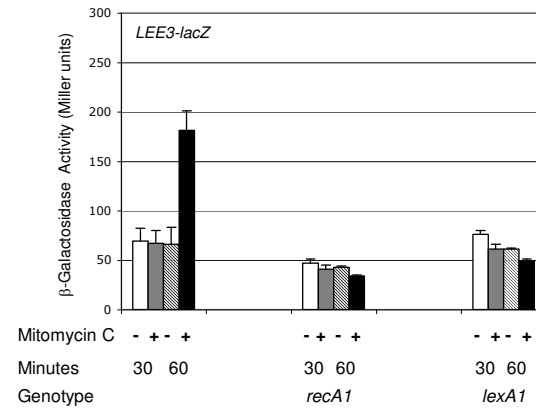
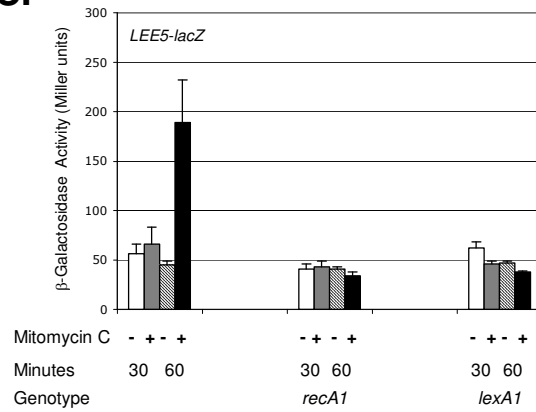
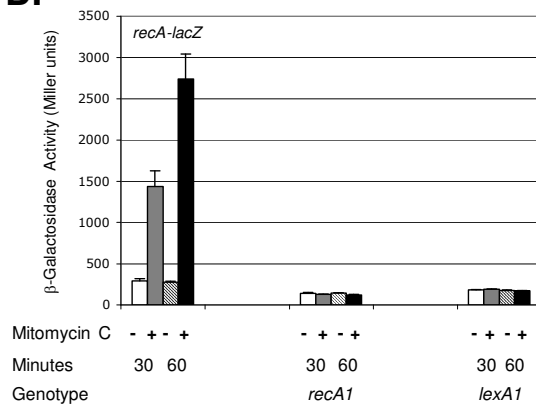
C.

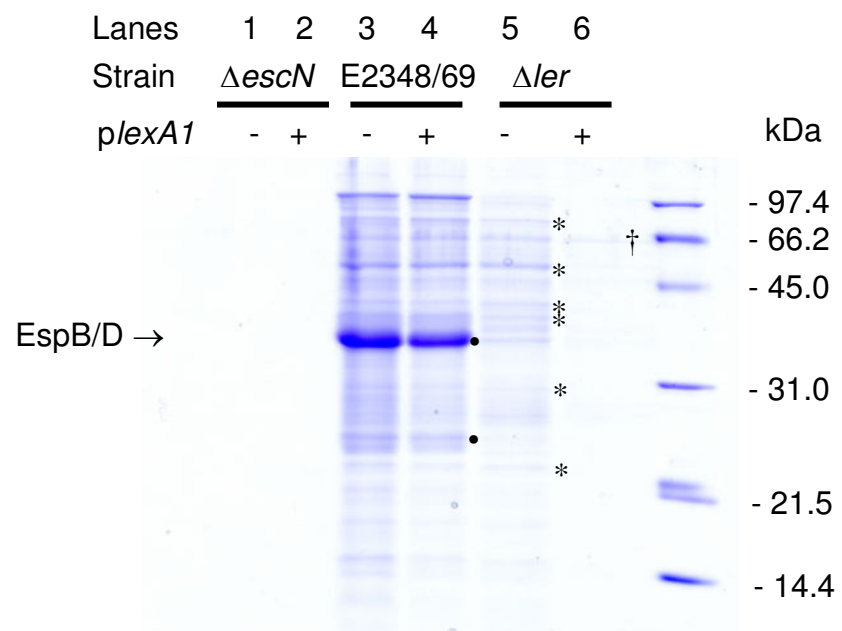
recA →

-35 -10 +1

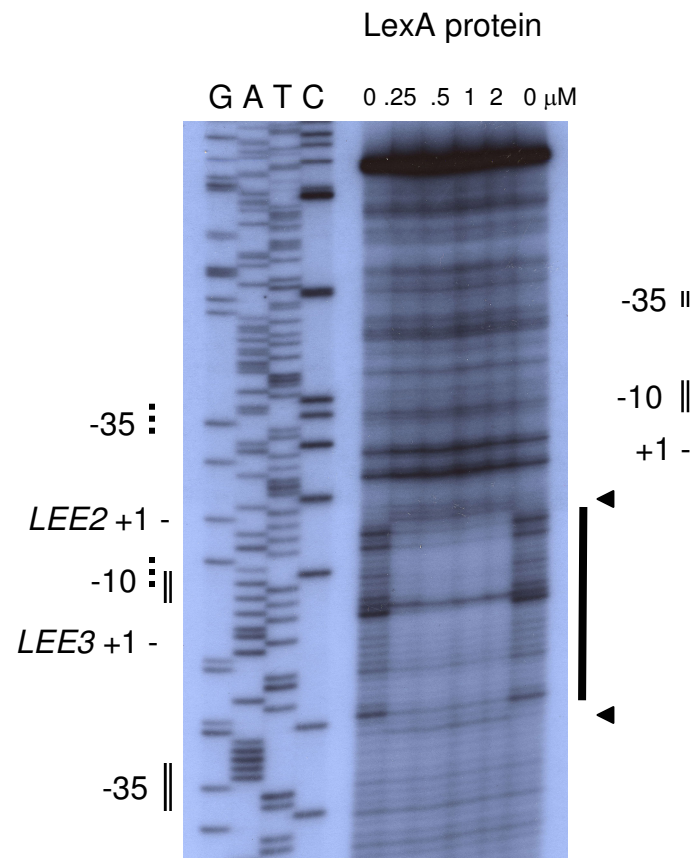
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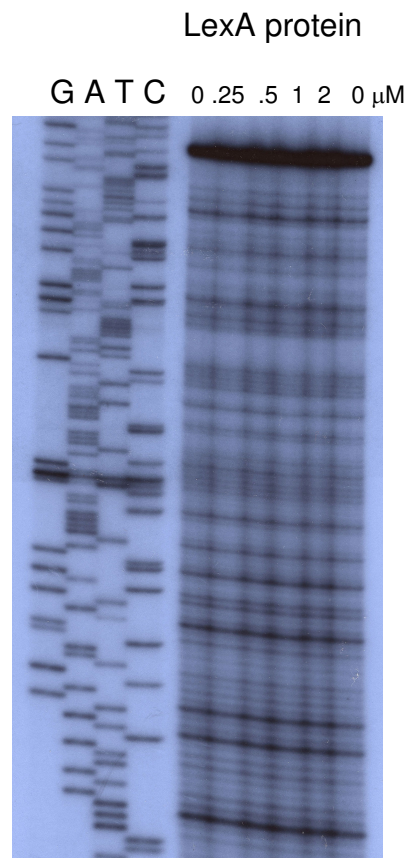
A.**B.****C.****D.**



A. *LEE2/LEE3*



B. *LEE5*



C. *recA*

