Phenethyl Isothiocyanate Inhibits Shiga Toxin Production in Enterohemorrhagic *Escherichia coli* by Stringent Response Induction

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The pathogenicity of enterohemorrhagic *Escherichia coli* (EHEC) depends on production of Shiga toxins, which are encoded by *stx* genes located in the genomes of lambdoid prophages. Efficient expression of these genes requires prophage induction and lytic development of phages. Treatment of EHEC infections is problematic due to not only the resistance of various strains to antibiotics but also the fact that many antibiotics cause prophage induction, thus resulting in high-level expression of *stx* genes. Here we report that *E. coli* growth, Shiga toxin-converting phage development, and production of the toxin by EHEC are strongly inhibited by phenethyl isothiocyanate (PEITC). We demonstrate that PEITC induces the stringent response in *E. coli* that is mediated by massive production of a global regulator, guanosine tetraphosphate (ppGpp). The stringent response induction arises most probably from interactions of PEITC with amino acids and from amino acid deprivation-mediated activation of ppGpp synthesis. In mutants unable to synthesize ppGpp, development of Shiga toxin-converting phages and production of Shiga toxin are significantly enhanced. Therefore, ppGpp, which appears at high levels in bacterial cells after stimulation of its production by PEITC, is a negative regulator of EHEC virulence and at the same time efficiently inhibits bacterial growth. This is in contrast to stimulation of virulence of different bacteria by this nucleotide reported previously by others.

Shiga toxin-producing *Escherichia coli* (STEC) organisms are a group of strains of this bacterium that express genes coding for specific toxins, called verotoxins or Shiga toxins (1). Most STEC strains are pathogenic to humans, including a subset of strains classified as enterohemorrhagic *E. coli* (EHEC) (2, 3). High level of EHEC pathogenicity requires production of Shiga toxins (1). Hemorrhagic colitis is the primary symptom of infection of humans by EHEC, but complications may include hemolytic-uremic syndrome (HUS), which is a severe disorder (2). This syndrome is characterized by acute renal failure, anemia, and thrombocytopenia. Other organs such as the lung, pancreas, and heart may also be damaged. Moreover, some patients additionally suffer from nervous system dysfunctions that can include lethargy or disorientation. Children and elderly people are groups at the highest risk for complications. Among patients infected with EHEC, 3 to 15% develop HUS (3). The mortality rate among patients who develop HUS is estimated to be as high as 10% if plasmapheresis treatment is conducted and significantly higher without treatment (2, 3). A recent EHEC outbreak in Germany, which occurred in 2011, corroborated the opinion about severity of infections with Shiga toxin-producing *E. coli* (4–8). In fact, among over 4,000 severe infections there were more than 50 fatal cases. The German outbreak was unique, as adults rather than children were predominantly affected and HUS incidence was especially high (over 20% of treated patients) (5–8).

In all STEC strains analyzed to date, genes coding for Shiga toxins (*stx* genes) are localized in the genomes of lambdoid bacteriophages integrated into the bacterial chromosome, thus forming Shiga toxin-converting prophages (9, 10). The *stx* genes are under the control of the late phage promoter pR' (according to phage λ genetic nomenclature) (11, 12), which is inhibited in the prophage state by the phage-encoded cI repressor, as in all lambdoid phages (13, 14). Hence, in *E. coli* lysogenic for Shiga toxin-converting bacteriophages, production of the toxin is blocked. Efficient expression of *stx* genes and release of the toxin require

prophage induction, followed by excision of the phage genome, several rounds of its replication, synthesis of phage-encoded proteins (including Shiga toxins), assembly of progeny virions, and lysis of the host cell (10, 15, 16).

Induction of lambdoid prophages depends on initiation of the bacterial SOS response, which occurs after DNA damage (13, 14). Various antibiotics may directly or indirectly interfere with DNA replication, resulting in DNA lesions, which are signals to initiate the SOS response, and subsequent prophage induction and Shiga toxin production (reviewed in references 10 and 17). Therefore, treatment of EHEC infections is problematic not only due to resistance of various strains to many antibiotics but also because of antibiotic-mediated prophage induction, which indirectly causes a high-level expression of *stx* genes. On one hand, an enhanced release of Shiga toxins following antibiotic treatment of EHEC is a phenomenon known for many years (18, 19), but on the other hand, enhanced production of these toxins was observed mainly in experiments with subinhibitory concentrations of antibiotics, and there are reports indicating that some antibiotics did not cause such effects (summarized in reference 20). Nevertheless, recommendations postulating that the use of antibiotics against STEC (including EHEC) should be avoided because of a potential increase in the risk of HUS development were published (21–23). Although it is plausible that some antibiotics could be used as

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effective anti-EHEC drugs, according to the predominant opinion, it is still premature to endorse antibiotic treatment for EHEC infection (20).

In the light of a lack of an effective treatment of EHEC infection that would also prevent HUS development, a search for bactericidal agents inhibiting also production of Shiga toxins is desirable. Hence, it is interesting that lytic development of Shiga toxin-converting bacteriophages, and particularly phage DNA replication, is strongly inhibited by guanosine tetraphosphate (ppGpp), an alarmone of the stringent response (24, 25). This response has been discovered as a bacterial reaction to amino acid starvation, but subsequent studies revealed that this response also occurs for other stresses (26). Under such conditions, ppGpp is produced rapidly and efficiently, and thus its intracellular concentration increases many times within a few minutes. In *E. coli*, there are two enzymes responsible for ppGpp synthesis. The *relA* gene product, ppGpp synthetase I, is a ribosome-associated enzyme, which is activated in amino acid-starved cells upon binding of uncharged tRNA to the ribosome. The SpoT protein, ppGpp synthetase II, is a bifunctional enzyme acting also as a ppGpp hydrolase, thus being responsible for ppGpp turnover in the cell (26). During amino acid starvation, major changes in transcription of many genes occur due to direct interactions of ppGpp with *E. coli* RNA polymerase; nevertheless, this nucleotide can also interact with some other proteins (26, 27). The alterations in gene expression are aimed toward saving energy and resources at times of stress and nutrient limitation. Generally, the stringent response is a global control system of bacterial physiology, as it affects directly or indirectly the majority, if not all, of crucial cellular processes (26).

Isothiocyanates, compounds derived from the hydrolysis of glucosinolates, found in cruciferous vegetables, have received increasing attention due to their chemopreventive, anticancer as well as antimicrobial activities, coupled with moderate toxicity toward human monocytes (28, 29). One of the most potent isothiocyanates against Gram-positive and Gram-negative bacteria is phenethyl isothiocyanate (PEITC). It has been shown, using disc-diffusion assay, that PEITC dose dependently inhibits the growth of human oral bacteria or bacterial strains isolated from human gastrointestinal tract (30, 31). The mechanism by which PEITC inhibits the growth of bacteria is not fully elucidated. Recently it has been suggested that phenyl isothiocyanate, a compound structurally similar to PEITC, disturbs the bacterial membrane function, which leads to loss of its integrity and cell death, although covalent binding to other cellular targets through the isothiocyanate moiety cannot be excluded (32).

In this report, we demonstrate that growth of PEITC-treated STEC bacteria is inhibited, as is the lytic development of Shiga toxin-converting bacteriophage after prophage induction. Moreover, *stx* expression was strongly impaired under these conditions. Our studies indicated that these effects arise from PEITC-mediated induction of the stringent response, most probably due to interactions of this isothiocyanate with amino acids, rather than from disruption of cell membrane integrity.

MATERIALS AND METHODS

***Escherichia coli* strains and bacteriophages.** *E. coli* strains and bacteriophages are listed in Table 1.

Media and growth conditions. Bacteria were cultured in liquid LB broth (Sigma) or minimal MOPS (morpholinepropanesulfonic acid) medium (33) at 37°C in shake flasks with agitation. LB medium supple-

TABLE 1 *Escherichia coli* strains and bacteriophages

<i>E. coli</i> strain or bacteriophage	Relevant genotype or description	Reference
<i>E. coli</i> K-12 laboratory strains		
MG1655	F ⁻ λ ⁻ <i>ilvG rfb-50 rph</i>	62
ppGpp ⁰	As MG1655 but <i>relA256 spoT212</i>	25
CF1652	As MG1655 but <i>relA251::kan</i>	63
SCUC34	933W Δ <i>stx</i> ₂ :: <i>catGFP</i> lysogen	64
MG1655 (933W)	MG1655 lysogenic with 933W Δ <i>stx</i> ₂ :: <i>catGFP</i>	25
ppGpp ⁰ (933W)	ppGpp ⁰ lysogenic with 933W Δ <i>stx</i> ₂ :: <i>catGFP</i>	25
CF1652 (933W)	CF1652 lysogenic with 933W Δ <i>stx</i> ₂ :: <i>catGFP</i>	25
<i>E. coli</i> O157:H7 clinical isolates		
86-24	O157:H7 lysogenic with ST2-8624	65
86-24Δ <i>tox</i>	As 86-24 but lysogenic with ST2-8624 Δ <i>stx</i> ₂ :: <i>GFP</i> instead of wild-type phage	34
PT22Δ <i>stx</i> ₂ ^a	PT22 lysogenic with P22 Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
PT27Δ <i>stx</i> ₂ ^a	PT27 lysogenic with P22 Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
PT32Δ <i>stx</i> ₂ ^a	PT32 lysogenic with P32 Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
PT38aΔ <i>stx</i> ₂ ^a	PT 38a lysogenic with P38a Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
PT38bΔ <i>stx</i> ₂ ^a	PT38b lysogenic with P38b Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
PT39aΔ <i>stx</i> ₂ ^a	PT39a lysogenic with P39a Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
Bacteriophages		
933W	933W but Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
ST2-8624	ST2-8624 but Δ <i>stx</i> ₂ :: <i>GFP</i>	34
P22Δ <i>stx</i> ₂ ^a	P22 but Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
P27Δ <i>stx</i> ₂ ^a	P27 but Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
P32Δ <i>stx</i> ₂ ^a	P32 but Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
P38aΔ <i>stx</i> ₂ ^a	P38a but Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
P38bΔ <i>stx</i> ₂ ^a	P38b but Δ <i>stx</i> ₂ :: <i>catGFP</i>	64
P39aΔ <i>stx</i> ₂ ^a	P39a but Δ <i>stx</i> ₂ :: <i>catGFP</i>	64

^a Clinical *E. coli* O157:H7 strains, bearing wild-type Shiga toxin-converting prophages, were isolated at the Cincinnati Children's Hospital Medical Center, and their Δ*stx*₂::*catGFP* derivatives were constructed for safety reasons.

mented with 1.5% bacteriological agar was used as a bottom agar for plating. Media were supplemented with antibiotics (chloramphenicol at 2.5 or 20 μg/ml; kanamycin at 50 μg/ml). Phage titration was carried out by a modified double-layer agar assay (34) with the top agar consisting of 1% Tryptone (Difco), 0.5% NaCl (POCH, Poland), and 0.7% bacteriological agar (Becton, Dickinson).

Monitoring of prophage induction and phage lytic development. Overnight cultures of relevant *E. coli* lysogenic strains were diluted 1:100 in 50 ml fresh LB or minimal medium and cultured with shaking at 37°C until *A*₆₀₀ reached 0.1. Then, the culture was treated with either mitomycin C (1 μg/ml) or hydrogen peroxide (1 μM) as an induction agent, and in addition, serine hydroxamate (SHX) (0.5 mg/ml) or PEITC (at indicated concentrations) was added at this time point. Samples (0.5 ml) were withdrawn every 30 min, 30 ml of chloroform was added to each sample, and the mixture was vortexed and centrifuged for 5 min (5,000 × *g*). Supernatants were diluted in TM buffer (10 mM Tris, 10 mM MgSO₄, pH 7.2), and 100 μl of each dilution was mixed with 0.5 ml of a culture of the indicator strain. Then, 2 ml of a prewarmed (to 45°C) top nutrient agar was added to each mixture, which was poured onto an LB agar plate supplemented with 2.5 μg/ml chloramphenicol, according to a previously published procedure (34). Results of experiments performed in triplicate are shown as mean values with standard deviations (SD).

Measurement of RNA and DNA synthesis efficiency. Synthesis of nucleic acids was assessed by measurement of incorporation of radioactive precursors, [³H]thymidine and [³H]uridine (for DNA and RNA, respectively), according to a previously described procedure (35). Briefly, overnight bacterial cultures were diluted in fresh LB medium (1:100) and

cultivated to A_{600} of 0.1. [^3H]thymidine or [^3H]uridine was added at 5 μCi . Samples (50 μl) were withdrawn, placed onto Whatman no. 3 filter papers, and then transferred immediately to ice-cold 10% trichloroacetic acid (TCA) for 10 min. Following sequential washing in 5% TCA and twice in 96% ethanol, the filters were dried, and radioactivity was measured in a scintillation counter MicroBeta (Wallac; PerkinElmer). Results (expressed in cpm) from three independent experiments were normalized to bacterial culture density (A_{600}) and presented as mean values with SD.

Determination of bacterial growth inhibition. The MIC test was performed by the broth microdilution method according to the British Society for Antimicrobial Chemotherapy (BSAC) standard methodology (36). Minimal MOPS medium (or its variant with one of 20 indicated amino acids, 20 mM each) was used to prepare bacterial inoculum and to dilute the PEITC stock solution (20 mM). Bacterial growth in the medium containing various final concentrations of PEITC was monitored by measurement of A_{600} . The PEITC MIC₅₀ was calculated on the basis of results.

Estimation of levels of ppGpp and pppGpp. (p)ppGpp levels were measured as previously described, with minor modifications (33). Briefly, bacteria were grown overnight in minimal MOPS medium and then washed and resuspended in low-phosphate (0.4 mM) MOPS labeling medium at A_{600} of 0.02. Cultures were grown until they reached A_{600} of 0.2 and diluted again (1:10) in the same medium. [^{32}P]orthophosphoric acid was added to 150 $\mu\text{Ci}/\text{ml}$, and then bacteria were cultured for at least 2 generations before the first sample was taken. SHX (0.5 mg/ml) or 20 μM PEITC was added at time zero. Samples (50 μl) were collected at indicated time points and then extracted with ice-cold formic acid (13 M) by three cycles of freeze-thaw. Samples were centrifuged (5,000 $\times$ g, 4°C, 5 min), and nucleotides present in the supernatant were separated by thin-layer chromatography (TLC) on polyethyleneimine (PEI) cellulose TLC plates in 1.5 M potassium phosphate buffer and analyzed in a phosphorimager (Typhoon; GE Healthcare). The spots corresponding to ppGpp and pppGpp were identified according to previously reported characteristics (33, 37); this identification was confirmed by a low intensity or absence of these spots in samples from nonstarved bacteria and by their complete lack in samples from *relA* or *relA spoT* mutants.

Microscopic analyses. Bacteria were cultured as described in "Monitoring of prophage induction and phage lytic development" above, and samples were collected at the indicated times. The cells were immobilized on 1% agarose pads set on a glass slide surface as described elsewhere (25). For membrane and chromosome visualization, cells were stained with Synaptored (Sigma-Aldrich; 5 $\mu\text{g}/\text{ml}$ final concentration) or DAPI (4',6-diamidino-2-phenylindole dihydrochloride; Sigma, Germany), respectively. Images were taken using a Leica DMI4000B microscope fitted with a DFC365FX camera (Leica). The following Leica filter sets were used: N2.1 (FM4-64), GFP (green fluorescent protein), and A4 (DAPI). Images were collected and processed using LAS AF 3.1 software (Leica).

Determination of toxicity of bacterial lysates to human and simian cells. Human HeLa or simian Vero cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco), pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1 $\times$ Antibiotic and Antimycotic solution (all purchased from Sigma, Germany), and incubated at 37°C in a humidified 5% CO₂ atmosphere.

In order to evaluate the Shiga toxin activity to HeLa or Vero cells, variants of bacterial lysates were prepared. Overnight cultures of *E. coli* O157:H7 and O157:H7 Δtox were cultivated in LB medium. After 100-fold dilution in fresh MOPS minimal medium (containing 0.5% glucose), the cultivation was continued until A_{600} reached 0.1. Then, mitomycin C, PEITC, and/or SHX was added to the cultures to various final concentrations, and cultures were incubated with shaking (170 rpm) for 30 min. For preparation of cell-free lysates, cultures were centrifuged (6,000 $\times$ g, 5 min, 4°C) and lysates were filtrated using low-protein-binding polyvinylidene difluoride (PVDF) syringe filters (0.22 μm ; Roth). Lysates were serially diluted in DMEM.

Cytotoxicity was assessed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, performed as described

FIG 1 Inhibition of *E. coli* growth by PEITC in a rich medium. *E. coli* strain MG1655 was grown in LB medium at 37°C without treatment (closed circles) or in the presence of PEITC, added at time zero to a final concentration of 10 (open circles), 20 (open triangles), 50 (open diamonds), or 100 (crosses) μM . A_{600} was measured at the indicated times. The results presented are mean values from 3 independent experiments with error bars indicating SD.

previously (38). HeLa or Vero cell lines were seeded at 1×10^4 cells/well in 96-well plates and incubated overnight. Then, the growth medium was substituted with fresh medium supplemented with tested compounds (5 μl of lysates was added to 95 μl of the DMEM). After 48 h of incubation, the medium was changed to an MTT solution (Sigma-Aldrich) (MTT was diluted in phosphate-buffered saline [PBS] to 1 mg/ml, and 100 μl of this mixture was added to each well). Plates were incubated at 37°C for 4 h, and the medium was removed. Purple formazan product was dissolved in 100 μl dimethyl sulfoxide (DMSO) and quantified by measuring A_{570} . Cytotoxicity data are presented as means (with SD) from at least three independent experiments, determined relative to nontreated cultures.

Statistical analyses. The significance of differences between mean values of two measured parameters was assessed by the *t* test. In experiments devoted to determine toxicity of bacterial lysates to human or simian cells, one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test was also employed. Differences were considered significant when *P* values were <0.05 .

RESULTS

Inhibition of *E. coli* growth and Shiga toxin-converting bacteriophage lytic development by PEITC. We found that PEITC inhibits growth of *E. coli*, including the strain bearing a derivative of one of the best-investigated Shiga toxin-converting prophages, phage 933W (Fig. 1). This inhibition revealed a dose-response correlation, with complete halting of the bacterial culture growth in LB medium at a 0.1 mM concentration of this isothiocyanate. Moreover, we found that PEITC effectively inhibited the growth of several clinical EHEC isolates (Table 2).

Then, we tested the effects of PEITC on Shiga toxin-converting prophage induction and phage lytic development upon prophage induction forced by chemical agents. Mitomycin C is commonly used for induction of lambdoid prophages under laboratory conditions, and hydrogen peroxide may be the major factor responsible for Shiga toxin-converting prophage induction in the human intestine (10, 13, 14). Interestingly, addition of PEITC to the culture of bacteria lysogenic for a derivative of bacteriophage 933W not only did not cause induction of the prophage but also either severely impaired or completely inhibited phage lytic development after prophage induction provoked by mitomycin C (over 100-fold-lower phage burst size was observed in cultures treated

TABLE 2 Inhibition of growth of clinical isolates of EHEC by PEITC in minimal (MOPS) and rich (LB) media

Strain ^a	MIC (μ M)	
	Minimal MOPS medium	LB medium
MG1655	20	200
86-24	20	200
PT22	20	100
PT27	40	200
PT32	20	100
PT38a	40	200
PT38b	10	100
PT39a	20	200

^a MG1655 is an *E. coli* K-12 laboratory strain, and other strains are derivatives of clinical isolates of EHEC.

with both PEITC and mitomycin C relative to analogous cultures treated solely with mitomycin C) or hydrogen peroxide, respectively (Fig. 2). Development of various Shiga toxin-converting bacteriophages was also inhibited by PEITC upon mitomycin C-mediated prophage induction in several clinical EHEC isolates (Table 3).

PEITC induces the stringent response in *E. coli*. To learn about a mechanism by which PEITC inhibits *E. coli* growth and Shiga toxin-converting phage development, we have investigated

what processes are impaired in PEITC-treated cells. Under these conditions, a strong inhibition of stable RNA synthesis, relative to untreated bacteria, was observed (Fig. 3A). Such a phenomenon is characteristic for agents or conditions either directly interfering with the transcription process or causing the stringent response (26). Therefore, we tested the latter possibility by employing the *relA* mutant, which is unable to produce large amounts of ppGpp in amino acid-starved bacteria due to a lack of the major ppGpp synthetase, and the *relA spoT* double mutant, which lacks both ppGpp synthetases, thus being unable to produce the stringent response alarmone under any conditions (such a phenotype is called ppGpp⁰). Treatment of both *relA* and *relA spoT* mutant strains with PEITC did not result in stable RNA synthesis inhibition (Fig. 3B and C), strongly suggesting that the effect observed in wild-type cells is ppGpp dependent. Synthesis of DNA at relatively short times (up to 30 min in cultures growing in a minimal medium) after addition of the tested compound was not significantly inhibited in all tested strains (wild type, *relA* mutant, and *relA spoT* double mutant), indicating that DNA replication elongation is not affected (Fig. 3D, E, and F). Such a phenotype, in combination with the RNA synthesis inhibition profile (Fig. 3A, B, and C), is also characteristic for the stringent response that in *E. coli* results in a negative regulation of DNA replication initiation rather than the elongation stage (39).

To test whether PEITC can induce the stringent response, we

FIG 2 Effects of PEITC on lytic development of bacteriophage 933W after prophage induction with either 1 μ g/ml mitomycin C (A and C) or 1 mM hydrogen peroxide (B and D) at time zero. *E. coli* strain MG1655 (933W) was grown in LB medium at 37°C without treatment (closed circles), with an induction agent (closed squares), with 50 μ M PEITC (open circles), or with an induction agent and PEITC (open squares). Phage titer (A and B) or A_{600} of the culture (C and D) was measured at the indicated times. The results presented are mean values from 3 independent experiments, with error bars indicating SD. Statistically significant differences ($P < 0.05$ in the *t* test) were obtained in following cases: the culture treated with mitomycin C versus any other culture at times 120, 150, 180 and 240 min, and the culture treated with mitomycin C and PEITC versus any other culture at times 180 and 240 min (A); the culture treated with hydrogen peroxide versus any other culture at times 120 min and later (B); the control culture versus the culture treated with mitomycin C at time points 150 min and later, the control culture versus the culture treated with PEITC or the culture treated with mitomycin C and PEITC at time points 60 min and later (C); the control culture versus the culture treated with PEITC or the culture treated with hydrogen peroxide and PEITC at time points 60 min and later, the control culture versus the culture treated with hydrogen peroxide at time points 150 min and later, and the culture treated with hydrogen peroxide versus the culture treated with PEITC or the culture treated with hydrogen peroxide and PEITC at time points 90 min and later (D).

TABLE 3 Efficiency of development of Shiga toxin-converting bacteriophages in clinical isolates of EHEC after mitomycin C-mediated prophage induction and treatment with PEITC

Phage titer (PFU/ml) obtained:					
Strain	At time zero	At 4 h in cultures with indicated treatment ^a			
		–MitC, –PEITC	–MitC, +PEITC	+MitC, –PEITC	+MitC, +PEITC
PT22	1.6 × 10 ³	1.2 × 10 ⁴	3.9 × 10 ³	2.2 × 10 ⁹	1.5 × 10 ⁴
PT27	2.2 × 10 ³	5.5 × 10 ³	2.8 × 10 ³	7.9 × 10 ⁸	3.9 × 10 ⁴
PT32	1.2 × 10 ³	4.3 × 10 ⁴	1.4 × 10 ⁴	6.8 × 10 ⁸	<10 ²
PT38a	<10 ²	7.7 × 10 ³	4.8 × 10 ³	4.5 × 10 ⁷	6.2 × 10 ⁴
PT38b	<10 ²	1.7 × 10 ⁴	<10 ²	9.2 × 10 ⁶	2.8 × 10 ³
PT39a	<10 ²	8.6 × 10 ³	<10 ²	4.8 × 10 ⁷	<10 ²

^a Bacteria were cultured in LB medium at 37°C, and mitomycin C (+MitC) and/or PEITC (+PEITC) was added to a final concentration of 1 μg/ml or 50 μM, respectively, at time zero; a minus sign (–) indicates absence of the indicated agent. Results represent mean values from 3 independent experiments; in each case, SD was <15%.

have monitored ppGpp levels in cells treated with either a potent inducer of serine starvation, serine hydroxamate (SHX), or this isothiocyanate and compared them to levels in untreated cells. We found that PEITC can cause accumulation of ppGpp and its precursor, pppGpp (guanosine pentaphosphate), almost as efficiently as SHX, which is routinely used in laboratories to induce the stringent response (Fig. 4). This effect was abolished by the *relA* mutation (Fig. 4), corroborating the conclusion that PEITC is a stringent response inducer.

We asked what mechanism underlies PEITC-mediated induction of the stringent response. It was reported previously that isothiocyanates may react with amino acids (40). In fact, we found that *E. coli* growth inhibition required significantly lower PEITC concentrations in a minimal medium devoid of amino acids (see Fig. S1 in the supplemental material) than in LB (Fig. 1). Therefore, to test the hypothesis that PEITC may titrate out particular

amino acids in *E. coli* cells, we cultured this bacterium in a medium containing particular tested amino acids (at final concentration of 20 mM) and determined the PEITC MIC₅₀ (estimated by measurement of A₆₀₀ of the culture). We found that some amino acids could considerably decrease the sensitivity of the tested *E. coli* strain to PEITC, with glycine and arginine being the most effective ones (Table 4). Thus, we tested whether these amino acids can restore the PEITC-inhibited bacterial growth. When the culture was treated with 20 μM PEITC, a strong growth inhibition was evident; however, subsequent supplementation of the medium with either 20 mM glycine or arginine resulted in efficient growth restoration (Fig. 5). Analogous supplementation with cysteine, alanine, or proline, amino acids that were not effective in decreasing the sensitivity of *E. coli* cells to PEITC, did not cause any improvement of the culture growth relative to bacteria treated solely with PEITC (Fig. 5).

FIG 3 Effects of PEITC on synthesis of stable RNA (A to C) and DNA (D to F). *E. coli* strain MG1655 (WT) (A and D) or its *relA spoT* (*ppGpp*⁰) (B and E) or *relA* (*relA*–) (C and F) derivatives were grown in a minimal medium at 37°C, either untreated (closed symbols) or in the presence of PEITC, added at time zero to a final concentration 50 μM (open symbols). [³H]uridine (A to C) or [³H]thymidine (D to E) was added to 5 μCi, and incorporation into TCA-precipitable material was measured. The results presented are mean values from 3 independent experiments with error bars indicating SD (error bars are not shown when they are smaller than the symbols). Statistically significant differences (*P* < 0.05 in the *t* test) were obtained only between results presented in panel A (at 15, 30, and 45 min), panel D (at 60 min), panel E (at 45 min), and panel F (at 45 and 60 min).

FIG 4 Induction of (p)ppGpp synthesis by PEITC or SHX. *E. coli* strain MG1655 (WT) or its *relA* (*relA*[−]) derivative was grown in MOPS labeling medium at 37°C in the presence of 150 μ Ci/ml [³²P]orthophosphoric acid. Samples were withdrawn either before (0 min) or after (15 or 30 min) addition of PEITC or SHX to a final concentration of 20 μ M or 0.5 mg/ml, respectively. Following cell lysis, nucleotides were separated by thin-layer chromatography on PEI cellulose TLC plates in 1.5 M potassium phosphate buffer and analyzed in a phosphorimager. The positions of ppGpp and pppGpp are indicated by arrows.

Inhibition of *stx* gene expression in bacteria treated with PEITC. We found that the PEITC-mediated stringent response is responsible for inhibition of Shiga toxin-converting bacteriophage development after mitomycin C- and hydrogen peroxide-provoked prophage induction, as such inhibition was abolished in the ppGpp⁰ host (Fig. 2). Since it was demonstrated previously that ppGpp-mediated negative regulation of phage DNA replication resulted in impairment of *stx* expression (25), we tested effects of PEITC on the latter process.

TABLE 4 MIC₅₀ of PEITC for *E. coli* MG1655 growth in a minimal medium supplemented with 20 mM amino acid

Amino acid present in the medium at 20 mM	PEITC MIC ₅₀ (μ M) ^a
None	<15
Alanine	<15
Arginine	40
Asparagine	<15
Aspartic acid	<15
Cysteine	<15
Glutamic acid	<15
Glutamine	<15
Glycine	50
Histidine	<15
Isoleucine	<15
Leucine	<15
Lysine	15
Methionine	15
Phenylalanine	15
Proline	<15
Serine	15
Threonine	15
Tryptophan	<15
Tyrosine	<15
Valine	<15

^a Presence of 20 mM asparagine, leucine, tyrosine, or valine caused inhibition of the culture growth even in the absence of PEITC.

FIG 5 Restoration of PEITC-treated *E. coli* culture growth by addition of amino acids to the growth medium. *E. coli* MG1655 was grown in a minimal medium at 37°C either untreated (closed circles) or in the presence of 20 μ M PEITC, added at time zero (open circles). At 60 min, the PEITC-treated culture was divided into 6 parts, which were further cultured either under the same conditions (open circles) or supplemented with different amino acids (20 mM each): L-glycine (closed triangles), L-arginine (closed inverted triangles), or L-cysteine, L-alanine, or L-proline (the last three experiments are shown by closed squares, as results were very similar; the differences were smaller than the symbols). The results presented are mean values from 3 independent experiments, with error bars indicating SD (error bars are not shown when they are smaller than the symbols).

A derivative of phage 933W, bearing a gene coding for GFP instead of *stx*, has been employed, and its expression was estimated using fluorescence microscopy. As expected, we found that mitomycin C-mediated prophage induction in the wild-type host caused both filamentation of cells (which is characteristic for intracellular phage lytic development) and effective expression of GFP (Fig. 6). Simultaneous treatment with PEITC efficiently alleviated cell filamentation and reduced GFP synthesis to an undetectable level (Fig. 6). A strong filamentation phenotype (suggesting enhanced efficiency of phage lytic development) and massive GFP production was observed in ppGpp⁰ mutants treated with mitomycin. Although these mutants did not form filaments after combined treatment with mitomycin C and PEITC, they produced considerable amounts of GFP (Fig. 6, white arrows). We conclude that phage development and expression of *stx* genes are impaired by PEITC in a ppGpp-dependent manner (note that similar results were obtained in bacteria treated with mitomycin C and SHX [Fig. 6]).

When prophage induction occurs in *E. coli* capable of Shiga toxin production, lysates of such bacterial cultures are toxic for human and simian cells (HeLa or Vero cell lines are routinely used in such tests [41]). Therefore, we tested whether PEITC can alleviate the toxicity of lysates of induced lysogens of STEC (the O157:H7 strain bearing a prophage ST2-8624 with native *stx* genes). We have chosen conditions of prophage induction (by mitomycin C), preparation of bacterial culture lysate, and treatment of HeLa or Vero cell cultures that caused a decrease of viability of human or simian cells by about 50% (Fig. 7). We found that treatment of bacterial cells with PEITC together with mitomycin C caused a statistically significant alleviation of toxicity of lysates to human and simian cells at the isothiocyanate concentrations of 5 and 10 μ M and a complete abolition of toxicity at 20 μ M (Fig. 7). Effects similar to those caused by PEITC were observed when the stringent control was induced by addition of SHX. Ad-

FIG 6 PEITC and SHX inhibit *stx* gene expression in a ppGpp-dependent manner in a STEC strain. The effects of prophage induction and addition of PEITC or SHX on cells of wild-type (WT, MG1655) and ppGpp⁰ (*relA spoT*) hosts lysogenic with phage 933W Δ *stx*₂::*catGFP* were assessed. Bacteria were cultivated in LB medium at 37°C. Prophage induction was provoked by addition of mitomycin C up to 1 μ g/ml to the bacterial culture at time zero. Serine starvation was caused by addition of serine hydroxamate (SHX) up to 0.5 mg/ml at time zero. PEITC (50 μ M) was added at time zero. The presence (+) or absence (–) of each of these agents in a particular sample is indicated above microscopic pictures showing representative results. Samples were withdrawn 3 h after addition of the indicated compound(s). Cell membranes were stained with SynaptoRed/FM4-64 (panels marked as Membrane), and DNA was stained with DAPI (Chromosome). GFP fluorescence [Toxin (GFP)] corresponds to the strength of transcription and translation signals for expression of *stx* genes in native phages. Panels with combined (merged) pictures are labeled Merge. Arrows indicate production of GFP, which corresponds to activity of signals for expression of *stx* genes, in the *relA spoT* mutant treated with mitomycin C and either PEITC or SHX. All microphotographs were made with the same magnification and are presented on the same scale; white bar, 10 μ m.

ditional control experiments confirmed that the observed abolition of toxicity of bacterial lysates was specific to PEITC and SHX (see Fig. S2 in the supplemental material). Effects of PEITC and SHX on GFP production in the otherwise isogenic strain but bearing the ST2-8624 Δ *stx*₂::*cat GFP* prophage were analogous to those observed in the host lysogenic with 933W Δ *stx*₂::*cat GFP* (compare Fig. 6 and 8). Therefore, we conclude that PEITC is able to abolish the toxic effects caused by STEC on human and simian cells.

DISCUSSION

Infections by *E. coli* strains producing Shiga toxins are a serious medical problem, not only because of a relatively high morbidity and mortality among untreated patients but also due to a lack of efficient treatment that could prevent development of severe complications (1–8). In many countries, the use of antibiotics is not recommended if infection with EHEC is confirmed or even suspected, since various antibiotics may stimulate induction of Shiga toxin-converting prophages, enhancing production of the toxin and increasing the severity of the disease symptoms (reviewed in references 3 and 10). Despite such recommendations, there are still contradictory opinions about a possible use of antibiotics for treatment of patients infected with EHEC (or more generally, STEC). Although increased production of Shiga toxins has been demonstrated after antibiotic treatment of STEC strains cultured under laboratory conditions (reviewed in references 17, 20, 22, and 23), it is worth noting that the vast majority of such experi-

ments were performed with the use of subinhibitory concentrations. For example, concentrations between 1/2 and 1/64 MIC were used to demonstrate enhanced *stx* gene expression and/or release of Shiga toxins following addition of norfloxacin, ciprofloxacin, ampicillin, levofloxacin, streptomycin, chloramphenicol, and erythromycin (42–46). Moreover, in similar experiments, some antibiotics (e.g., rifaximin, gentamicin, cefotaxime, fosfomycin, and kanamycin) were found to decrease, rather than increase, the toxin production (43, 44). The effects of some antibiotics were strongly dependent on their concentrations, causing either an increase or decrease in efficiency of Shiga toxin production (44–46). In this light, it is important to note that it was reported recently that STEC strain O104:H4, which caused a recent outbreak in Germany, did not release Shiga toxin in response to therapeutic concentrations of ciprofloxacin, meropenem, fosfomycin, and chloramphenicol (47).

Definitely, different classes of antibiotics differentially influence Shiga toxin production (48), and effects of particular antibiotics are dependent on their concentrations. However, such a conclusion complicates the problem rather than solves it. For example, when considering the use of a particular antibiotic for treatment of an EHEC-infected patient, one should consider that—in contrast to laboratory conditions—after administration, the drug concentration at the target tissue or organ does not reach its therapeutic threshold immediately, and perhaps its subinhibitory levels can last for certain periods of time. Without extensive

FIG 7 Effects of PEITC and SHX on viability of human and simian cells treated with lysates of the EHEC strain (after Shiga toxin prophage induction). HeLa (A and B) and Vero (C and D) cell lines were treated with lysates of *E. coli* strain 86-24 (lysogenic with Shiga toxin-converting prophage ST2-8624, bearing wild-type *stx* genes) prepared from cultures: untreated (control), treated with 1 μ g/ml mitomycin C (to induce the prophage) and either various concentrations of PEITC (as indicated) or 0.5 mg/ml SHX. The viability of human and simian cells was assessed as described in Materials and Methods. Results shown in panels A and C represent mean values from 3 independent experiments assayed in triplicate (with error bars indicating SD). Statistical significance values, obtained in the *t* test, are shown as *P* values, and the statistical significance values of one-way ANOVA with Bonferroni's *post hoc* test are shown (***, $P < 0.001$). Panels B and D show representative micrographs from experiments labeled like those in panels A and C, respectively. White bars, 100 μ m.

studies, it would be difficult to estimate if this might be sufficient for induction of Shiga toxin-converting prophage and for effective production of the toxin.

Another problem is a lack of sufficient data from clinical studies on the effects of antibiotic therapies on patients suffering from EHEC infection. Most of the published results are either inconclusive regarding this dilemma (49) or intriguing (50). In the latter case, it is interesting that contrary to U.S. Food and Drug Administration guidelines, the Ministry of Health and Welfare of Japan recommended early administration of fosfomycin, norfloxacin, and kanamycin for medical treatment of STEC infection. Despite this recommendation, the incidence of HUS in children infected with STEC was not higher in Japan than in other countries (50, 51). Therefore, the controversy as to whether the use of antibiotics in treatment of STEC-infected patients can be effective or harmful still exists.

In the light of this controversy, it is worth mentioning that a common mechanism for cellular death induced by bactericidal antibiotics, which is based on stimulation of production of highly

deleterious hydroxyl radicals in bacterial cells, has been proposed (52). Such a mechanism includes generation of DNA breaks in bacterial chromosomes that can be mediated by antibiotic-stimulated intracellular oxidation of nucleotides (53). This might provide an interesting explanation for increased production of Shiga toxins in antibiotic-treated patients suffering from STEC infections, especially in the light of reports indicating that hydrogen peroxide may be the major factor responsible for Shiga toxin-converting prophage induction in human intestine (54, 55). Although it has been recently questioned whether antibiotic-mediated production of hydroxyl radicals in bacterial cells is a common mechanism for killing bacteria by bactericidal compounds (56, 57), direct or indirect stimulation of the bacterial SOS response upon treatment with antibiotics and the resultant prophage induction are an unquestioned fact. Therefore, finding potential factors or agents that would inhibit STEC growth and at the same time prevent either lambdoid prophage induction or phage development is highly desirable.

FIG 8 PEITC and SHX inhibit *stx* gene expression in the EHEC strain. The effects of prophage induction and addition of PEITC or SHX on O157:H7 strain lysogenic with phage ST2-8624 $\Delta stx_2::catGFP$ were assessed. Bacteria were cultivated in minimal medium at 37°C. Prophage induction was caused by addition of mitomycin C up to 1 μ g/ml to bacterial culture at time zero. Serine starvation was caused by addition of serine hydroxamate (SHX) up to 0.5 mg/ml at time zero. PEITC (20 μ M) was added at time zero. The presence (+) or absence (–) of each of these agents in a particular sample is indicated above the microscopic pictures, showing representative results. Samples were withdrawn 4 h after addition of indicated compound(s). Cell membranes were stained with Synptored/FM4-64 (panels labeled Membrane), and DNA was stained with DAPI (Chromosome). GFP fluorescence [Toxin (GFP)] corresponds to the strength of expression of *stx* genes in native phages. Panels with merged pictures are labeled Merge. All microphotographs were made with the same magnification and are presented on the same scale; white bar, 10 μ m.

In this report, we demonstrate that PEITC can severely impair both STEC growth and lytic development of Shiga toxin-converting bacteriophage. Abolition of the phage development upon hydrogen peroxide-mediated prophage induction may be of particular importance since it is likely that this agent plays a crucial role in such a process occurring in STEC-infected human intestine (54, 55, 58). PEITC also caused inhibition of *stx* expression and abolition of toxicity to human and simian cells of lysates of STEC cultures treated with mitomycin C, a prophage-inducing agent. We found that PEITC did not provoke production of reactive oxygen species in bacterial cultures growing under conditions employed in our experiments (see Fig. S3 in the supplemental material).

Three lines of evidence led to the conclusion that PEITC affects *E. coli* growth, development of Shiga toxin-converting prophages, and expression of *stx* genes due to induction of the stringent response. First, the above-mentioned effects were not observed in mutants unable to produce ppGpp. Second, synthesis of stable RNA species is inhibited in PEITC-treated cells only if they are

able to produce ppGpp. Third, addition of PEITC to an *E. coli* culture caused a rapid increase in the amounts of pppGpp and ppGpp, to levels comparable with those observed in amino acid-starved cells. It is likely that the stringent response induction is due to direct interactions of PEITC with particular amino acids and out-titration of these protein synthesis precursors. Such interactions with amino acids were described previously for other isothiocyanates (40), and we demonstrated that excess of glycine or arginine could restore growth of bacterial cultures treated with PEITC. Moreover, *E. coli* growth inhibition required significantly lower PEITC concentrations in a minimal medium that is devoid of amino acids (see Fig. S1 in the supplemental material) than in LB (Fig. 1), which contains amino acids and peptides. Under conditions employed in our experiments, we could not observe any significant effects of PEITC on membrane integrity (Fig. 6 and 8), which was suggested previously as a mechanism for the antibacterial action of another, structurally related isothiocyanate (32). We do not suggest an absence of PEITC-mediated changes in

membranes, but we conclude that such a mechanism is not essential for inhibition of *E. coli* growth, Shiga toxin-converting prophage development, and *stx* gene expression under the conditions employed in this work.

Previous studies indicated that Shiga toxin-converting bacteriophages cannot develop efficiently under conditions of amino acid deprivation (24). A subsequent report demonstrated that this developmental inhibition is due to ppGpp-dependent negative regulation of bacteriophage DNA replication, which resulted also in inefficient expression of *stx* genes (25). It is therefore likely that the mechanism of PEITC-mediated impairment of phage development and production of Shiga toxin is based on inhibition of phage DNA replication that is caused by ppGpp as an effect of induction of the stringent response by this isothiocyanate.

It is tempting to speculate that compounds inducing the stringent response and thus inhibiting expression of *stx* genes in STEC, like PEITC, might be considered as potential anti-EHEC drugs. From this point of view, it is worth to note that although selection of cells deficient in ppGpp production is likely, such mutants were found to survive very poorly under conditions simulating a natural environment (59). Therefore, propagation of PEITC-resistant mutants in their natural habitats seems rather unlikely.

It is intriguing that pathogenicity of STEC is significantly decreased by ppGpp (as demonstrated in this report), while some previous works (by others) demonstrated positive regulation of virulence factors in different bacteria by this nucleotide. For example, the stringent response alarmone is required for production of pneumolysin toxin in *Streptococcus pneumoniae*, quorum-sensing-dependent infection by *Pseudomonas aeruginosa*, adhesion and biofilm formation by *Listeria monocytogenes*, regulation of pathogenicity island expression in *Salmonella enterica* serovar Typhimurium, survival of *Helicobacter pylori* during infection, and survival of *Mycobacterium tuberculosis* (26, 60). Moreover, ppGpp-deficient mutants of many pathogenic bacteria have serious virulence defects, including STEC, which requires ppGpp for expression of genes coding for proteins involved in intestine colonization (26, 60, 61). Thus, it appears that ppGpp may be either a positive or negative regulator of virulence, depending on particular features and origins of bacterial virulence, i.e., whether they are bacterial pathogenicity islands or bacteriophage-derived genes. On the other hand, it is worth noting that conclusions on involvement of ppGpp in bacterial virulence came from studies employing (i) mutants unable to produce this nucleotide, (ii) measurement of expression of particular genes in (rather than measurement of virulence of) starved bacteria, and (iii) *in vitro* transcription systems (summarized in reference 60). To study the effects of increased intracellular ppGpp levels on virulence, cultures of bacteria entering stationary phase, rather than those expressing the “true” stringent control (defined as a response to complete amino acid deprivation), were investigated (61). Although those studies demonstrated an enhanced adherence capacity of EHEC and increased levels of gene expression in the locus of enterocyte effacement under conditions of either a downshift in nutrients or entry into the stationary growth phase (when levels of ppGpp were increased a few times), the virulence levels of these bacteria were not tested during the stringent response (i.e., at intracellular ppGpp concentrations increased by about 2 orders of magnitude) (61). Therefore, it seems likely that under natural conditions, virulence of various bacterial pathogens requires moderate (at most a few times higher than the basal level), rather

than highly elevated, intracellular levels of ppGpp. In fact, under laboratory conditions, moderate ppGpp concentrations are found in the stationary growth phase, while concentrations of this nucleotide over 100-fold higher than the basal level occur during the stringent response, when bacterial growth is severely inhibited. Therefore, if a putative treatment of STEC-mediated or other infectious diseases with compounds (like PEITC) provoking intracellular production of ppGpp is considered, the efficiency of the stringent response induction should be high enough to both inhibit bacterial growth and avoid virulence expression. On the other hand, any attempts to develop an antibacterial drug interfering with ppGpp production should take into consideration the fact that the absence of this nucleotide in STEC cells leads to enhancement of expression of genes coding for Shiga toxins.

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