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

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Genomes of bacterial pathogens contain 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 TTSS 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 responded to SOS signaling and that this regulation was LexA dependent. As determined by a 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 obtained evidence that the cryptic phage-located *nleA* gene encoding an effector molecule is SOS regulated. Thus, we demonstrated, for the first time to our knowledge, that genes encoding components of a TTSS are regulated by the SOS response, and our data might explain how a subset of EPEC effector proteins, encoded in cryptic prophages, are coordinately regulated with the LEE-encoded TTSS necessary for their translocation into host cells.

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

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

EPEC diarrhea results from a loss of absorptive tissue due to destruction of the microvilli during the formation of AE lesions, alteration of host cell signaling events that causes active ion secretion, increased intestinal permeability, loosening of

tight junctions, and inflammation at the site of infection (for reviews, see references 7 and 23). Effector molecules translocated into the host cell by the TTSS apparatus include the LEE-encoded Tir, EspF, EspG, EspH, EspZ, and Map proteins, as well as NleA (also designated EspI), EspD, EspJ, EspG2, and Cif, which are encoded outside the LEE pathogenicity island (16). EspG2 is encoded in the EspC pathogenicity island (34), and interestingly, the NleA, NleD, EspJ, and Cif proteins are encoded in cryptic prophage (10, 19, 40).

Maximal secretion of EPEC effector molecules occurs during the early exponential phase of growth at 37°C at pH 7 and is influenced by medium conditions (24, 25, 48, 58). To date, however, we do not understand how the prophage-encoded effector molecules are coordinately regulated with the components of the LEE-encoded TTSS.

A number of regulatory proteins have been shown to control expression of the EPEC LEE (for a review, see <http://www.ecosal.org/ecosal/toc/index.jsp>); these proteins include the master regulator Ler, which is encoded in the *LEE1* operon (13, 33). PerC, encoded in the *E. coli* attachment factor virulence plasmid, and integration host factor 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 the *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 finally, the GrlRA (global reg-

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

Strain, plasmid, or phage	Genotype and/or description	Reference or source
Strains		
E2348/69	Wild-type EPEC pathogen	27
CVD452	E2348/69 Δ <i>escN::aphT</i>	22
SE796	E2348/69 Δ <i>ler::aphA3</i>	13
MG1655	Wild-type laboratory K-12 strain	2
DH5 α	<i>supE44</i> Δ (<i>argF-lac</i>) <i>U169</i> ϕ 80 <i>dlacZ</i> Δ <i>M15</i> <i>deoR</i> <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Laboratory stock
MC4100	<i>araD139</i> Δ (<i>argF-lac</i>) <i>U169</i> <i>rpsL150</i> <i>relA1</i> <i>flbB5301</i> <i>deoC1</i> <i>ptsF25</i> <i>rbsR</i>	4
USL10	MC4100 <i>recA1</i> <i>srl::Tn10</i>	8
DM803	<i>F</i> [−] <i>metA28</i> <i>lacY1</i> or <i>-Z4</i> λ ⁺ <i>thi-1</i> <i>xyl-5</i> or <i>-7</i> <i>galK2</i> <i>tsx-6</i> <i>lexA1</i>	39
TL229	MC4100 <i>malT1</i> Δ <i>malE44</i> <i>zjb-729::Tn10</i>	26
JLM166	MC4100 Φ <i>LEE2-lacZ</i> (positions −682 to 206)	33
JLM172	MC4100 Φ <i>LEE3-lacZ</i> (positions −434 to 262)	33
KMTIR3	MC4100 Φ <i>LEE5-lacZ</i> (positions −303 to 172)	20
JLM281	MC4100 Φ <i>recA-lacZ</i> (positions −97 to 827)	This study
JLM803	<i>F</i> [−] <i>metA28</i> <i>lacY1</i> or <i>-Z4</i> λ ⁺ <i>thi-1</i> <i>xyl-5</i> or <i>-7</i> <i>galK2</i> <i>tsx-6</i> <i>lexA1</i> <i>zjb-729::Tn10</i>	This study
KH4151	JLM281 <i>recA1</i> <i>srl::Tn10</i>	This study
KH4155	KMTIR3 <i>recA1</i> <i>srl::Tn10</i>	This study
KH4166	JLM166 <i>recA1</i> <i>srl::Tn10</i>	This study
KH4172	JLM172 <i>recA1</i> <i>srl::Tn10</i>	This study
JLM166A	JLM166 <i>lexA1</i> <i>zjb-729::Tn10</i>	This study
JLM172A	JLM172 <i>lexA1</i> <i>zjb-729::Tn10</i>	This study
JLMTIR3A	KMTIR3 <i>lexA1</i> <i>zjb-729::Tn10</i>	This study
JLM281A	JLM281 <i>lexA1</i> <i>zjb-729::Tn10</i>	This study
Plasmids		
pBluescript II KS	Cloning vector, <i>bla</i>	Stratagene
pRS551	(Promoterless) <i>lacZ</i> reporter fusion vector, <i>bla</i>	50
pKH281	<i>recA-lacZ</i> (positions −97 to 827) in pRS551	This study
pKH284	<i>LEE2</i> (positions −83 to 75)/ <i>LEE3</i> (positions −94 to 64) promoter in pBluescript	This study
pKH286	<i>recA</i> (positions −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 the first ATG in pCR2.1-TOPO	This study
pKH <i>LEE2</i>	297 bp of <i>sepZ</i> coding sequence starting from the first ATG in pCR2.1-TOPO	This study
pKH <i>LEE3</i>	300 bp of <i>escV</i> coding sequence 400 bp downstream from the first ATG in pCR2.1-TOPO	This study
pKH <i>LEE5</i>	301 bp of <i>tir</i> coding sequence starting from the first ATG in pCR2.1-TOPO	This study
pKH <i>nleA</i>	300 bp of <i>nleA</i> coding sequence starting from the first ATG in pCR2.1-TOPO	This study
pDG588	<i>LEE5</i> (positions −75 to 88) promoter in pBluescript	This study
Phages		
P1 <i>vir</i>	Generalized transducing phage	Laboratory stock
λ RS45	Specialized transducing phage	50

ulator of LEE repressor and activator) 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 the 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). In this study we demonstrate that expression of the TTSS of EPEC is regulated by the SOS response in a LexA-dependent manner, and below we discuss the implica-

tions 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 bacteriophages 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 at the following final concentrations: ampicillin, 100 μ g/ml; kanamycin, 50 μ g/ml; chloramphenicol, 30 μ g/ml; and tetracycline, 15 μ g/ml.

Generation of regulatory fragments. Promoter fragments for transcriptional studies were generated by PCR by standard protocols (21) utilizing *Taq* polymerase (Sigma), oligonucleotide primers listed in Table 2 (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 (Table 2). Amplified DNA fragments were gel isolated using a Qiaquick gel extraction kit (QIAGEN) and were subsequently cloned with a TOPO TA cloning kit (Invitrogen) used according to the manufacturer's instructions. Regulatory fragments were verified by restriction mapping and DNA sequence analysis.

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5' to 3') ^a
Tir5.....	CCGGAATTCGTTGGAAATACAGACATGCA
TIR+88.....	CCGGATCCATACATATATCCTTTTATTT AGAA
5' <i>LEE3</i>	CCGGAATTCACCTGGTTATAAATAACCA
3' <i>LEE3</i>	CCGGGATCCGATTTCATCTGACAGGCTCTGA
5'- <i>recA</i>	GTGCGTCGTCAGGCTACTG
3'- <i>recA</i>	GGATGTTGATTCTGTCTATGGC
5'- <i>rec-foot</i>	CCGGAATTCGTGCGTCGTCAGGCTACTG
3'- <i>rec-foot</i>	CCGGATCCCGTCGATAGCCATTTTAC
Slot 16S rRNA 5'For.....	AAATTGAAGAGTTTGATCATGGCTC
Slot 16S rRNA 3'Rev.....	TCTCAGACAGCTAGGGATCGTCGCG
Slot <i>LEE2</i> 5'For.....	ATGGAACGACGAAATTTAAG
Slot <i>LEE2</i> 3'Rev.....	TTAGGCATATTTTCATCGCTA
<i>LEE3</i> #2 For.....	GGGCCGAGCGTGTGTCTGAA
<i>LEE3</i> #2 Rev.....	CAGAAAACAGGCTAAGTGCC
Slot <i>LEE5</i> 5'For.....	ATGCCTATTGGTAACCTTGG
Slot <i>LEE5</i> 3'Rev.....	GAATATCAAGTGGCCCCCTTA
Slot <i>nleA</i> 5'For.....	ATGAACATTCAACCGATCGTAACAT
Slot <i>nleA</i> 3'Rev.....	GAATAAACCAACATATCCAATGCT
Slot <i>recA</i> 5'For.....	ATACAGGAGTAAAAATGGC
Slot <i>recA</i> 3'Rev.....	GTACGCGTGGTTTACCGGA
BluescriptNotI.....	GGCCGCTCTAGAACTAGTG
M13For.....	CCCAGTCACGACGTTGTAAACG

^a Restriction sites used in cloning are underlined.

Construction of a chromosomally encoded, single-copy *recA* transcriptional fusion. The *recA* transcriptional reporter gene fusion was constructed by excising the *recA* regulatory fragment (positions -97 to 827) from the TOPO TA plasmid vector with restriction endonuclease EcoRI and was cloned upstream of the promoterless *lacZ* gene in pRS551 previously cleaved with EcoRI and dephosphorylated with shrimp alkaline phosphatase (Roche), creating plasmid pKH281. Electrocompetent DH5 α was transformed by using a Bio-Rad Micropulser electroporation apparatus and selecting for ampicillin resistance. Plasmid DNA was isolated using an UltraClean mini plasmid prep kit (Mo Bio) according to the manufacturer's instructions and was subjected to restriction enzyme digestion and DNA sequence analysis for verification of the specific and *recA-lacZ* transcriptional fusions.

In order to isolate the chromosomally encoded, single-copy *recA-lacZ* transcriptional fusion, the 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 using established protocols (33, 50).

Enzymatic assays. β -Galactosidase assays were performed as described by Miller (36), except that saturated overnight cultures were diluted 50-fold and allowed to grow to the exponential phase (optical density at 600 nm [OD₆₀₀], ~0.5). Individual cultures were subsequently diluted to obtain OD₆₀₀ of ~0.1 to begin the time course. These cultures were then divided in two, and mitomycin C was added to one of the cultures at a final concentration of 1 μ g/ml at time zero. Aliquots were removed after 30 and 60 min of growth and kept on ice until β -galactosidase assays were performed. The data presented below are expressed in Miller units and are the means of at least two independent experiments performed in triplicate.

Generalized P1vir transduction. Strains containing *recA1* or *lexA1* alleles were constructed by P1vir transduction (36). P1vir lysates were generated from donor strains that contained Tn10 insertions, which encoded tetracycline resistance, linked to mutant *recA1* or *lexA1* alleles, which conferred UV sensitivity (6, 39). To isolate a Tn10 insertion linked to the *lexA1* allele, we transduced strain DM803 to tetracycline resistance by using a P1vir lysate from strain TL229 that contained Tn10 linked to *lexA*, *zjb-729::Tn10*. By selecting for 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 died when they were exposed to 30 s of UV light from a germicidal STERILAMP [Westinghouse].) Once strains containing Tn10 insertions linked to *lexA1* (JLM803) or *recA1* (USL10) were isolated, we infected the appropriate recipient strains with P1vir lysates generated from strains JLM803 and USL10, selecting for tetracycline resistance. Transductants that had inherited the Tn10 and 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 medium with and without mitomycin C for 60 min during expo-

nential growth. Strains E2348/69 and MC4100 were inoculated into 2 ml of LB medium, grown overnight at 37°C with aeration, and diluted 50-fold in LB medium the next morning. Cultures were grown at 37°C with shaking until the A_{600} was between 0.5 and 1.0. These cultures were diluted to obtain an A_{600} of ~0.1 in LB medium with and without 1 μ g/ml of mitomycin C. After 60 min of growth, cells were harvested by centrifugation and stored at -80°C. Total RNA was isolated from each culture by using Trizol (Invitrogen) according to the manufacturer's instructions.

Total RNA was then treated with 10 U of DNase I (Sigma) at 37°C for 30 min in buffer containing 10 mM Tris (pH 8.0), 2.5 mM MgCl₂, and 0.5 mM CaCl₂. The reaction was quenched by addition of EDTA to a final concentration of 5 mM and heating to 75°C for 10 min. RNA integrity and the absence of contaminating DNA were analyzed by agarose gel electrophoresis, followed by staining with ethidium bromide. Clear, sharp 23S and 16S RNA species were observed, and no detectable genomic DNA was present. The concentration of RNA was determined spectrophotometrically by determining the absorbance at 260 nm.

Total RNA (10 μ g/dot) in transfer buffer (2% formaldehyde, 2.5 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) was heated at 75°C for 15 min, vacuum blotted onto a Hybond-N membrane (GE Healthcare), and dried completely at 80°C for 2 h. The blots were then rinsed with 2 $\times$ SSC and incubated with prehybridization solution (5 $\times$ SSC, 5 $\times$ Denhardt's reagent, 0.5% sodium dodecyl sulfate [SDS], 100 μ g/ml salmon sperm DNA [Invitrogen]) at 65°C with agitation for more than 2 h. 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 were subsequently cloned by TOPO TA cloning (Invitrogen) performed according to the manufacturer's 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 to verify their nucleotide sequences. Each probe fragment of DNA was excised from the plasmid, gel isolated (Qiaquick gel extraction kit), and radioactively labeled with [α -³²P]dATP (NEBlot kit) according to the manufacturer's instructions. Unincorporated nucleotides were removed with a spin column (Probe Quant G-50; GE Healthcare), and the DNA was denatured at 100°C for 5 min and chilled on ice. The probes were then added individually to each prehybridization solution and membrane and incubated at 65°C with agitation overnight to allow hybridization. The hybridization solution was removed, and the membranes were washed twice in 2 $\times$ SSC-0.1% SDS at 65°C for 15 min, twice in 1 $\times$ SSC-0.1% SDS at 65°C for 15 min, and three times in 1 $\times$ SSC-0.1% SDS at 65°C for 30 min. The membranes were then wrapped in Saran Wrap, mounted on Kodak X-OMAT LS X-ray film, and exposed for various 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 determine specific RNA levels (Packard TRICARB 2900 TR liquid scintillation analyzer). The cpm for each RNA dot under each condition were normalized to the 16S rRNA cpm, and the relative increase (expressed as a percentage) in the transcript level was calculated for each operon or gene by comparing the signal in the presence of the DNA-damaging agent mitomycin C and the signal in the absence of mitomycin C. The results for two independent RNA isolations performed in triplicate were subjected to statistical analysis (Student's paired *t* test), and a *P* value of <0.05 was considered significant.

Cloning and expression of the *lexA1* allele. The *lexA1* 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 open reading frame corresponding to the *lexA* gene from *E. coli* strain MG1655, including the ribosome binding sequence. Genomic DNA from strain JLM281A (Table 1), which contains the mutant uncleavable *lexA1* allele that was verified to confer a non-SOS-inducible phenotype in this work, was used as the template DNA for PCR amplification. A single fragment was observed, gel isolated, and cloned directly into pBAD33 using the XbaI/HindIII sites engineered into the primer sequences, creating plasmid pKH296. The pBAD33 plasmid contained the arabinose-inducible P_{BAD} promoter, which drove transcription of the cloned open reading frame in the presence of arabinose. Using DNA sequence analysis, this plasmid was verified to contain the G-to-A transition mutation that resulted in a G80D amino acid substitution that rendered the protein encoded by the *lexA1* allele uncleavable (28).

Analysis of TTSS-dependent protein secretion. EPEC strains were inoculated into 2 ml of LB medium containing chloramphenicol for plasmid pBAD selection and 0.2% glucose to inhibit expression of the *lexA1* allele cloned in pKH296. The cultures were incubated overnight at 37°C with shaking. The cultures were then diluted 50-fold into Dulbecco modified Eagle medium (DMEM) containing 50 mM HEPES buffer (pH 7.4), chloramphenicol, and 0.1% arabinose to induce expression of the *lexA1* allele. The cultures were incubated at 37°C with shaking

TABLE 3. LexA binding sites

Gene or operon	LexA binding site ^a
LexA consensus site ^b	TACTGTATATATACAGTA
<i>recA</i>	TACTGTATGCTC TACAGTA
<i>LEE2/LEE3</i>	TACTGTATATAT TATCCAAT
<i>LEE5</i>	TACTGTGATTTATTTGGTTT

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

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

until the optical density at 600 nm was between 0.8 and 1.0. Bacteria were removed from the medium by centrifugation at 12,000 × g for 30 min. The equivalent of 4.5 OD₆₀₀ units of medium was removed with a serological pipette, and proteins were precipitated as described previously (24). Protein pellets were resuspended in Laemmli SDS-polyacrylamide gel electrophoresis loading buffer and resolved on a 15% SDS-polyacrylamide gel. Proteins were visualized by overnight staining with 0.25% Coomassie blue R-250 in 40% methanol and 10% acetic acid. Gels were destained 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 (positions -97 to 62) was amplified using the 5'-*recA*-foot and 3'-*recA*-foot primers and genomic DNA isolated from strain MG1655. The 159-bp fragment was gel isolated, cleaved with EcoRI and BamHI, and ligated into pBluescript II KS cut with the same restriction endonucleases, creating pKH286. The *LEE2* (positions -83 to 75)/*LEE3* (positions -94 to 64) and *LEE5* (positions -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-bp fragment that encoded the overlapping *LEE2/LEE3* promoters, including the putative LexA binding site. Primers TIR5 and TIR+88 were used to amplify the 163-bp fragment encoding the *LEE5* promoter and putative LexA binding site. The fragments were gel isolated and cloned using a 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 containing the *LEE2/LEE3*, *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 5 min. The polymerase was then heat inactivated at 70°C for 15 min. The labeled fragment was then released from the vector by cleavage with EcoRI and gel isolated. LexA binding reactions were performed by incubating 70,000 cpm of probe DNA, 40 μ g/ml nonspecific DNA (pBluescript II KS), and various amounts of purified LexA protein in binding buffer (20 mM Tris HCl [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, 50 mM NaCl) (30) at room temperature for 10 min. After LexA binding, 40 mU of DNase I (Sigma) was added, and the preparations were incubated at room temperature for 2 min. The reactions were terminated by addition of stop solution (200 mM NaCl, 2 mM EDTA, 1% SDS). The reaction mixtures were then extracted once with phenol and once with chloroform, followed by ethanol precipitation (2 volumes of 100% ethanol, 0.1 volume of 7.5 M ammonium acetate [pH 7.5], 1 μ l of glycogen [Invitrogen]). The pellets were resuspended in 2.5 μ l water and 2.5 μ l Sequenase stop solution (USB), denatured at 75°C for 2 min immediately prior to loading, and separated in a denaturing 6% polyacrylamide-Tris-borate-EDTA gel. The gel was then transferred to Whatman paper, dried at 80°C for 1 h, and exposed to Kodak X-Omat LS film. Sequencing reaction mixtures containing each plasmid and the BluescriptNotI primer were loaded adjacent to the DNase I reaction mixtures to determine the exact positions of protected DNA sequences.

RESULTS

Putative LexA binding sites identified in the *LEE2*, *LEE3*, and *LEE5* promoters. We identified a putative LexA binding site in the overlapping EPEC *LEE2/LEE3* promoters extending from position -4 to position -23 and from position -13 to

position 7 in relation to the start of transcription for the *LEE2* and *LEE3* promoters (33), respectively (Table 3 and Fig. 1A). Similarly, we identified a putative LexA binding site in the *LEE5* promoter, extending from position -20 to position -1 in relation to the start of transcription (12) (Table 3 and Fig. 1B). These sequences contained the nearly invariant CTG motif of the canonical LexA recognition sequence (9, 61) and were located directly within the *LEE2*, *LEE3*, and *LEE5* promoters. In comparison, the canonical LexA binding site in *recA* spans from position -30 to position -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 determined β -galactosidase activities 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 cross-links (56, 57), causing double-strand breaks, which induces the SOS response (47, 61). Because genes of the SOS regulon are induced at various times after DNA damage occurs, we monitored β -galactosidase activities at 30 and 60 min after the addition of mitomycin C. At 60 min, mitomycin C caused ~4-fold-increased expression from the *LEE2-lacZ* fusion (expression increased from 50 to 213 Miller units) (Fig. 2A). Similarly, mitomycin C caused ~3-fold- and ~4-fold-increased expression from

FIG. 1. Putative and established LexA binding sites in the *LEE2/LEE3*, *LEE5*, and *recA* promoters. (A) The putative LexA binding site (61) in the *LEE2* promoter spans from position -4 to position -23, corresponding to positions -13 to 7 in the *LEE3* promoter. (B) The putative LexA binding site in the *LEE5* promoter spans from position -20 to position 1. (C) The LexA binding site (SOS box) (61) in the *recA* promoter spans from position -30 to position -11, as 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) are indicated by bold type, while the direction of transcription for each promoter is indicated by an arrow. The observed binding of purified LexA protein to the *LEE2/LEE3* promoter region in panel A is indicated by a dashed line.

FIG. 2. DNA damage-associated responses of single-copy *LEE-lacZ* fusions occur in a RecA-LexA-dependent manner in *E. coli* K-12-derived strains. β -Galactosidase activities derived from *LEE2-lacZ* (A), *LEE3-lacZ* (B), *LEE5-lacZ* (C), and *recA-lacZ* (D) fusions were monitored 30 and 60 min after 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. The error bars indicate one standard deviation. The values are the means of at least two experiments performed in triplicate.

the *LEE3-lacZ* and *LEE5-lacZ* fusions, respectively (expression increased from 66 to 181 Miller units and from 45 to 181 Miller units, respectively) (Fig. 2B and C). We did not observe mitomycin C-dependent increased expression in the *LEE2-lacZ*, *LEE3-lacZ*, and *LEE5-lacZ* fusion strains containing the mutant *recA1* allele (Fig. 2A to C), as mutations in *recA* prevented the bacteria from inducing the SOS response (61).

As a positive control, we constructed a *recA-lacZ* single-copy fusion in strain MC4100, and mitomycin C caused expression to increase from 288 to 1,435 Miller units and from 275 to 2,740 Miller units at 30 and 60 min, respectively (Fig. 2D). The ~10-fold increase in activity observed with the *recA* transcriptional fusion at 60 min 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 whose construction was identical to that of all other fusions in strain MC4100 increased less than twofold in the presence of mitomycin C. This fusion contained the *lacZYA* promoter, as well

as the *lacI* gene encoding the Lac repressor, and exhibited >100-fold induction in the presence of the inducer isopropyl- β -D-thiogalactopyranoside (IPTG) (data not shown). We also observed that expression from single-copy *LEE1-lacZ* and *LEE4-lacZ* fusions increased less than twofold in the presence of mitomycin C, and we 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 on functional LexA protein. Based on our observation that increased LEE transcription in response to DNA damage was dependent on a functional RecA protein, we predicted that this signaling was also dependent on a functional LexA protein. We therefore transduced the *lexA1* allele, which encodes a mutant LexA protein unable to undergo autocatalytic cleavage in the presence of the coprotease RecA and thus renders the cell unable to induce the SOS response (39), into the *LEE-lacZ* fusion

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

Operon or gene	% Increase (SD) ^a	P
<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 The values indicate the increases in transcription of the operons or genes when the signal in the presence of mitomycin C was compared with the signal in the absence of mitomycin C 60 min after addition of mitomycin C. EPEC strain E2348/69 was grown in LB medium at 37°C to the mid-exponential phase prior to treatment. The values are the data for two independent RNA isolations probed in triplicate.

strains and monitored the β -galactosidase activity in response to DNA damage. Reporter gene assays in the presence of the *recA1* and *lexA1* alleles were performed with derivatives of K-12 strain MC4100 because the wild-type 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 wild-type EPEC. As predicted, addition of mitomycin C did not result in increased activity from the *LEE2-lacZ*, *LEE3-lacZ*, and *LEE5-lacZ* fusions in the presence of the *lexA1* allele (Fig. 2A to C). 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 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 wild-type EPEC, we performed an RNA dot blot analysis. Briefly, whole-cell RNA was isolated from strain E2348/69 in the exponential phase in the absence and in the presence of mitomycin C at 60 min, conditions which were identical to the conditions used for monitoring β -galactosidase activities resulting from the *LEE-lacZ* single-copy fusions in the K-12-derived strains. Ten-microgram aliquots of RNA were dotted onto a nylon membrane and hybridized with radiolabeled DNA probes generated by PCR using the oligonucleotide primers shown 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 the results for the K-12-derived strains, transcription of *LEE2* and *LEE3* increased 60 min after the addition of mitomycin C (127% [$P < 0.001$] and 136% [$P = 0.038$], respectively) (Table 4). Surprisingly, we did not observe a significant increase in *LEE5* transcription in EPEC in 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 gene, *recA*, also increased in the presence of mitomycin C (503%; $P < 0.001$). The negative control *lacZ* gene, the *LEE1* and *LEE4* operons, and the cryptic prophage-located effector molecule-encoding *nleD* and *espJ* genes did not exhibit 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 has 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 control of the arabinose-inducible P_{BAD} promoter in plasmid pBAD33 (the resulting plasmid was designated pKH296), and we transformed the plasmid into EPEC strains E2348/69, CVD452 with *escN* (the gene encoding the ATPase of the TTSS [14]) deleted, and SE796 with the global regulator *ler* gene deleted (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, and harvested in the late exponential phase, and secreted proteins were precipitated and then separated by polyacrylamide gel electrophoresis. As expected, we observed no secretion of proteins from strain CVD452 with *escN* deleted in the presence of either pBAD33 or pKH296 (Fig. 3, lanes 1 and 2). For wild-type EPEC strain E2348/69 we observed modest decreases in the quantities 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 EPEC virulence, we also monitored protein secretion in strain SE796 containing a *ler* deletion, which dampened expression of the LEE and resulted in a better understanding of the contribution of the SOS response control of TTSS function. In strain SE796, expression of the uncleavable LexA protein eliminated secretion of all proteins except one protein comigrating with the 66-kDa molecular mass marker, compared to the pBAD33 plasmid control (Fig. 3, lanes 5 and 6).

FIG. 3. LexA-dependent secretion by the EPEC TTSS. Strains were grown to an A_{600} 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 *lexA1* expression. Bacterial cultures were centrifuged, supernatants were removed, and secreted proteins were precipitated, washed, and separated on a 15% polyacrylamide-SDS gel. Secretion was monitored for 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 at the top. The dots indicate the positions of proteins, including the predominantly observed EspB/D protein, whose secretion was partially reduced by the *lexA1* allele encoding the uncleavable LexA in wild-type EPEC strain E2348/69. The asterisks indicate the positions of proteins whose secretion was reduced in the Δler strain SE796 in the presence of the uncleavable LexA. The dagger indicates the position of a ~ 66 -kDa protein that required EscN but neither Ler nor a functional LexA transcriptional regulator for secretion. The molecular masses of standards are indicated on the right.

FIG. 4. DNase I protection assays to establish purified LexA protein binding in *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 a 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 included as a positive control, as LexA binding in vitro at this locus was established previously (30). The region of protection is indicated by a 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 are indicated by dashed lines. The results for DNA sequencing ladders are shown to the left of the results of DNase I protection assays, in which various amounts of purified LexA protein were used, as indicated above the lanes.

These results were consistent with the alterations in transcriptional activities of LEE operons observed in both K-12 and wild-type 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/LEE3* and *LEE5* regulatory fragments containing the putative LexA binding sites (Fig. 1 and 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, results which were nearly identical to results described previously (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 (Fig. 1C and 4C).

Purified LexA protein bound the *LEE2/LEE3* regulatory fragment from position -2 to position -25 in relation to the *LEE2* start of transcription (Fig. 1A and 4A). This site corre-

sponds to positions -15 to $+9$ in relation to the overlapping *LEE3* promoter (Fig. 1A and 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 (Fig. 1A and 4A and Table 3). LexA protein bound discretely in the -10 hexamers of both *LEE2* and *LEE3* promoters, even at a relatively high protein concentration, $2 \mu\text{M}$ (Fig. 4A). Although 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 as determined 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, which encode 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 prevented 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 wild-type 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 obtained for the K-12-derived strains.

Using a DNase I protection assay, we found that purified LexA protein specifically bound a *LEE2/LEE3* regulatory fragment. We observed that LexA binding protected the *LEE2/LEE3* overlapping, divergent promoter fragment under conditions identical to those where LexA protected a *recA* promoter fragment from DNase I digestion (Fig. 4). We therefore concluded that 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 *umuCD/hlyE* 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 as determined by RNA dot blot analysis (Table 4), we did not observe binding of the 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 wild-type pathogen is that an EPEC-specific repressor dampens transcription of *LEE5* and does not allow a response to the DNA damage signal under the conditions tested. We hypothesized that without an EPEC-specific repressor in the K-12 strain, SOS regulation of the *LEE5* operon is demonstrable. Evidence that a non-H-NS, negative regulator acts at the *LEE5* operon in EPEC has been reported previously (49, 58). In addition, we predicted that if the *LEE5* operon of EPEC were subject to SOS regulation, the regulation would 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 SOS induction of the *recA* gene in response to DNA damage. The *recA* gene was induced 5-fold and 10-fold 30 and 60 min after the addition of mitomycin C, respectively, while the *LEE2* and *LEE3* operons required 60 min of exposure to the DNA-damaging agent before induction was observed (Fig. 2). We attributed this difference in induction kinetics to the relative strength of induction; the *LEE2* and *LEE3* operons were induced 3- to 4-fold, while the *recA* gene was induced 10-fold at 60 min.

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 other bacteria. 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_{STX2}

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, and we found 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 in defective prophages, including Cif, NleA, NleD, and EspJ (16), and the genes encoding these proteins are likely to be coordinately regulated with the TTSS, which is necessary for their secretion so that they can play a role in pathogenesis. The CP-933P prophage-encoded NleA protein colocalizes with the Golgi apparatus in the host cell and is not required for AE lesion formation, but it does play an important, although 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 coprotease RecA leads to autocatalytic cleavage and release of the λ CI repressor (29, 41), expression of phage genes, and thus phage induction. Some phages 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 coprotease 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 in 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 in the invading bacterium (59). Antibiotics, such as β -lactams, that damage the bacterial cell wall also induce the SOS response (35). EPEC inhibited expression of inducible nitric oxide synthase, which produces the antibacterial agent nitric oxide (NO), in human intestinal epithelial cells in culture (31). This activity was dependent on attachment to and delivery of effector molecules in the host cell, and these findings provide further evidence that EPEC perceives and responds to DNA damage signals in 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 in the host, including 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 findings underscore the need to diversify our therapeutic arsenal directed against EPEC and related pathogens.

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