

Toxin Gene Expression by Shiga Toxin-Producing *Escherichia coli*: the Role of Antibiotics and the Bacterial SOS Response

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Toxin synthesis by Shiga toxin-producing *Escherichia coli* (STEC) appears to be coregulated through induction of the integrated bacteriophage that encodes the toxin gene. Phage production is linked to induction of the bacterial SOS response, a ubiquitous response to DNA damage. SOS-inducing antimicrobial agents, particularly the quinolones, trimethoprim, and furazolidone, were shown to induce toxin gene expression in studies of their effects on a reporter STEC strain carrying a chromosome-based *stx2::lacZ* transcriptional fusion. At antimicrobial levels above those required to inhibit bacterial replication, these agents are potent inducers (up to 140-fold) of the transcription of type 2 Shiga toxin genes (*stx2*); therefore, they should be avoided in treating patients with potential or confirmed STEC infections. Other agents (20 studied) and incubation conditions produced significant but less striking effects on *stx2* transcription; positive and negative influences were observed. SOS-mediated induction of toxin synthesis also provides a mechanism that could exacerbate STEC infections and increase dissemination of *stx* genes. These features and the use of SOS-inducing antibiotics in clinical practice and animal husbandry may account for the recent emergence of STEC disease.

The associations between *Escherichia coli* O157:H7 infection, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) were established in the early 1980s (1,2). Shiga toxin-producing *E. coli* (STEC) strains have since been recognized as the cause of both outbreaks and sporadic cases of diarrhea and HUS, involving thousands of cases and numerous deaths (3). Shiga toxins are key virulence factors in the pathogenesis of STEC disease (3). The term Shiga toxin (Stx) refers to two families of related toxins, Stx/Stx1, which includes the classical Shiga toxin produced by *Shigella dysenteriae*, and Stx2 (4). The *stx* genes carried by STEC strains are, with one possible exception (*stx2e*), encoded on bacteriophage genomes integrated into the bacterial chromosome. Stx2-producing STEC

strains are more closely associated with HUS than are strains that produce only Stx1 (5,6).

Because antimicrobial agents may play a role in the pathogenesis of severe STEC disease, chemotherapy for STEC infections remains controversial (7,8). The location of *stx* genes (predominantly on λ -like bacteriophage genomes integrated into the chromosome of their host bacterium) has important implications because the induction of the SOS response, an extensively characterized genetic regulatory mechanism, induces high-level expression of previously silent bacteriophage genes (9). *Stx* genes are coexpressed with genes of the bacteriophage (10,11), and certain quinolones (known to be potent SOS inducers) induce increases in toxin (12,13) and bacteriophage production (13) of two to three orders of magnitude within 2 to 4 hours. The potential importance of a link between the SOS response and prophage induction for *Stx1* and *Stx2* expression has been reinforced by recent sequencing and pathogenicity studies (11,14,15).

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We have constructed a genetically modified derivative of a clinical isolate in which the genes encoding both elements of the toxin (*stx2AB*) were partially replaced with a *lacZ* reporter gene. The product of this gene, β -galactosidase, is easily assayed and detected; its expression reflects the transcriptional activity of the *stx2* gene and can be visualized in simple agar plate assays and quantified in biochemical assays. We have extended our earlier observations on the effects of quinolones on reporter expression by this strain to include a wider range of antimicrobial agents and the modulating effects of different environmental conditions. Our results show that several agents could increase the amounts of toxin produced and that SOS-inducing agents could play an important role in the epidemiology of STEC infections.

Methods

Bacterial Strain and Growth Conditions

RV31, a strain of *E. coli* O157:H7 isolated locally from a patient with hemorrhagic diarrhea, was used to construct the reporter strain, PK552 (*stx2A::lacZ*), which contains a copy of the *E. coli lacZ* gene transcriptionally fused to the promoter region of *stx2* on the chromosome. The reporter strain construction, which involved allelic exchange with a series of suicide plasmid vectors, will be described elsewhere. In the first step, the entire indigenous *lac* operon and adjacent *lacI* gene were deleted to create a Lac⁻ strain. Then, most of the coding sequence of the *stx2A* gene was replaced with a promoterless *E. coli lacZ* gene, resulting in a transcriptional fusion in which transcription of the *lacZ* reporter gene is controlled by *stx2* regulatory mechanisms. The structure of the final construct was confirmed by diagnostic polymerase chain reaction, Southern blotting, and nucleotide sequencing across the *lacZ* insertion points.

Bacterial growth in the presence of antibiotics was monitored by the Bioscreen C system and associated Biolink software (Labsystems, Basingstoke, UK). This procedure allowed the simultaneous measurement of growth in up to 200 tests by recording changes in optical density. The *stx2::lacZ* reporter strain PK552 was grown overnight at 37°C in Antibiotic Medium No. 3 (Oxoid, Basingstoke, Hampshire, UK). A 2-mL aliquot of overnight culture was added to 100 mL

of prewarmed medium and incubated with shaking at 37°C to an optical density at 600 nm (OD_{600nm}) of approximately 0.4. The culture was then divided, and test antibiotics were added to these aliquots from stock solutions. Aliquots of these cultures (200 μ L, 4 replicates) were then injected into wells of honeycomb plates (Lab-systems). The Biolink software was used to program the Bioscreen C system to specify wavelength, incubation temperature, length of experiment, timing of readings, and the rate of shaking of the cultures. The cultures were incubated in the Bioscreen C system at 37°C with continuous shaking, and OD_{600nm} was recorded every 15 min. MICs for selected antibiotics were determined in the Bioscreen system by using the inoculation pattern described above and by the conventional agar dilution method.

Detection of β -galactosidase Activity

An agar plate assay was used to screen the responses of the reporter strain, PK552 (*stx2A::lacZ*), in producing β -galactosidase activity in the presence of antibiotics. The assays were performed by using Luria-Bertani (LB) agar plates containing 20 μ g/mL of the chromogenic lactose analog X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; Sigma, Poole, Dorset, UK). An agar overlay of the PK552 reporter strain was made by mixing 100 μ L of an overnight culture with 3 mL of molten LB top agar, cooled to 48°C, and pouring the mixture over the surface of an LB agar plate. The plate was incubated with antibiotic discs (see below) placed on the surface, under the conditions specified. Expression of β -galactosidase was indicated by the enzymatic cleavage of X-gal, resulting in a blue color, the intensity of which related to the amount of enzyme produced.

Microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) at 37°C were produced in a VAIN incubator (Don Whitley Scientific, Shipley, Yorkshire, UK). Microaerobic conditions at 42°C and anaerobic conditions were produced in gas jars by using Oxoid gas-generating kits according to the manufacturer's instructions.

Antibiotic discs (Oxoid/Unipath, UK) were as follows: ofloxacin (5 μ g), nalidixic acid (30 μ g), cinoxacin (100 μ g), enrofloxacin (5 μ g): flumequine (30 μ g), ciprofloxacin (1 μ g), perfloxacin (5 μ g), norfloxacin (10 μ g), amoxycillin/clavulanic acid (20/10 μ g), imipenem (10 μ g), aztreonam (30 μ g), ceftazidime (30 μ g), cefotaxime

(30 µg), cefuroxime (5 µg), piperacillin/tazobactam (10/75 µg), ampicillin (10 µg), cephalexin (30 µg), chloramphenicol (30 µg), doxycycline (30 µg), erythromycin (15 µg), trimethoprim (5 µg), sulphamethoxazole (25 µg), furazolidone (50 µg), amoxicillin (25 µg), novobiocin (30 µg), rifampin (25 µg), gentamicin (10 µg), fosfomycin (200 µg) (oral and systemic salts), polymyxin B (300 IU), and metronidazole (50 µg).

The Bioscreen C system was also used to measure total β -galactosidase activity in whole cultures of strain PK552 by orthonitrophenyl- β -D-galactoside (ONPG) assay (16). Aliquots (20 µL, 4 replicates) of culture were removed from wells of the honeycomb plate and added to a fresh plate. To this, 180 µL of Z buffer (60 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 40 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 10 mM KCl, 1 mM MgSO_4 , and 50 mM β -mercaptoethanol, pH 7.0) containing 2 mg/mL lysozyme, 0.01% sodium dodecyl sulfate (SDS), and 100 µg/mL chloramphenicol was added to the cells. The plate was incubated at 37°C for 30 min to lyse the cells and placed on ice until use. The Bioscreen C system was prewarmed to 28°C, and 40 µL of 4 mg/mL ONPG solution (made up in Z buffer) was added to each well. Plates were incubated in the Bioscreen C system at 28°C for 4 hours, and OD was recorded at both 420 nm and 540 nm every 10

min. β -galactosidase activity was determined by using the linear portion of the corrected OD_{420} / time relationship by the Miller formula, adjusted for the sample volume (16). Replicate samples (at least four in all assays reported) yielded mean values with coefficients of variation <10% in all cases.

Results

All the quinolones induced reporter expression, while only a few of the other agents had this effect (Figure 1). The induction occurred in three general patterns: a defined zone within the zone of growth inhibition (quinolones), a defined zone at the growth/no growth interface (furazolidone), or a diffuse zone within the zone of inhibition (trimethoprim). In addition, the incubation conditions appeared to produce a background level of induction or suppression. Microaerobic conditions and to a lesser extent incubation at 30°C were associated with background induction, while anaerobic conditions and incubation at 42°C had a suppressive effect as determined by the intensities of the blue zones. However, some quinolone-mediated induction was always detectable, even under the most suppressive condition (42°C). Induction by furazolidone and trimethoprim was, in general, similarly enhanced and

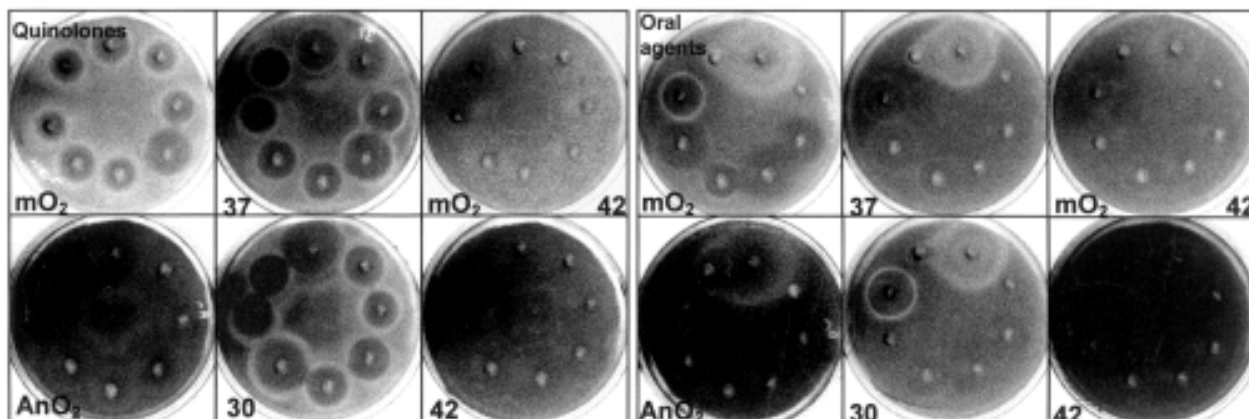


Figure 1. Zones of *stx2* expression induced by various antimicrobial agents under different incubation conditions. The disc-diffusion assay plates demonstrate background levels and zones of blue coloration related to reporter gene β -galactosidase activity produced by our reporter strain. Digital images were acquired and processed identically. Color was encoded by the CMYK system, and only the cyan component is displayed as a gray-scale image. Blue zones appear as lighter areas of the plate, the intensity of which represents the blue intensity as judged by direct comparison. Abbreviations: mO_2 , microaerobic and AnO_2 , anaerobic incubation conditions; numbers denote incubation temperatures (°C). Left panel, quinolones (clockwise from top): ofloxacin, nalidixic acid, cinoxacin, enrofloxacin, flumequine, ciprofloxacin, perfloxacin, and norfloxacin. Right panel, other oral agents (clockwise from top): trimethoprim, erythromycin, doxycycline, chloramphenicol, cephalexin, amoxycillin, furazolidone, and sulphamethoxazole.

suppressed, although 42°C abolished all induction, and anaerobic conditions did not suppress trimethoprim/sulphamethoxazole induction.

As a group, only the agents that inhibit prokaryotic translation failed to induce reporter expression under any condition tested (Table). Like furazolidone, although less intensely, several β -lactam agents induced expression at the growth/no growth interface. Imipenem (a carbapenem) not only failed to induce expression but also inhibited induction by the adjacent monobactam, aztreonam (not shown), at subgrowth-inhibitory levels. We subsequently observed that this suppressive effect was confined to β -lactam-mediated induction (i.e., imipenem showed no suppressive effect against

quinolones, trimethoprim, or furazolidone). We also noted an apparent inhibitory effect of clavulanic acid on amoxycillin in the co-amoxycylav combination and a requirement for potentiating conditions (30°C, mAO₂ 37°C) for detectable induction by novobiocin, polymyxin B, and rifampin.

We examined some of the features of induction by quantitative assay of β -galactosidase in cultures induced by exposure to antibiotics in liquid medium (Figures 2 and 3). The relationship of induction to MIC was consistent with that observed in the plate assays. Cefuroxime and furazolidone showed induction at sub-MIC levels, while the highest levels of induction with ofloxacin and trimethoprim were

Table. Induction of *stx2* expression by antibiotics under different incubation conditions^a

Target of action Agent (disc content mg/L)	Incubation conditions ^b						Zone pattern ^c
	mO ₂ /37	O ₂ /37	mO ₂ /42	AnO ₂ /37	O ₂ /30	O ₂ /42	
Background	+	-	-	-	+	-	NA
DNA Gyrase							
Quinolones ^d	+	+	+	+	+	+/-	≥MIC
Novobiocin (30)	+	-	-	-	+	-	≥MIC
Folate metabolism							
Trimethoprim (5)	+	+	-	+	+	-	≥MIC
Smx (25)	+	+	-	+	-	-	≥MIC
DNA							
Metronidazole (50)	-	-	-	-	-	-	NA
Furazolidone (50)	+	+	-	-	+	-	=MIC
Cell envelope							
Cephalexin (30)	+	+	-	-	+/-	-	=MIC
Amoxycillin (25)	+	+	-	-	-	-	=MIC
Amoxy/Clav (20/10)	+/-	-	-	-	-	-	=MIC
Ampicillin (10)	+	+	-	-	-	-	=MIC
Pip/Taz (10/75)	+	+	-	-	-	-	=MIC
Imipenem (10)	-	-	-	-	-	-	NA
Aztreonam (30)	+	+	-	-	-	-	=MIC
Cefuroxime (5)	+	+	-	-	-	-	=MIC
Ceftazidime (30)	+	+	-	-	-	-	=MIC
Cefotaxime (30)	+	+	-	-	-	-	=MIC
Fosfomycin (200)	-	-	-	-	-	-	NA
Polymyxin B (300 IU)	+/-	-	-	-	+	-	=MIC
Translation							
Gentamicin (10)	-	-	-	-	-	-	NA
Chloramphenicol (30)	-	-	-	-	-	-	NA
Doxycycline (30)	-	-	-	-	-	-	NA
Erythromycin (15)	-	-	-	-	-	-	NA
Transcription							
Rifampin (25)	-	-	+/-	-	-	-	NA

^a*Stx2* induction effects (zones of blue coloration, Figure 1): -, not detected, +/-, borderline induction, +, definite induction, ≥MIC, zone of induction within the zone of growth inhibition; =MIC, induction on the edge of the zone of inhibition; NA, not applicable; Amoxy/Clav, amoxycillin/clavulanic acid; Pip/Taz, piperacillin/tazobactam; Smx, sulphamethoxazole.

^bO₂, aerobic; mO₂, microaerobic; AO₂, anaerobic/incubation temperature °C

^cNote: The patterns indicated were detected in three separate experiments and recorded by two independent observers.

^dThe quinolones tested are listed in the Materials and Methods section.

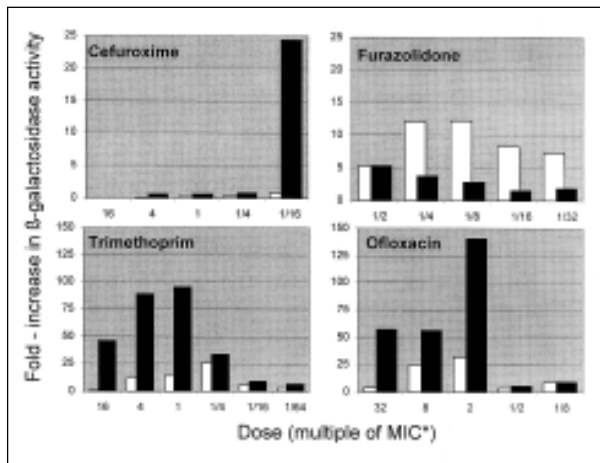


Figure 2. Dose-response characteristics of antibiotic-induced expression of *stx2::lacZ*. Exponential-phase inocula were exposed to antibiotics for 4 h (white bars) or 24 h (black bars), and β -galactosidase activity was determined at the end of these periods. Fold-increase = Test activity (+ antibiotic)/control activity (- antibiotic). Note the difference in scale between upper and lower panels. *MICs refer to the antibiotic concentrations required to inhibit growth in the Bioscreen system, using the same inoculation pattern as in the antibiotic induction experiments. The values were cefuroxime 10 g/L, furazolidone 32 g/L, trimethoprim 0.6 g/L and ofloxacin 0.05 g/L.

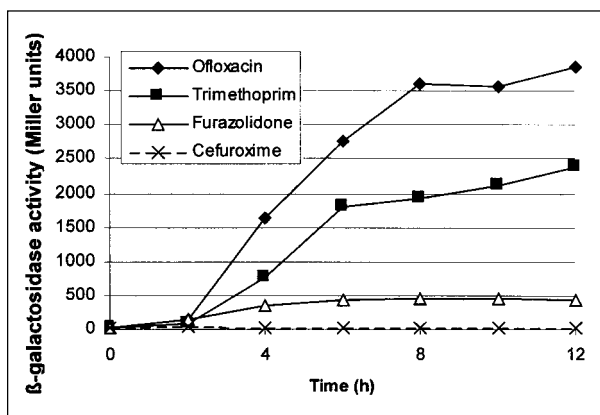


Figure 3. Time course of *stx2::lacZ* induction by antibiotics. Exponential-phase cultures were exposed to the antibiotics indicated for up to 12 h, and whole culture β -galactosidase activities were determined. The exposure levels were cefuroxime 0.625 g/L (MIC \times 1/16), furazolidone 8 g/L (MIC \times 1/4), trimethoprim 0.6 g/L (1 \times MIC) and ofloxacin 0.1 g/L (2 \times MIC).

seen at supra-MIC exposure levels; the latter two agents were clearly more potent inducers (Figures 2 and 3). We also examined the effects of fosfomycin (0.03-80 g/L) in this assay; no inducing effect was detected.

The time course experiments (Figure 3) confirm the relative inducing potencies (Figure 2) and show that furazolidone-mediated induction is complete by 8 h. Levels of furazolidone-induced enzyme activity were lower at 24 h than at 4 h or 12 h. In contrast, trimethoprim and ofloxacin induction rapidly increased up to 6 h and 8 h, respectively, then continued to increase more slowly until the final measurement at 12 h. Additional expression induced by these two agents continued up to 24 h (Figure 2), while cefuroxime-induced expression occurred only between 12 h and 24 h.

Discussion

These patterns of reporter gene expression demonstrate that several antimicrobial agents have the capacity to increase the amount of Stx2 synthesized by our STEC strain. Levels of β -galactosidase expression in the reporter strain are closely correlated with biologically active toxin produced by the parental wild type strain (12). The demonstrated link to de novo gene expression distinguishes our study from earlier work in which it could not be determined whether increased toxin levels reflected release of preformed toxin by cell lysis or increased synthesis (17-20). The inductions we observed show how certain agents and environmental conditions may increase the amount of toxin in infected persons and that, in the absence of definitive clinical data, they provide a rational basis for avoiding certain agents in treating patients who may have STEC infections.

Several of the agents tested here—the quinolones (21), trimethoprim (22), furazolidone, and metronidazole (23)—have a recognized capacity to induce bacterial SOS response, which is initiated when damaged bacterial DNA interacts with and activates the multifunctional RecA protein. Activated RecA, in turn, causes the degradation of two key repressor proteins, LexA and CI. The resulting de-repression of genes regulated by LexA leads to the temporary arrest of DNA synthesis and cell division and the activation of error-prone DNA repair. In strains carrying an integrated λ -like bacteriophage, cleavage of the CI phage repressor/activator

protein leads to the induction of previously silent phage-encoded genes (in this case including *stx2A* and *stx2B*), followed by production of phage particles and host bacterial cell lysis (9).

The most potent SOS inducers, the quinolones and trimethoprim, produced the most prominent effects in both agar and liquid culture assays. Metronidazole did not induce reporter expression under the conditions tested; however, this agent would not normally be expected to affect *E. coli*. Another potent SOS inducer, mitomycin C, which is known to increase toxin levels in vitro (24,25), tests positive in agar and broth induction tests with our reporter strain (results not shown). The potential importance of the SOS response to pathogenesis in vivo has recently been underlined by a study in which STEC strains with mutations in *recA*, a critical gene for SOS induction, were rendered avirulent in a mouse 50% lethal dose test (15).

The importance of antibiotic-induced Stx expression would be reinforced if the implicated agents were in some way associated with occurrence or severity of STEC disease. The results of available studies have conflicted with regard to the influence of antibiotics. The age groups studied, the timing of antibiotic therapy, and the range of agents used complicate the analyses. Nonetheless, use of quinolones and trimethoprim/sulphamethoxazole (26,27) has been implicated as a risk factor for HUS. In a recent clinical study, children treated with cephalosporins or trimethoprim-sulphamethoxazole had, respectively, 13.4- and 17.7-fold increases in risk for HUS (28). Our observations suggest that the net effect of one of these agents on the exposure of an infected patient to toxin depends on the stage of the infection, the number of organisms present at the time antibiotics are administered, the immediate environmental conditions of those organisms, and the time-concentration profile and bactericidal effect of the drug. This complex interplay of factors could render an SOS-inducing antibiotic clinically beneficial (e.g., if the numbers of infecting organisms were insufficient to produce substantial amounts of toxin and they were all killed by the first dose), neutral, or disadvantageous in different situations. Moreover, exposure of patients to other potential SOS-inducing agents (cf. 9,23) could further complicate the relationship between antibiotic use and severe STEC disease. A strong association between mitomycin

C administration and HUS was detected in a study of "cancer-associated" HUS (29).

Grif and colleagues reported on the effects of sub-MIC levels of 13 antibiotics on release of biologically active toxin (as distinct from toxin gene expression) by three STEC strains into culture supernatants after overnight incubation (20). These authors observed substantial interstrain differences in the responses, as well as increased levels of toxin after exposure to several agents that had not previously been associated with SOS-inducing activity. Grif et al. did not distinguish between enhanced release of pre-formed toxin and increased toxin production, and the most potent inducing effects we have observed were at suprainhibitory levels of exposure. Nonetheless, we cannot rule out interstrain differences in susceptibility to SOS activation and the possibility that other induction mechanisms may be involved. Our observations on SOS induction by several β -lactam agents and the inhibitory effects of imipenem appear pertinent. Suppression of toxin expression by imipenem supports previous observations on toxin release (30). Paired disc-diffusion assays involving imipenem and other recognized SOS-inducing antibiotics indicated that this effect was confined to induction by β -lactam agents (Kimmitt, Harwood, and Barer, unpubl data). This finding, combined with the different time course of induction with cefuroxime, reinforces the view that there may be induction pathways distinct from the SOS response. Although the nature of the putative alternate induction pathway(s) remains obscure, the clear evidence for β -lactam-mediated induction may contraindicate use of the implicated agents in treating patients with STEC infection.

Although any increase in exposure of STEC-infected persons to Shiga toxins seems undesirable, SOS-mediated induction seems particularly hazardous because of its potential rapid effects. We report kinetics (Figure 3) somewhat slower than those we observed previously in the shaken conical flask incubations (12). The rapid buildup in toxin levels associated with SOS induction could facilitate entry of toxin into the bloodstream and subsequent disseminated effects on the kidneys and other organs. SOS-mediated induction also leads to dissemination of the toxin-encoding bacteriophage.

Matsushiro et al. observed parallel increases in toxin and bacteriophage counts in response to

norfloxacin (13), and we have made similar observations with ofloxacin and trimethoprim (Kimmit, Harwood and Barer, unpubl data). Moreover, sequencing studies clearly indicate that expression of *stx2* and bacteriophage genes is coordinately regulated (11,14). Hemorrhagic colitis and HUS attributable to STEC were recognized after trimethoprim and the 4-quinolones were introduced into human and veterinary clinical practice, and the substantial recent increase in reports of STEC disease follows expanded use of fluoroquinolones.

Fluoroquinolones and trimethoprim have been recommended for prophylaxis and treatment of travelers' diarrhea (31), and the former are often used to treat severe bacterial enteric infections. Our findings indicate that this approach may be inappropriate if STEC infection is a possibility. Furthermore, reports that certain antimicrobial agents may ameliorate or reduce symptoms or the frequency of life-threatening complications in STEC infections provide an incentive to find a rational basis for selection (8,27). Our results suggest that agents with SOS-inducing activity, antimicrobial or otherwise, should be avoided (Table).

We conclude that SOS-mediated induction of Shiga toxins and toxin-encoding bacteriophages may contribute to the emerging epidemiologic pattern of STEC disease. Many other bacterial virulence determinants are encoded on lysogenic bacteriophage genomes (32), and the issues raised here may have public health and clinical implications beyond the understanding of STEC disease.

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Dr. Kimmitt is trained in medical microbiology and molecular biology. He is currently studying the molecular biology of chlamydia infection at the London School of Hygiene and Tropical Medicine.

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