INFECTION AND IMMUNITY, Feb. 1996, p. 495–502 Vol. 64, No. 2
0019-9567/96/$04.00+0

Regulation of the Shiga-Like Toxin II Operon in *Escherichia coli*

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Received 28 July 1995/Returned for modification 16 September 1995/Accepted 21 November 1995

Investigations of the regulation of the bacteriophage-encoded Shiga-like toxin II (SLT-II) in *Escherichia coli* demonstrated that bacteriophages exhibit a regulatory impact on toxin production by two mechanisms. Firstly, replication of the toxin-converting bacteriophages brings about an increase in toxin production due to concomitant multiplication of toxin gene copies. Secondly, an influence of a phage-encoded regulatory molecule was demonstrated by using low-copy-number plasmid pADR-28, carrying a translational gene fusion between the promoter and proximal portion of *slt-IIA* and the structural gene for bacterial alkaline phosphatase (*phoA*). PhoA activity, reflecting the *slt-II* promoter activity, was significantly enhanced in *E. coli* strains which had been lysogenized with an SLT-I- or SLT-II-converting bacteriophage (H-19B or 933W, respectively) or bacteriophage λ. Both mechanisms are dependent on bacteriophage induction and hence are *recA* dependent. Moreover, the study revealed that the DNA-binding protein H-NS has a regulatory impact on both bacteriophage-mediated SLT-II synthesis and the activity of the *slt-II* promoter of plasmid pADR-28. While a slight impact of growth temperature on SLT-II expression was observed, no impact of either osmolarity, pH, oxygen tension, acetates, iron level, or utilized carbon source could be demonstrated.

Shiga-like toxins (SLTs), produced by enterohemorrhagic *Escherichia coli*, and Shiga toxin (ShT), produced by *Shigella dysenteriae* type 1, constitute a family of evolutionarily related toxins. All members of the Shiga toxin family are structurally very similar and exhibit the same biological activities, i.e., neurotoxicity following intravenous injection into mice and rabbits, enterotoxicity in rabbit ileal loops, and cytotoxicity to certain tissue culture cells (2, 23, 24, 46). They possess multiple B subunits, mediating binding to glycolipid receptors containing Galα1-4Gal disaccharide (10, 11, 19, 58), and a single A subunit, acting as an rRNA *N*-glycosidase on a specific site of the 28S rRNA of the 60S ribosomal subunit (12). While SLT-I cross-reacts with polyclonal antiserum raised against ShT, SLT-II and its variants are immunologically distinct from ShT and SLT-I (46, 56). The genes for the expression of SLT-I and -II are phage mediated, while ShT is chromosomally encoded (18, 42, 44–46, 61). The genes for the SLT-II variants appear to be chromosomally encoded (33, 46). Sequence comparisons revealed that the genes encoding ShT and SLT-I are essentially identical (55). However, their overall homology to the genes encoding SLT-II and the SLT-II variants is only about 55% (20, 46, 60). The roles of SLTs in disease have still not been fully elucidated yet. It appears, however, that SLT-II may play a more crucial role than SLT-I in the development of hemorrhagic colitis and the hemolytic-uremic syndrome (HUS) (46).

The promoter activity of SLT-I and ShT but not of SLT-II or its variants is repressed by high levels of iron (6, 57, 59). Repression of ShT expression at a growth temperature of 30°C (59) and the effects of different culture conditions on the production of phage-mediated SLT-I and -II and of SLT-II variants have also been reported (21, 25, 29, 43, 59). Recently, mouse intestinal mucus was shown to induce SLT-IIc expression in *E. coli* O91:H21 (34).

It has previously been shown that treatment of the lysogens harboring SLT-I- or SLT-II-converting phages with either certain antibiotics, such as mitomycin, or UV irradiation results in phage induction and a concomitant increase in toxin production (1, 3, 4, 16, 38). In these studies, mitomycin and/or UV irradiation had no effect on toxin production when the toxin genes were not carried on a phage. It was suggested that the RecA protein plays a key role in the underlying mechanism of mitomycin-induced toxin expression. However, Yee et al. (63) recently reported that mitomycin caused induction of a chromosomally encoded SLT-IIva in the enteropathogenic *E. coli* strain H.I.8 that was lysogenic with a non-toxin-carrying bacteriophage. Recently, Fujii et al. (13) also reported encephalopathy in mice induced by the SLT-IIv-producing *E. coli* O157:H$^-$ strain E32411/HSC following mitomycin treatment.

The objectives of this study were firstly to explore the mechanism of mitomycin induction and specifically to investigate the role of RecA in the induction of SLT-II production and secondly to determine whether there are environmental factors which have a regulatory effect on the *slt-II* operon. A translational gene fusion was constructed between the proximal portion of *slt-IIA*, including the promoter region, and the gene for bacterial alkaline phosphatase. The gene fusion-containing plasmid was used to investigate whether the *slt-II* operon is influenced by culture conditions, including temperature, osmolarity, pH, oxygen tension, carbon source, and the presence of acetates, as well as by the presence of bacteriophages, phage inducers, or the DNA-binding protein H-NS.

[A portion of this material was presented at the 2nd International Symposium and Workshop on "Verocytotoxin (Shiga-like toxin)-producing *Escherichia coli* infections" in Bergamo, Italy, 1994, and was published in reference 22a.]

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TABLE 1. Bacterial strains and plasmids used in the study

E. coli strain or plasmid	Description or genotype	Reference
Strains		
CC118	F$^-$ Δ(*ara-leu*)*7697 araD139* Δ(*lac*)X74 *phoA*Δ20 *galE galK thi rpsE rpoB argE*(Am) *recA1*	32
C600(933W)	*recA*$^+$; SLT-II-converting phage 933W	56
C600(H19B)	*recA*$^+$; SLT-I-converting phage H19B	53
MC1029	*araD139* D(*araABC-leu*)*7697* Δ(*lac*)X74 *galU galK trpB9601*(Am) *strA recA56*	7
HMG5	*hns* (*drdX*)-negative mutant of *E. coli* MC1029	14
Sm10λpir	*thi1 thr1 leuB6 supE44 tonA21 lacY1 recA*::RP4-2-Tc::Mu Km^r λ*pir*	36
Plasmids		
F42 *lac13 zzf-2*::Tn*phoA*	Tn*phoA* is Tn*5* IS*50*L::*phoA*	32
pDRM	Tc^r; 933W phage DNA; *slt-IIA*; in vector pBR322	This study
pADR-18	Tc^r Km^r; 933W phage DNA; *slt-IIA*::Tn*phoA*; in vector pDRM	This study
pADR-28	Tc^r; 933W phage DNA; *slt-IIA*::Tn*phoA*; in vector pLG339	This study
pLG339	Tc^r Km^r	54
pSB53	Ap^r; *phoA* gene of *E. coli*	52
pRSA87	Ap^r *hlyD*::Tn*phoA*; in vector pRSC4.1	52
pRSA122	Ap^r *hlyD*::Tn*phoA*; in vector pRSC4.1	52
pIM10	Ap^r *recA*; in vector pUC18	This study
pIM12	Ap^r Cm^r *recA*; in vector pUC18	This study
pIM14	Ap^r Cm^r *recA*; in vector pGP704	This study
pGP704	Ap^r *ori* R6K *mob*RP4	30

MATERIALS AND METHODS

Media, chemicals, and enzymes. Bacteria were grown in Luria-Bertani (LB) medium unless stated otherwise. Other growth media were LBON (28), brain heart infusion broth, and half-strength M63 (26) supplemented with the amino acids leucine and arginine. For certain experiments, the osmotic strength of half-strength M63 was increased by the addition of 0.1 to 0.6 M NaCl, KCl, or sucrose. As a carbon source, glucose, glycine, sucrose, or lactose was added at a final concentration of 0.5% to M63 medium supplemented with leucine and arginine. For pH experiments, brain heart infusion broth was adjusted to pH values of either 6.5, 7, 7.5, 8, or 8.5 and buffered with 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). Syncase medium (11) supplemented with 0 or 10 μg of $FeCl_3$ per ml was used for iron experiments. The effect of oxygen tension and acetates was examined following aerobic, microanaerobic, or anaerobic growth in brain heart infusion broth (pH 7.4) supplemented with 0, 25, 50, 75, or 100 mM sodium acetate. Microanaerobic culture conditions were obtained by growing the bacteria in 50-ml tubes which were filled with medium to the top and made airtight with screw tops. Anaerobic culture conditions were obtained by using the Merck Anaerocult A system. Strains carrying recombinant plasmids were cultivated under selective antibiotic pressure. Strains with relaxed plasmids were grown with ampicillin (60 μg/ml), kanamycin (30 or 300 μg/ml as indicated below), or tetracycline (50 μg/ml). The low-copy-number plasmid pLG339 (54) and its low-copy-number derivative pADR-28 were selected with tetracycline (10 μg/ml) and kanamycin (10 μg/ml). Chemicals were purchased from Sigma, Deisenhofen, Germany. Screening for PhoA-expressing *E. coli* strains required the addition of 5-bromo-4-chloro-3-indolylphosphate (40 μg/ml), a substrate for alkaline phosphatase. For certain experiments, mitomycin (25 to 200 ng/ml) was added to the cultures.

Bacterial strains, bacteriophages, and plasmids. The bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 1. *E. coli* CC118 and CC118(F42 *lac13 zzf-2*::Tn*phoA* [Tn*phoA* is Tn*5* IS*50*L::*phoA*]) were kindly provided by S. Calderwood (6, 32). The *hns*$^+$ and *hns E. coli* strains MC1029 and HMG5, respectively, were a generous gift of B. E. Uhlin (14).

Mitomycin treatment and UV irradiation of bacterial cultures. Five-milliliter bacterial cultures were grown overnight in LB medium. One percent inocula were made and grown in the same medium to an optical density at 600 nm (OD_{600}) of 0.7. The cultures were then divided, and one half was used as a control while the other half was exposed to either UV irradiation or mitomycin treatment.

For the treatment with mitomycin, an aliquot of the bacterial culture was put into an Erlenmeyer flask and aqueous, filter-sterilized mitomycin (25, 50, 100, or 200 ng/ml) was added to the culture. Both control and mitomycin-treated cultures were then shaken at 200 rpm at 37°C for various times.

Phage transfer and phage quantitation. Following growth of the bacteriophage-containing bacterial cultures, the phages were extracted by collection of 1-ml aliquots. Supernatants were obtained by centrifugation for 2 min in a microcentrifuge at maximum speed and were filter sterilized through a 0.45-μm-pore-size filter. Bacterial lysogens were constructed by infecting bacteria with a diluted phage extract. Following overnight incubation at 37°C, the few colonies growing in the centers of the phage plaques were picked and subcultured on LB plates. Lysogenization was screened by testing the bacteria for reinfection with the same bacteriophage and by colony hybridization with the corresponding gene probe. Bacteriophage release was determined by extracting dilutions of the sterile bacteriophage extracts made in LB medium containing 10 mM $CaCl_2$. One hundred microliters of each dilution was mixed with 100 μl of the indicator strain, which had been grown to stationary phase in LB medium containing 10 mM $CaCl_2$ and 0.2% maltose. The mixture was incubated for 20 min at 37°C with shaking. Following incubation, 2.5 ml of molten LB top agar (46°C) was added and the mixture was poured onto petri dishes containing regular LB agar. Plaques were counted following overnight incubation at 37°C.

DNA cloning techniques. Bacteriophage DNA was isolated from *E. coli* C600 (933W) by a standard procedure (31). Plasmid DNA isolation, DNA cleavage with restriction enzymes, agarose gel electrophoresis, elution of DNA fragments from agarose gels, transformation of *E. coli* strains with plasmid DNA, generation of DNA probes, colony and Southern hybridization, and PCR were performed by standard techniques (31, 48).

Construction of an *slt-IIA*-alkaline phosphatase translational fusion. Plasmid pDRM was constructed by ligating a 3.5-kb *Eco*RI-*Pst*I 933W phage DNA fragment, encoding 843 bp (95%) of the amino-terminal portion of the *slt-IIA* structural gene, signal sequence, and promoter as well as additional phage DNA (39–41), into an *Eco*RI- and *Pst*I-restricted 3.6-kb pBR322 fragment, conferring tetracycline resistance.

Plasmid pADR-18 was constructed by introducing the transposon Tn*phoA* from plasmid F42 *lac13 zzf-2*::Tn*phoA* into plasmid pDRM by the procedure of Manoil and Beckwith (6, 32). Tn*phoA* is a kanamycin resistance-encoding transposon that allows the generation of gene fusions between the amino-terminal portion of a target gene and the reporter gene of alkaline phosphatase. Briefly, competent cells of *E. coli* CC118(F42 *lac13 zzf-2*::Tn*phoA*) were transformed with the purified plasmid pDRM. The resulting transformants were selected on LB plates containing tetracycline and kanamycin (30 μg/ml). Following overnight incubation, the transformants were selected again on LB plates containing tetracycline and kanamycin (300 μg/ml). The higher concentration of kanamycin was used to select for transposition of Tn*phoA* onto the plasmid vector. All the colonies from each plate were pooled and grown overnight. Plasmid DNA was isolated from the overnight culture, and the plasmid mixture was used for the transformation of *E. coli* CC118 cells carrying a deletion of the chromosomal *phoA* gene (6, 32). The transformants were selected on LB plates containing tetracycline, kanamycin, and 5-bromo-4-chloro-3-indolylphosphate. Individual blue colonies contained in-frame fusions of Tn*phoA* to secreted gene products on pDRM. Colonies were purified by restreaking twice on the same medium, and plasmid DNA was isolated. The presence and location of Tn*phoA* within pDRM were determined by colony blot analysis using an *slt-II* gene probe (39, 60) and restriction mapping. *slt-IIA–phoA* fusions were confirmed by Western blot (immunoblot) analysis using polyclonal rabbit antisera raised against SLT-II (11) and alkaline phosphatase. The fusion point between *slt-IIA* and *phoA* was elucidated by analyzing the nucleotide sequence by the dideoxy chain termination method of Sanger et al. (8, 40, 41, 50).

Finally, plasmid pADR-28 was constructed. For this, plasmid pADR-18 was digested to completion with *Nhe*I, producing a 5.9-kb fragment which contained the *slt-IIA–phoA* fusion as well as upstream phage DNA. This fragment was blunt ended and cloned into the *Sma*I-linearized low-copy-number vector pLG339, conferring both tetracyline and kanamycin resistance (54). Insertion of a DNA

sequence into the *Sma*I site destroyed the vector-mediated kanamycin resistance. Transformation of *E. coli* CC118 followed, and the transformants were screened for tetracycline resistance and kanamycin sensitivity. Colony blot analysis, restriction mapping, Southern blot analysis, and alkaline phosphatase activity measurements (35) confirmed that plasmid pADR-28 carried the *slt-IIA–phoA* fusion in the transcriptional orientation opposite that of the kanamycin resistance gene in pLG339.

Determination of alkaline phosphatase (PhoA) activity. PhoA activity of pelleted bacterial cells and culture supernatants was determined by measuring the rate of hydrolysis of *p*-nitrophenyl phosphate (Sigma 104) by the procedure described previously (5, 35). When the PhoA activity in *phoA*$^+$ *E. coli* strains was measured, the background levels reached by those *E. coli* strains harboring the vector control plasmid pLG339 were subtracted from the levels reached by *E. coli* strains harboring plasmid pADR-28.

Determination of copy number of plasmid pADR-28. The relative copy number of plasmid pADR-28 was determined by making use of a 420-bp DNA probe specific for the gene encoding the tetracycline resistance marker of plasmid pADR-28. This probe was obtained by restriction of plasmid pBR322 with the restriction enzymes *Sal*I and *Nhe*I. Total DNA of bacteria harboring plasmid pADR-28 was extracted, and a series of dilutions of this DNA was blotted onto a nylon membrane. Hybridization with the above-described probe followed.

Determination of *slt-II* gene copy number. The relative copy number of the *slt-II* gene was determined as described for determination of plasmid pADR-28. However, an *slt-II*-specific probe was used (39).

PAGE and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was performed in slab gels (8 by 0.75 cm) (11). Western blot analysis, using the Promega Proto Blot alkaline phosphatase system, followed. For the detection of SLT-IIA and alkaline phosphatase (PhoA), polyclonal antibodies raised against SLT-II (11) and PhoA were used as primary antibodies. For the detection of H-NS, a monoclonal antibody was used.

Extraction of SLT from bacterial cultures. Following growth of the SLT-producing bacterial cultures, toxin concentrations in culture supernatants and in cell lysates, the latter prepared either by sonication or by polymyxin B treatment, were measured. One milliliter of the cell culture was collected and subjected to centrifugation for 2 min in a microcentrifuge. The supernatant was removed and retained. To obtain a sonicated cell lysate, the cell pellet was solubilized in phosphate-buffered saline (PBS) and sonicated. To confirm that more than 90% cell lysis was obtained, the OD_{600} was measured before and after sonication. The sonicated cell solution was subjected to centrifugation in a microcentrifuge for 2 min, and the supernatant was retained for toxin quantitation. Alternatively, treatment of the cell culture with the antibiotic polymyxin B was used to release toxin. For this, the cell pellets were washed with PBS and then incubated with polymyxin B in PBS (2 mg/ml) for 10 min at 4°C. The mixture was then microcentrifuged for 5 min, and the supernatant was again retained for toxin quantitation.

Quantitation of SLT production by enzyme-linked immunosorbent assay (ELISA). Nunc-Immuno Plates Maxi-Sorb were coated overnight at 4°C with either crude hydatid cyst material containing P_1-glycoprotein, mouse monoclonal antibody against SLT-I (4D3), or mouse monoclonal antibody against SLT-II (2B1 or 4D1) in PBS and then blocked for 1 h with 1% bovine serum albumin in PBS to saturate nonspecific protein binding sites at room temperature. Toxin-containing solutions diluted in PBS-Tween were added (100 μl/well), and the plates were incubated for 1 h at room temperature. After the plates were washed with PBS-Tween, bound toxin was detected with rabbit polyclonal antibodies against either SLT-I (1:5,000) or SLT-II (1:7,500) for another hour. After the plates were washed again with PBS-Tween, a goat anti-rabbit immunoglobulin G antibody conjugated to alkaline phosphatase (1:1,000) was added for 1 h. Following a final wash, the bound enzyme was detected by the Sigma 104 phosphatase substrate (*p*-nitrophenyl phosphate disodium) (1 mg/ml in alkaline phosphatase buffer, pH 9.5). A_{504} was measured. Purified SLT-II was used as a standard.

Construction of *recA*-negative mutant strains. The 1.375-kb *recA* gene (49) from *E. coli* C600 was amplified by PCR and cloned into the *Sma*I site of plasmid pUC18. The newly created 4.1-kb plasmid, designated pIM10, was restricted with *Eco*RV, deleting 186 bp of the *recA* gene, which was replaced by a 1.4-kb sequence carrying the chloramphenicol resistance gene (*cat*). In this plasmid, termed pIM12, the *cat* cassette is flanked by 369- and 820-bp *recA* sequences. Plasmid pIM12 was restricted with *Sph*I and *Kpn*I, blunt ended, and cloned into the *Eco*RV-restricted vector pGP704 to create plasmid pIM14. Plasmid pGP704 (30) is a λ-pir-dependent suicide vector conferring ampicillin resistance. The chromosomal *recA* gene in *E. coli* strains not possessing the π protein, encoded by the *pir* gene, was destroyed following conjugation with the plasmid donor strain *E. coli* Sm10λpir(pIM14) and a double-crossover event between the *recA* sequences on the plasmid and the chromosomal *recA* gene. *recA*-negative mutants were screened for by testing the resulting chloramphenicol-resistant strains for sensitivity to UV light, mitomycin, and methylmethanethiosulfonate and for loss of ampicillin resistance. Chromosomal DNA was then restricted with *Kpn*I and subjected to Southern blot analysis using a *recA* probe. Mutants differed from the wild-type *recA*-positive strains by having a 1.4-kb shift resulting from the integration of the *cat* cassette into the *recA* sequence.

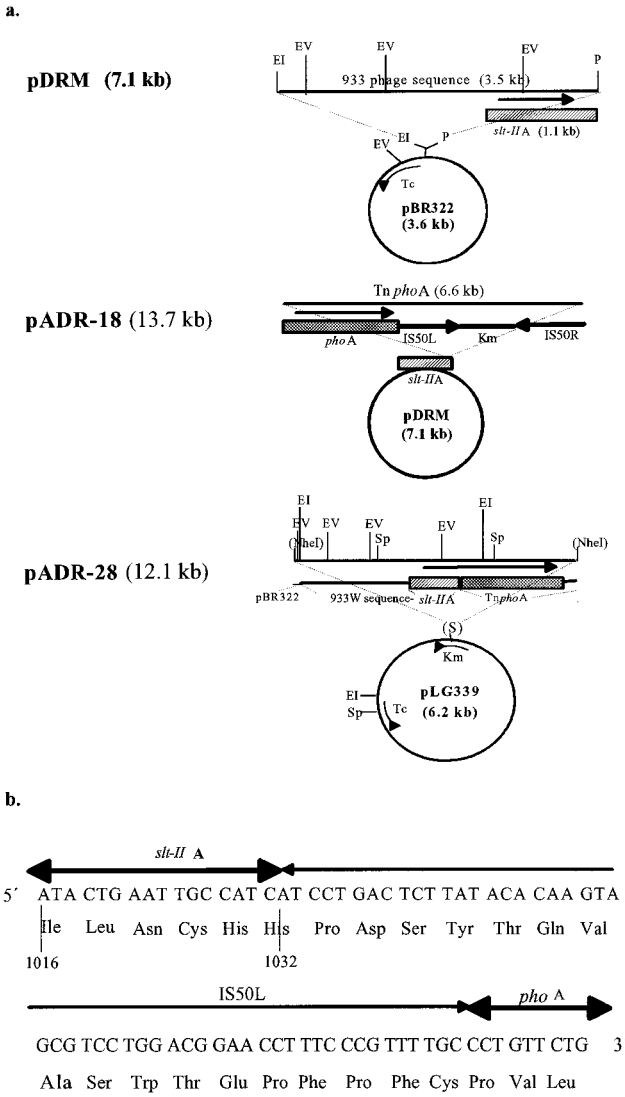

FIG. 1. (a) Structures of plasmids pDRM, pADR-18, and pADR-28. Plasmid pDRM contains 95% of the *slt-IIA* structural gene, signal sequence, and promoter as well as additional phage DNA (from *E. coli* bacteriophage 933W) in vector pBR322. Plasmids pADR-18 and pADR-28 contain a translational fusion between the truncated *slt-IIA* gene (82.3%) and the *phoA* gene in the high- and low-copy-number vectors pBR322 and pLG339, respectively. The position and direction of gene transcription are indicated by an arrow below each linearized map. Vectors, not shown to scale, are drawn as circles. The sites of insertion are indicated by dotted lines connecting the maps of the vectors and linearized inserts. Restriction sites and antibiotic resistances are abbreviated as follows: EI, *Eco*RI; EV, *Eco*RV; P, *Pst*I, S, *Sma*I; Sp, *Sph*I; Tc, tetracycline resistance; and Km, kanamycin resistance. The parentheses around the *Nhe*I sites indicate that these sites were blunt ended. (b) Nucleotide and deduced amino acid sequences of the fusion protein encoded by pADR-18 and pADR-28, from nucleotide position 1016 of the *slt-II* operon across the fusion junction with IS*50*L into the coding sequence of *phoA*. Tn*phoA* insertion occurred at nucleotide position 1033 of the *slt-IIA* gene sequence, thus fusing 731 bp (82.3%) of the *slt-IIA* structural gene with the *phoA* structural gene.

RESULTS

Construction of an *slt-IIA–phoA* translational fusion. A gene fusion between the promoter and proximal portion of *slt-IIA* and the gene for bacterial alkaline phosphatase was constructed and cloned into the low-copy-number vector pLG339. Figure 1 shows the plasmid constructs pDRM, pADR-18, and pADR-28 as well as the location of the insertion of Tn*phoA*

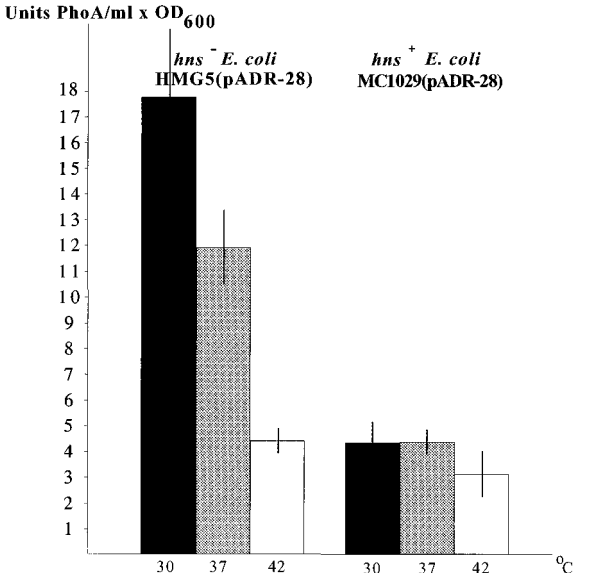

FIG. 2. Alkaline phosphatase (PhoA) activities in *hns E. coli* HMG5(pADR-28) and *hns*$^+$ *E. coli* MC1029(pADR-28) during aerobic, logarithmic growth at 30, 37, and 42°C in LB medium. Standard deviations are indicated by vertical lines ($n = 5$).

into the *slt-IIA* structural gene and the nucleotide sequence across the fusion joint. In plasmids pADR-18 and pADR-28, Tn*phoA* fused with 731 bp (82.3%) of the structural gene for SLT-IIA. Moreover, SLT-IIA–PhoA protein fusions were confirmed by Western blot analysis using polyclonal rabbit antisera against SLT-II and alkaline phosphatase (data not shown).

Impact of H-NS and environmental conditions on SLT-II expression. *E. coli* CC118(pADR-28) and the vector control strain CC118(pLG339) were examined for their alkaline phosphatase (PhoA) activities, indicating SLT-II expression, following growth under various culture conditions. Oxygen tension (aerobic, microaerobic, or anaerobic growth), pH (pH range of 6.5 to 8.5), carbon source (glucose, glycine, sucrose, or lactose), and the presence of acetate (0, 25, 75, or 100 mM sodium acetate) in the medium had no impact on SLT-II expression (data not shown). In order to investigate the impact of the H-NS protein as well as those of temperature and osmotic strength on SLT-II expression, plasmid pADR-28 as well as the low-copy-number vector control pLG339 was transformed into the isogenic *hns*$^+$ and *hns E. coli* strains MC1029 and HMG5, respectively (14). The resulting *E. coli* strains were examined for their PhoA activities following growth in LB medium at 37, 30, and 42°C as well as in LBON (LB medium prepared without NaCl) or half-strength M63 media, the osmotic strength of which was increased by the addition of 0.1 to 0.6 M NaCl, KCl, or sucrose. As indicated in Fig. 2, PhoA expression in the *hns* mutant *E. coli* strain HMG5(pADR-28) was clearly derepressed compared with that in the *hns* wild-type strain *E. coli* MC1029(pADR-28). The basal levels of PhoA activity found in *E. coli* MC1029(pLG339) and HMG5(pLG339) were equally low (data not shown). There was no influence of varying osmolarity on PhoA expression in the *hns*$^+$ or *hns* background (data not shown). Compared with those observed following growth at 37°C, the PhoA activities of *E. coli* HMG5(pADR-28) were increased following growth at 30°C and decreased following growth at 42°C. In the *hns*$^+$ *E. coli* MC1029(pADR-28), the levels of PhoA activity were similar at all growth temperatures tested. No marked differences in PhoA activities of *E. coli* MC1029(pADR-28) and HMG5(pADR-28) following growth at 42°C were observed (Fig. 2).

In order to exclude the possibility that H-NS and temperature influence the replication of plasmid pADR-28, total bacterial DNA was extracted from the *E. coli* strains HMG5, HMG5(pADR-28), MC1029, and MC1029(pϕADR-28) following growth at either 30, 37, or 42°C and hybridized with a DNA probe specific for the tetracycline-resistance-conferring gene of plasmid pADR-28. Neither H-NS nor any of the tested temperatures had any impact on the copy number of plasmid pADR-28 (data not shown).

In order to investigate whether bacteriophage-mediated SLT-II production is also influenced by the H-NS protein, we transfected the isogenic *E. coli* strains HMG5 and MC1029 with the SLT-II-converting phage 933W and tested the culture supernatants and the cell lysates, obtained by polymyxin B extraction, for toxin production following overnight growth at 30, 37, or 42°C by ELISA. SLT-II production was detectable by ELISA only in the *hns E. coli* strain HMG5(933W) and not in the isogenic *hns*$^+$ *E. coli* strain MC1029(933W) at the tested temperatures. The total toxin yield from cultures of HMG5(933W), grown at 30°C, was 4.11 ng of SLT-II per ml per OD_{600} unit, and this was about the same as that obtained from cultures grown at 37°C, which was 3.86 ng of SLT-II per ml per OD_{600} unit ($n = 3$). In cultures grown at 42°C, total SLT-II expression was only 0.80 ng of SLT-II per ml per OD_{600} unit ($n = 3$).

Impact of bacteriophage-mediated factors on SLT-II expression. Plasmid pADR-28 and the vector control pLG339 were transformed into *E. coli* C600 strains that were lysogenized either with the λ phage or with an SLT-I- or SLT-II-converting phage (H-19B or 933W, respectively). The resulting *E. coli* strains were tested for phage production and alkaline phosphatase (PhoA) expression following growth in LB medium and LB medium to which various concentrations of mitomycin (25 to 200 ng/ml) had been added.

A mitomycin-dose- and time-dependent increase in phage production was observed for all three phage types following 3 to 7.5 h of growth (data not shown).

Compared with the PhoA production of *E. coli* C600 (pADR-28), the enzyme activities in *E. coli* C600(H-19B/pADR-28) and *E. coli* C600(933W/pADR-28) increased in a mitomycin-dose-dependent manner (Fig. 3). The distribution of PhoA activity in culture supernatants and pelleted bacterial cells also changed for the lysogens in a mitomycin-dose-dependent manner (data not shown). With increasing concentrations of mitomycin, the relative amounts of activities found in the culture supernatants of the lysogens increased. At a concentration of 200 ng of mitomycin per ml, only 5.4% of the total PhoA activity of *E. coli* C600(pADR-28) was found in the culture supernatant while 32.2 and 33.0% of the total PhoA activities were found in the culture supernatants of the lysogens C600(H-19B/pADR-28) and C600(933W/pADR-28), respectively. The level of PhoA activity of *E. coli* C600(λ/pADR-28) was about threefold higher than that of the nonlysogenic strain, and it was not significantly further enhanced by the addition of mitomycin to the cultures (Fig. 3). In the absence of mitomycin, the level of PhoA production was identical to that of nonlysogenic *E. coli* C600. When mitomycin was added at the same concentrations to cultures of *E. coli* C600(pADR-28), no increase in PhoA enzyme activity occurred.

To guarantee that this induction phenomenon was specific for the *slt-II* operon and not a function of an increased copy number of plasmid pADR-28, total bacterial DNA was extracted from *E. coli* C600, C600(pADR-28), C600(H-19B), C600(H-19B/pADR-28), C600(933W), and C600(933W/pADR-28)

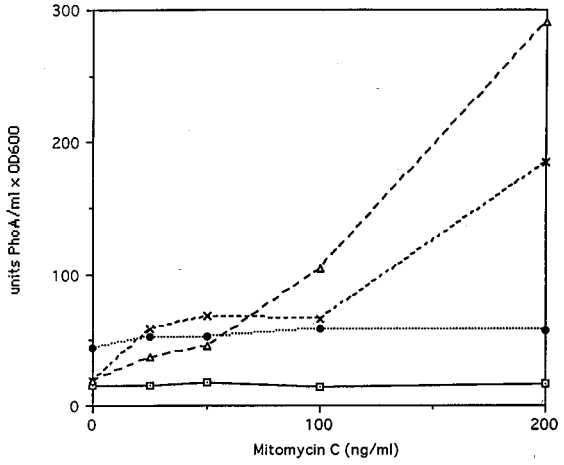

FIG. 3. Alkaline phosphatase (PhoA) activities in *E. coli* C600(pADR-28) (□), C600(H-19B/pADR-28) (×), C600(933W/pADR-28) (△), and C600(λ/pADR-28) (●) following 7.5 h of growth in LB medium or in LB medium or in LB medium supplemented with different concentrations (0 to 200 ng/ml) of mitomycin ($n = 5$).

following growth in the presence or absence of mitomycin (200 ng/ml). A series of dilutions of total DNA was blotted onto a nylon membrane and hybridized with the above-described DNA probe specific for the tetracycline-resistance-conferring gene of plasmid pADR-28. The copy number of plasmid pADR-28 did not increase following growth in the presence of mitomycin and the SLT-converting phage H-19B or 933W, confirming that the induction phenomenon was *slt-II* specific (data not shown).

Another confirmation of the specificity of the phage-mediated induction for the *slt-II* operon was acquired by transformation of *E. coli* C600 and the lysogenic *E. coli* strains C600 (933W) and C600(H19B) with plasmid pSB53 (52), carrying only the *phoA* gene, or the HlyD-PhoA fusion protein-encoding plasmid pRSA87 or pRSA122 (52), all of which were kindly provided by I. Gentschev. Table 2 shows that the presence of the phages did not have any impact on PhoA production mediated by these plasmids following mitomycin treatment.

TABLE 2. Effect of mitomycin on PhoA activities in nonlysogenic or lysogenic *E. coli* strains carrying plasmid pADR-28, pSB53, pRSA87, or pRSA122[a]

E. coli strain	PhoA activity (U of PhoA/ml × OD_{600})	
	Control	With mitomycin (200 ng/ml)
C600(pADR-28)	19.2	24.5
C600(H-19B/pADR-28)	16.9	726.9
C600(933W/pADR-28)	17.3	579.7
C600(pSB53)	1.7	1.4
C600(H-19B/pSB53)	1.3	1.4
C600(933W/pSB53)	0.9	1.5
C600(pRSA87)	10.9	10.5
C600(H-19B/pRSA87)	12.7	13.8
C600(933W/pRSA87)	11.8	11.0
C600(pRSA122)	18.5	19.9
C600(H-19B/pRSA122)	12.3	16.7
C600(933W/pRSA122)	14.7	12.2

[a] PhoA activities were measured following 24 h of growth ($n = 3$).

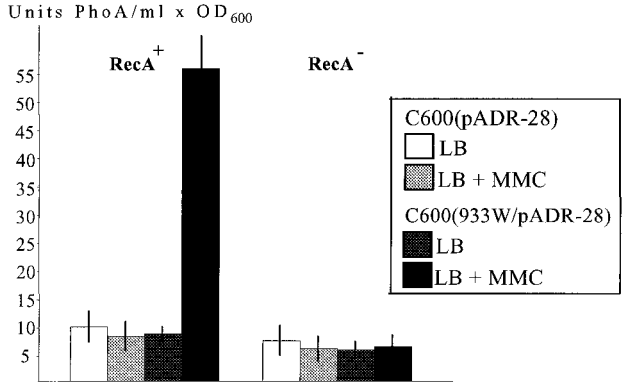

FIG. 4. Alkaline phosphatase (PhoA) activities in *recA*$^+$ and *recA* strains of *E. coli* C600(pADR-28) and C600(933W/pADR-28) following 6 h of growth in either LB medium only or LB medium supplemented with mitomycin (MMC) at a concentration of 50 ng/ml ($n = 4$).

Role of *recA* in phage-mediated SLT-II induction. In order to elucidate the role of *recA* in phage-mediated SLT-II induction, *recA*-negative mutants of *E. coli* C600(pADR-28) and the SLT-II-converting phage-harboring *E. coli* strains C600 (933W), C600(933W/pLG339), and C600(933W/pADR-28) were constructed.

In the *recA* mutant strain of *E. coli* C600(933W/pADR-28), mitomycin had no enhancing effect on PhoA expression, while significant induction was observed in its *recA*$^+$ parent strain (Fig. 4). Moreover, phage induction occurred only in the *recA*$^+$ *E. coli* strain. While for untreated cultures of the *recA*$^+$ *E. coli* strain C600(933W), the majority of SLT-II (83.5%) was found in the culture supernatant, the majority of the toxin (85.7%) of the isogenic *recA* strain was present in the cell lysate (Table 3). Figure 5 shows that mitomycin treatment resulted in a decrease in the number of toxin gene copies in the *recA E. coli* mutant strain of C600(933W) while a significant increase was found in the *recA*$^+$ parent strain.

DISCUSSION

In enterohemorrhagic *E. coli* the genes for SLT-I and -II are located on the DNA of temperate phages integrated into the host chromosome. In contrast, the genes for the SLT-II subtypes are located in the chromosome and are usually not phage associated (33, 45, 46). It is well known that SLT-I and Shiga toxin are regulated by the Fur protein in response to iron (6, 57, 59). Little is known about the regulation of SLT-II production. Hence, the aim of this study was to explore the regulation of SLT-II synthesis.

TABLE 3. Effect of mitomycin on SLT-II production in *recA*$^+$ *E. coli* C600(933W) and its isogenic *recA* mutant following 24 h of growth

E. coli C600(933W) genotype	Amt of SLT-II produced (ng/ml)			
	Control		With mitomycin (50 ng/ml)	
	SN[a]	PB[b]	SN	PB
recA$^+$	28.4	5.6	2,528.0	78.0
recA	0.2	1.2	0.5	2.9

[a] SN, culture supernatant.
[b] PB, polymyxin B extract.

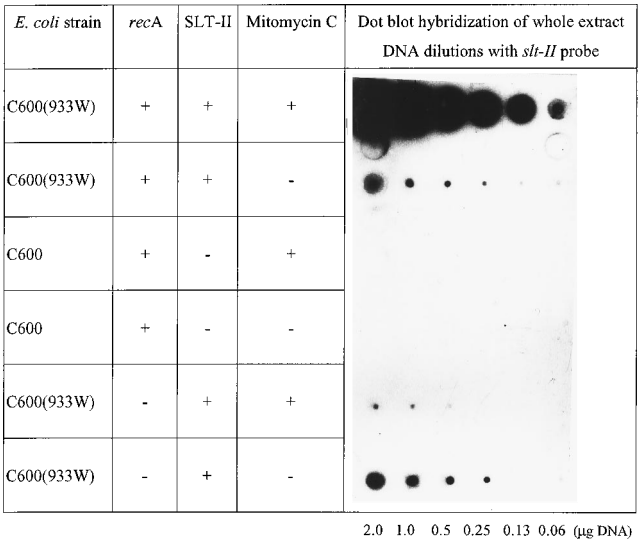

FIG. 5. Dot blot hybridization of *slt-II*-specific probe with dilutions of total cellular DNA (2 to 0.06 μg) extracted from isogenic *recA*$^+$ and *recA E. coli* C600(933W) and nontoxigenic *E. coli* C600 grown in LB medium with and without mitomycin (50 ng/ml).

We constructed a plasmid, pADR-28, containing a translational gene fusion between *slt-IIA*, including its promoter and proximal portion, and the structural gene for bacterial alkaline phosphatase (PhoA). Expression of PhoA in an *E. coli* strain harboring the plasmid should reflect SLT-II expression. In addition to providing a relatively easy enzymatic assay for toxin synthesis, construction of this plasmid allows studies of SLT-II regulation independent of the converting-phage induction state. Plasmid pADR-28 was transformed into the PhoA-negative *E. coli* strain CC118 as well as into the isogenic *E. coli* strains MC1029 (*hns*$^+$) and HMG5 (*hns*). H-NS is a histone-like DNA-binding protein, highly conserved among members of the family *Enterobacteriaceae* (62). It plays a central role in the organization of the nucleoid and in the regulation of various unlinked genes, including virulence determinants. Both upregulation and downregulation of the expression of numerous proteins are observed in *hns* mutants (9, 17, 28, 37, 47, 51). The effects on the expression of the fusion protein with alterations in culture conditions, such as temperature, osmolarity, oxygen tension, pH, the level of iron, the presence of acetates, and carbon source, were examined. For both *hns*$^+$ strains, CC118(pADR-28) and MC1029(pADR-28), no alteration in growth conditions produced any significant change in alkaline phosphatase expression. In the *hns E. coli* HMG5(pADR-28) strain, PhoA expression was derepressed when the bacteria were grown at 30°C. Growth at 37°C resulted in lower-level PhoA activity, but this activity was still greater than that expressed in the *hns*$^+$ background. At 42°C the *hns*-negative and -positive strains produced comparable levels of PhoA. The fact that PhoA activity following growth at 30, 37, and 42°C is significantly different only in the *hns E. coli* background (HMG5) and not in the isogenic *hns*$^+$ *E. coli* background (MC1029) may be due to the H-NS protein repressing PhoA expression so severely, i.e., to a minimal level, that temperature regulation cannot be detected. The presence of H-NS in enterohemorrhagic *E. coli* O157:H7 strains 933 and 86-24 was confirmed by Western blot analysis using a monoclonal antibody raised against H-NS (data not shown). However, SLT-II regulation by H-NS in a wild-type enterohemorrhagic *E. coli* strain remains to be demonstrated. In order to determine whether H-NS also influences bacteriophage-mediated SLT-II production, we transfected the isogenic *recA*-negative *E. coli* strains HMG5 and MC1029 with the SLT-II-converting phage 933W and compared the levels of SLT-II production following growth at 30, 37, and 42°C. H-NS also repressed bacteriophage-mediated SLT-II production. Toxin was present in amounts measurable by ELISA only in the *hns E. coli* strain. Its *recA*-negative background accounts for the low level of toxin expression. In contrast to expression of the *slt-II* operon from the low-copy-number plasmid pADR, SLT-II expression occurred at about equal levels following growth at 30 and 37°C but at a lower level following growth at 42°C. It has been shown that H-NS is involved in the repression of P fimbriae at temperatures below 30°C (14). H-NS also represses the expression of S fimbriae (37). Similarly, H-NS might be involved in the thermoregulation of SLT-II production. Further experiments are clearly necessary to determine the underlying mechanism of this regulation phenomenon.

In order to examine the impact of phage-mediated factors on SLT-II expression, plasmid pADR-28 was transformed into *E. coli* C600 and into *E. coli* strains that were lysogenized with either SLT-I- or SLT-II-converting phages (H19B or 933W, respectively). In the uninduced state the levels of PhoA expression from pADR-28 in C600, C600(933W), and C600 (H19B) were comparable. Treatment with mitomycin resulted in phage induction and also dramatic increases in the expression of PhoA. Because of phage-mediated lysis, PhoA activity was found to increase in a mitomycin-dependent manner not only in the bacterial cell pellets but also in the culture supernatants. Since mitomycin treatment of the nonlysogen C600 (pADR-28) had no effect on PhoA expression, mitomycin itself was not responsible for the increase in PhoA synthesis. The fact that PhoA induction in strains harboring bacteriophages occurs only in a *recA*$^+$ *E. coli* background indicates that the transition from lysogenic to lytic phage growth is a prerequisite for the above-described induction phenomenon. In order to determine whether the upregulation of PhoA expression was specific to phages carrying the toxin genes, we lysogenized *E. coli* C600(pADR-28) with the λ phage. The level of expression of PhoA in the lysogen was threefold greater than that in C600 (pADR-28). This increase in PhoA activity in the absence of mitomycin, which was not seen with the SLT-converting-phage lysogens, may have been due to a higher spontaneous induction rate in the λ lysogen.

It has also been shown that when the toxin gene is carried on the phage of a *recA*$^+$ lysogen, mitomycin treatment of the bacterial culture leads to a concomitant increase in phage numbers and numbers of toxin gene copies as the phage DNA is replicated and consequently to an increase in toxin synthesis. Induction of SLT-II production and phage replication occurred only in the *recA*$^+$ background strain, since RecA is necessary for the initiation of the lytic phage cycle. Interestingly, in the uninduced condition, the *recA*$^+$ lysogen produced about 24-fold more toxin than its isogenic *recA* mutant strain. While 83.5% of the total SLT-II production of the uninduced *recA*$^+$ lysogen was found in the culture supernatant, about the same amount (85.7%) of total toxin production was found to be cell associated for the uninduced *recA* lysogen. These differences in both total toxin production and toxin location might result from the occurrence of spontaneous phage induction in the *recA*$^+$ lysogen but not in the *recA* lysogen.

The data lead to the conclusion that bacteriophages exhibit a regulatory impact on SLT-II production by two mechanisms. Firstly, increased replication of the toxin-converting phages, elicited by phage induction, brings about an increase in toxin

production because of concomitant multiplication of toxin gene copies. Secondly, a phage-encoded factor, which is also dependent on phage induction, has a positive regulatory impact on the *slt-II* operon. Further experiments will determine the groups of phages which carry the regulatory factor and the nature of the factor. Consistent with the data presented, Yee et al. have shown that induction of cultures of *E. coli* H.I.8 with mitomycin resulted in a dramatic increase in levels of a chromosomally encoded SLT-IIva and in production of a colicin and of bacteriophage particles (63).

The fact that mitomycin is capable of inducing SLT synthesis in wild-type *E. coli* strains, such as those of the O157:H7 serotype (22), may have important clinical consequences in relation to the possible role of SLTs in the development of HUS. Mitomycin is used in the clinical treatment of neoplasia but has severe adverse effects. Approximately 10% of all mitomycin-treated patients develop renal failure (15), and these may go on to develop the so-called cancer-associated HUS (27). It was reported that 98.8% of the cancer-associated HUS patients in a study had been treated with mitomycin in the preceding months (27). These findings and the fact that SLT production has been proposed as a major cause of HUS suggest a possible link between mitomycin therapy, increased SLT production, and the subsequent development of HUS (2).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-20325 (A.D.-R.) and P30DK-34928 (from the Center for Gastroenterology Research on Absorptive and Secretory Processes) from the National Institutes of Health as well as by the Fonds der Chemischen Industrie, the Hans-Böckler-Stiftung, and the Graduiertenkolleg "Infektiologie."

We thank Joachim Morschhäuser, Institut für Molekulare Infektionsbiologie, Würzburg, Germany, for stimulating discussions and Wiebke Streckel, Robert Koch-Institut, Wernigerode, Germany, for providing the isogenic *hns*$^+$ and *hns*$^-$ *E. coli* strains MC1029(933W) and HMG5(933W), respectively. We are also grateful to Andrew Wright, Tufts University, Boston, Mass., for generously providing the polyclonal antiserum against alkaline phosphatase and to Erhard Bremer, Max-Planck-Institut, Marburg, Germany, for kindly providing the monoclonal antiserum against H-NS.

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Editor: A. O'Brien