The effect of two ribonucleases on the production of Shiga toxin and stx-bearing bacteriophages in Enterohaemorrhagic Escherichia coli

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Enterohaemorrhagic Escherichia coli (EHEC) comprise a group of intestinal pathogens responsible for a range of illnesses, including kidney failure and neurological compromise. EHEC produce critical virulence factors, Shiga toxin (Stx) 1 or 2, and the synthesis of Stx2 is associated with worse disease manifestations. Infected patients only receive supportive treatment because some conventional antibiotics enable toxin production. Shiga toxin 2 genes (stx2) are carried in λ-like bacteriophages (stx2-phages) inserted into the EHEC genome as prophages. Factors that cause DNA damage induce the lytic cycle of stx2-phages, leading to Stx2 production. The phage Q protein is critical for transcription antitermination of stx2 and phage lytic genes. This study reports that deficiency of two endoribonucleases (RNases), E and G, significantly delayed cell lysis and impaired production of both Stx2 and stx2-phages, unlike deficiency of either enzyme alone. Moreover, scarcity of both enzymes reduced the concentrations of Q and stx2 transcripts and slowed cell growth.

Enterohaemorrhagic Escherichia coli (EHEC) comprise a group of enteric pathogens that cause a spectrum of clinical manifestations, including diarrhea, bloody diarrhea or the more serious hemolytic uremic syndrome (HUS), and multi-organ disease1. A critical virulence trait of EHEC is the production of Shiga toxin2,3. Stx primarily targets renal microvascular endothelial cells, but other cell types such as neurons are also susceptible4,5. There is no available antidote to neutralize the toxin, which inhibits protein synthesis by cleaving the N-glycosidic bond at adenine 4324 in 28S ribosomal RNA6.

There are two immunologically distinct Stx types, Stx1 and Stx2. The Stx1 and Stx2 types are further classified into three and seven known subtypes, respectively7. The presence of Stx2-encoding genes (stx2 genes) and the production of Stx2 have been linked to worse disease outcomes in animal models and epidemiological studies8–11. In addition, the Stx2a subtype is highly associated with the development of HUS12. The stx2 genes are located in the genome of resident lambdoid prophages, which share a similar developmental cycle with the λ phage of nonpathogenic E. coli13,14. The stx2-harboring phages (stx2-phages) usually exist as prophages, but exposure to DNA-damaging agents, such as mitomycin C (MMC), induces the initiation of the lytic growth cycle15–17. After DNA damage occurs, the cell DNA damage response is triggered, resulting in an increase in the transcription of the host recA gene18,19 and the formation of single-stranded DNA-RecA complexes. These complexes mediate the self-cleavage of the phage transcriptional repressor cl20, resulting in transcription of additional phage genes, among them, the gene Q21. The Q protein acts as a transcriptional antiterminator that allows read-through of a terminator leading to stx2 expression21. In the final step of the lytic cycle, the bacterial cells lyse and release phage particles and toxin outside the cell22. While Stx reaches systemic circulation and the target organs, the stx phages can potentially infect phage-sensitive bacteria, contributing to the dissemination of stx genes to new hosts23–26.

The prevention of EHEC transmission is currently the best strategy to avoid infection and disease27. The administration of conventional antibiotics is not recommended, and available treatment options rely on fluids, electrolyte management, and dialysis in cases of kidney failure28–30. Some antibiotics (e.g., quinolones, trimethoprim-sulfamethoxazole) induce the stx-phage lytic cycle and high Stx production31–33. However, in vitro and
in vivo studies indicate that other antibiotics (e.g., rifaximin, azithromycin) do not trigger the synthesis of toxin in certain Stx-producing E. coli. Enzymes involved in RNA turnover, such as the well-characterized endoribonuclease (RNase) E, influence the expression of virulence traits and have been proposed as potential antibacterial targets. RNase E is present in Gram-negative bacteria and has a central role in degradation of mRNAs, cleavage of precursor rRNAs and tRNAs, and degradation or cleavage of small regulatory RNAs. The RNase E N-terminal catalytic domain is essential and shares about 32% sequence identity with its homolog RNase G. In contrast to RNase E, RNase G is dispensable for viability and controls the stability of a limited number of mRNAs. However, RNase G may have a global role in mRNA stability in some bacteria other than E. coli. Because RNase E is required for viability, advances concerning its role in virulence have been hampered. To overcome this impediment, we previously reported the construction of an EHEC strain in which RNase E synthesis is controlled by addition of a chemical (isopropyl β-D-1-thiogalactopyranoside [IPTG]) to the culture medium. Depletion of RNase E produced lower stx2-phage yields and an initial delay in Stx2 production; however, final Stx2 concentrations were as high as the control. In this study, the RNase G-encoding gene (rng) was deleted and its effect on the production of stx2-phages and Stx2 was examined under normal and deficient RNase E backgrounds.

**Results**

**Growth profile of EHEC with deficiency in RNase E, RNase G, or both enzymes in MMC-treated cultures.** The RNase G-encoding gene (rng) was deleted in the EHEC strains TEA028 (parental strain) and its RNases E and G derivatives to generate the strains TEA028-Δrng and TEA028-rne-Δrng, respectively (Supplementary Table S1). RNase E is IPTG-inducible in TEA028-rne, producing normal levels of RNase E in medium supplemented with 100 µM IPTG, but low levels of RNase E at or below 1 µM IPTG. Because RNase E is required for viability, advances concerning its role in virulence have been hampered. To overcome this impediment, we previously reported the construction of an EHEC strain in which RNase E synthesis is controlled by addition of a chemical (isopropyl β-D-1-thiogalactopyranoside [IPTG]) to the culture medium. Depletion of RNase E produced lower stx2-phage yields and an initial delay in Stx2 production; however, final Stx2 concentrations were as high as the control. In this study, the RNase G-encoding gene (rng) was deleted and its effect on the production of stx2-phages and Stx2 was examined under normal and deficient RNase E backgrounds.

To induce the stx2-phage lytic cycle, cultures of the TEA028 parental strain and its RNase E and G derivatives were treated with subinhibitory concentrations of MMC (1 µg/mL). The strains were grown to optical density at 600 nm (OD600) of 0.30–0.35 (time 0), and then MMC was added to an aliquot of the cultures. Thereafter, the turbidity of the cultures was determined at various time intervals (Supplementary Fig. S2). The difference of turbidity readings between MMC-treated and non-treated cultures is a measure of cell lysis progression after MMC addition due to activation of the phage lytic cycle. As reported previously, RNase E deficiency resulted in slower rate of lysis (Fig. 1, compare TEA028 cells vs. TEA028-rne cells at 0.1 µM IPTG). The absence of RNase G in RNase E-deficient cells (TEA028-rne-Δrng at 0.1 µM IPTG) provoked a substantial delay in cell lysis; in contrast, the absence of RNase G alone (TEA028-Δrng) had no effect. Complementation of the rng deletion with the rng gene expressed from a plasmid in the TEA028-rne-Δrng (prng) strain resulted in a similar rate of cell lysis to TEA028-rne.

Supplementation with 100 µM IPTG did not affect significantly the response to MMC treatment of the strains TEA028 or TEA028-Δrng (Supplementary Fig. S3). Likewise, the strains TEA028-rne at 100 µM IPTG (normal
versus TEA028-rne study50; however, plaques of phages, which were detected by plaque hybridization under the same experimental conditions in a previous E and G derivatives after MMC-treatment for 6 h. After treatment with MMC, EHEC TEA028 produces stx2Δrng of TEA028-rne eightfold difference in the mean of Stx2 levels between cell lysates of the control (TEA028) at 6 h and cell lysates 1.7-fold at 10 h and 2.2-fold at 24 h when compared with the 6-h time point. However, there was still about 0.1 µM IPTG resulted in 245 ± 36 ng/mg of protein (mean ± standard error) contrasting with the production of 4,677 ± 173 ng/mg of protein (mean ± standard error) by TEA028. Because of the slower kinetics of growth and impaired the production of infectious phages in agreement with our previous findings (Fig. 2) while TEA028-rne-Δrng (p rng) reduced phage yields even further, whereas the absence of only RNase G caused no effect. Complementation of the rng deletion partially restored the production of phages (TEA028-rne-Δrng (p rng) vs. TEA028-rne-Δrng at 0.1 µM IPTG).

**Figure 2.** Production of phages by EHEC containing normal levels of RNases E and G versus derivative strains deficient in either or both enzymes. Cultures of TEA028 (parental strain) and its RNases E and G derivatives were grown in LB medium to an optical density at 600 nm of 0.30–0.35 at which point the cultures were treated with MMC (1 µg/mL) to induce the phage lytic cycle. Supernatants were collected at 6 h, and phage titers were determined by the double agar assay as described in “Methods” section. The strains TEA028-rne, TEA028-rne-Δrng, and TEA028-rne-Δrng (prng) underproduce RNase E when the medium is supplemented with 0.1 µM IPTG. Means and standard errors of 3–4 biological replicates are shown. Abbreviations: rng: TEA028-Δrng; rne: TEA028-rne; rne-rng: TEA028-rne-Δrng; rne-rng (prng): TEA028-rne-Δrng (prng); p.f.u.: plaque forming units. * Adjusted P value versus TEA028 = 0.0095; ** adjusted P value versus TEA028 = 0.0001; # adjusted P value versus TEA028-rne = 0.0007; & adjusted P value versus TEA028-rne-Δrng = 0.0015.

levels of RNase E) and the parental strain TEA028 behaved similarly after MMC addition (Supplementary Fig. S4) in agreement with our previous results59.

The delayed kinetics of cell lysis after induction of the phage lytic cycle suggested that deficiency of RNases E and G could impair the production of stx2-phages, Stx2, or both.

**Production of stx-phages in EHEC with deficiency in RNase E, RNase G, or both enzymes in MMC-treated cultures.** Phage yields were determined in culture supernatants of TEA028 and its RNases E and G derivatives after MMC-treatment for 6 h. After treatment with MMC, EHEC TEA028 produces stx2-phages, which were detected by plaque hybridization under the same experimental conditions in a previous study50; however, plaques of stx1-phages were not detected. RNase E deficiency (TEA028-rne at 0.1 µM IPTG) impaired the production of infectious phages in agreement with our previous findings (Fig. 2) while TEA028-rne at 100 µM IPTG (normal RNase E levels) produced similar phage yields to the control (Supplementary Fig. S4). Deficiency of both RNase E and RNase G (TEA028-rne-Δrng at 0.1 µM IPTG) reduced phage yields even further, whereas the absence of only RNase G caused no effect. Complementation of the rng deletion partially restored the production of phages (TEA028-rne-Δrng (p rng) vs. TEA028-rne-Δrng at 0.1 µM IPTG).

**Production of toxin in EHEC with deficiency in RNase E, RNase G, or both enzymes in MMC-treated cultures.** Next, Stx2 production was measured in extracts prepared from MMC-treated cultures of TEA028 and its RNase E and RNase G derivatives. Specifically, the EHEC strain TEA028 and its derivatives produce Stx2a subtype. At 6 h after MMC addition, lysates from the strains lacking RNase G (TEA028-Δrng) or underproducing RNase E (TEA028-rne at 0.1 µM IPTG) had toxin levels that were not significantly different from TEA028 (control) (Fig. 3). Similarly, strain TEA028-rne at 100 µM IPTG (normal RNase E levels) produces toxin as the control (Supplementary Fig. S4, and Ref. 49). Deficiency of RNase E and G (TEA028-rne-Δrng at 0.1 µM IPTG) resulted in 245 ± 36 ng/mg of protein (mean ± standard error) contrasting with the production of 4,677 ± 173 ng/mg of protein (mean ± standard error) by TEA028. Because of the slower kinetics of growth and lysis in TEA028-rne-Δrng at 0.1 µM IPTG, Stx2 was also measured in cell extracts at later time points (10 and 24 h) after MMC addition. Toxin production by TEA028 was not measured beyond the 6-h period because at this point cells are mostly lysed, and thus, a small increment or no increment in Stx2 accumulation would be expected at later time points. In cultures of TEA028-rne-Δrng at 0.1 µM IPTG, toxin accumulation increased 1.7-fold at 10 h and 2.2-fold at 24 h when compared with the 6-h time point. However, there was still about eightfold difference in the mean of Stx2 levels between cell lysates of the control (TEA028) at 6 h and cell lysates of TEA028-rne-Δrng at 0.1 µM IPTG at 24 h. For comparison, Stx2 concentrations under RNase E scarcity alone (TEA028-rne at 0.1 µM IPTG) were still high at 10 h. Complementation of the rng deletion in the TEA028-rne-Δrng (p rng) strain, partially restored Stx2 production at 6, 10, and 24 h after MMC addition. Partial complemen-
levels, and fold changes were above 1 at all time points in most of the experiments (Fig. 4e, h). In particular, complementary Fig. S7a, S7b, and S7c). Depletion of RNase E or of RNases E and G did not reduce recA transcript levels in nonpathogenic E. coli strains, with high levels at 30 min and falling afterwards to a fold change below 1 in some experiments (Supplementary Fig. S7c). Under RNase E deficiency, high transcript levels were observed at 2.5 h when compared with the control (TEA028) (Fig. 4a, g).

Quantification of mRNA levels of the stx2, recA, and Q mRNAs in EHEC and its RNases E and G derivatives. Reverse transcription real-time PCR (RT-qPCR) was used to examine whether the reduction in Stx2 levels under RNases E and G deficiency could be explained by a concomitant reduction in stx2 mRNA levels. In addition, recA and Q transcript levels were also examined since expression of these genes is essential for initiation of the stx2 phage lytic cycle and Stx2 production when DNA damage occurs. Cultures of each strain were grown to OD600 of 0.3–0.35 (time 0) at which point MMC (1 µg/mL) was added; thereafter, aliquots were collected at the indicated time intervals, and toxin concentrations were determined in whole cell lysates. The strains TEA028, TEA028-rne, TEA028-rne-Δrng, and TEA028-rne-Δrng (prng) underproduce RNase E when the medium is supplemented with 0.1 µM IPTG. Means and standard errors of at least 4 biological replicates are graphed. Abbreviations: rng: TEA028-Δrng; rne: TEA028-rne; rne rng: TEA028-rne-Δrng; rne rng (prng): TEA028-rne-Δrng (prng). * Adjusted P value versus TEA028 = 0.0001; ** adjusted P value versus TEA028 < 0.0001; & adjusted P value versus TEA028 at 10 h = 0.0001; & adjusted P value versus TEA028-rne at 10 h = 0.0024.

In the TEA028 strain, the stx2B transcript reached high levels 1 h after the addition of MMC (Fig. 4a). Similar stx2B mRNA kinetics were observed in strains TEA028-rne at 100 µM IPTG (Supplementary Fig. S7b) and TEA028-Δrng (Supplementary Fig. S7c). Under RNase E deficiency, high transcript levels were observed at 2.5 h and later time intervals, after an initial delay (Fig. 4d). In contrast, deficiency of both RNases reduced significantly the levels of stx2 transcripts, with 50-fold decrease at 2.5 h when compared with the control (TEA028) (Fig. 4a, g). The recA transcripts showed similar kinetics in the TEA028, TEA028-rne at 100 µM IPTG, and TEA028-Δrng strains, with high levels at 30 min and falling afterwards to a fold change below 1 in some experiments (Supplementary Fig. S7a, S7b, and S7c). Depletion of RNase E or of RNases E and G did not reduce recA transcript levels, and fold changes were above 1 at all time points in most of the experiments (Fig. 4e, h). In particular, recA transcript levels stayed significantly elevated at three time points under RNases E and G scarcity when compared with the control (TEA028) (Fig. 4b, h).

The kinetics of Q transcript levels were similar in TEA028, TEA028-rne at 100 µM IPTG, and TEA028-Δrng (Supplementary Fig. S7a, S7b, and S7c), resembling the expression pattern of stx2 mRNA. Under RNase E scarcity, there was a slight delay in the Q mRNA peak, but the levels were as high as in the parental strain at later time points (Fig. 4f). Under RNases E and G deficiency, the Q transcript levels were significantly lower at all time points with about 42-fold reduction at 2.5 h when compared with TEA028 (Fig. 4c, i). These findings indicate...
that RNases E and G scarcity significantly impaired Q expression, which could have contributed to reduction in stx2 transcription and Stx2 production.

**Determination generation times in EHEC and its RNases E and G derivative strains.** In addition to the reduction in Q transcript levels, the impairment of Stx2 production under RNases E and G deficiency may be an indirect consequence of increased generation times from the burden of slowed RNA metabolism. In support of this hypothesis, the generation time was significantly increased in TEA028-rne-Δrng at 0.1 µM IPTG when compared with the control or deficiency of RNase E alone (Supplementary Fig. S8).

**Discussion**
This study establishes that depletion of RNases E and G significantly impair the production of both Stx2 and stx2-phages in EHEC. In contrast, the absence of RNase G does not impair the production of either toxin or phages, and deficiency of RNase E impairs phage production only (this study and Ref. 50). The inducing agent used in this
study, MMC, strongly triggers the cellular response to DNA damage. Thus, cells deficient in both RNases still undergo the initiation of the phage lytic cycle with concomitant toxin production although at a slower rate and resulting in lower Stx2 yields even at 24 h after MMC addition. RecA and Q proteins are critical for the initiation of the phage lytic cycle and toxin production. The reduced stx2 transcript levels under RNase E and G deficiency cannot be explained by a change in recA transcript levels, which remained higher than in the control. In contrast, RNase E and G scarcity caused a drastic reduction in transcript levels of the Q antiterminator, which is required for transcription to continue to stx2 genes. This result can explain, at least partially, the observed decrease in stx2 transcript concentrations. In addition, the impaired expression of Q could have affected the transcription of late genes required for the assembly of phage particles. The reduction of Q transcript levels could be the result of impaired Q gene transcription or transcript stability. If the latter scenario is correct, RNases E/G effect on Q mRNA lifetimes is probably indirect because they are required for Q transcripts to reach high levels.

Deficiency of both RNases likely creates metabolic burden by affecting the stability and processing of many RNAs. As a consequence, the growth rate is decreased, probably having an indirect effect on the production and release of toxin and phages. The mechanisms underlying these effects are probably many and complex since depletion of RNase E alone changes the half-life of thousands of transcripts in nonpathogenic E. coli55. In EHEC, direct RNase E or G degradation or processing of transcripts encoding virulence factors, such as Shiga toxin, has been scarcely explored37,38,54.

The importance of the production of actual phage particles in disease progression is debatable. Some studies indicate that infection of commensal bacteria by stx phages may amplify the production of toxin55,56, potentially worsening disease outcomes. However, subsequent work with a microbiome-replete murine model of EHEC infections indicates that the actual production of phage particles is negligible for disease57. Nevertheless, potential transmission of stx genes to naive bacterial hosts is problematic, as exemplified by the novel and highly virulent Stx2-producing E. coli serotype O104:H4 that caused an outbreak in Europe in 201158. Therefore, the reduction of both toxin and infective phage particles produced under RNases E and G scarcity is important.

Despite remarkable advances in the understanding of EHEC pathogenesis and its virulence factors, specific therapeutics against EHEC infections are still lacking. The neutralization of Stx by a binding agent (Ssynsorb P) was ineffective in a multicenter randomized controlled clinical trial, and the authors concluded that success of similar strategies was doubtful59. Although humanized monoclonal antibodies against Stx1 or Stx2 showed efficacy at controlling fatal complications in animal models60–63, clinical trials have not advanced beyond the determination of safety and pharmacokinetic parameters64–65. In the United States, antibiotics are contraindicated for treatment of Stx-producing E. coli infections24. This topic remains controversial since there are some conflicting data from in vitro, animal model and clinical studies (reviewed in66). Nevertheless, it is clear that the effect of antibiotics on Stx production depends on the antimicrobial compound and the particular Stx-producing E. coli strain. Given the complexity of the problem, the therapeutic potential of the findings reported here is unknown, at this moment. Inhibitors of purified RNase E/G have been isolated67,68; however, there are currently no reported inhibitors of those enzymes that can be tested in vivo, i.e., on the EHEC capacity to produce toxin.

**Methods**

**Strains and culture conditions.** Supplementary Table S1 describes the strains used in this study. For each experiment, the strains were freshly streaked on plates directly from glycerol stocks maintained at −70 °C. To prepare the inoculum, one colony was inoculated into Luria Bertani (LB) medium and incubated for 12–13 h at 37 °C with agitation (130 rpm). The cells were collected by centrifugation, resuspended in phosphate-buffered saline or LB, and inoculated into LB medium at a ratio 1:500. The cultures were incubated at 37 °C with agitation to OD600 of 0.30–0.35, at which point MMC (1 µg/mL) was added; thereafter, samples were collected at various time intervals. The LB medium was supplemented as follows: tetracycline (tet) (6 µg/mL) in the case of TEA028 and TEA028-Δrng strains; and tet (6 µg/mL), ampicillin (amp) (100 µg/mL), chloramphenicol (25 µg/mL), and isopropyl β-D-1-thiogalactopyranoside (IPTG) (100 µM or 0.1 µM) in the case of TEA028-rnc, TEA028-rnc-Δrng, TEA028-rnc-Δrng (prrg), and TEA028-rnc-Δrng (pGTG) strains.

**Oligonucleotides.** The oligonucleotide sequences used in this study are described in Supplementary Table S2.

**Construction of TEA028-Δrng and TEA028-rnc-Δrng strains.** The rng gene from strains TEA028 and TEA028-rnc was deleted following the gene-doctoring procedure69. The kanamycin (kan) resistance gene was amplified with primers f-EcoRI-rng and r-Xhol-rng (Supplementary Table S2) using plasmid pDOC-K as the template. The PCR fragment was cloned between the EcoRI and Xhol sites of plasmid pDOC-C (or pDOC-C-Gen [see below]), which carries an amp resistance marker, and the sacB gene that confers resistance to sucrose. The resulting plasmid was then introduced into the strain TEA028. Next, TEA028 was transformed with the plasmid pACBSCE, which carries the genes encoding for the λ-Red proteins and the gene encoding for the restriction enzyme I-SceI. Cultures were grown for 2 h at 37 °C, and then the cells were pelleted and resuspended in LB supplemented with 0.5% L-arabinose to induce the expression of λ-Red proteins. Appropriate dilutions were plated to select for kan resistant and sucrose insensitive recombinants. The colonies were also checked for loss of the donor plasmids. Next, the recombinants were screened with PCR primers CC1 and CC2 and with complementary primers to the rng gene flanking region as described by Lee et al.69. To eliminate the kan resistance marker inserted into the rng gene, the FLP recombinase was produced from the pCP20 plasmid (E. coli Genetic Stock Center), which was then eliminated by incubation at the restrictive temperature. To delete the rng gene in the TEA028-rnc strain, which is amp resistant, the pDOC-C plasmid was modified by introducing a gentamicin resistance gene (aacC1). The aacC1 gene was amplified with PCR primers f-SphI-gen and r-SphI-gen.
Construction of plasmid prng and site-directed mutagenesis. The prng gene was amplified using genomic DNA as template and primers f-XbaI-rng and r-HindIII-term-rng. The M13 transcriptional terminator, which has high termination efficiency, was added at the end of the primer r-HindIII-term-rng19. The prng sequence was cloned into XbaI and HindIII restriction sites of plasmid pLacI49 and confirmed by Sanger sequencing (Genewijz, Inc.). The resultant plasmid (prng) was introduced into the EHEC strain TEA028-rne-Δrng. The Q5 Site-Directed Mutagenesis Kit (New England Biolabs) was used to change the ATG start codon to GTG of the cloned rng gene into plasmid prng. The change in the rng sequence was confirmed by Sanger sequencing.

Shiga toxin quantification. Stx2 was quantified in whole cell extracts by the receptor ELISA technique (RELISA), as described in Supplementary Information.

Plaque assays. Culture supernatants were collected after centrifugation at 15,000 x g for 5 min, treated with one-tenth volume of chloroform, and then mixed with an equal volume of 4 M (NH4)2SO4 solution before storage at 4 °C. Phages were quantified by the double agar overlay method or the drop method as described by Thuraissamy and Lodato50. Plaque numbers were log-transformed before statistical analysis.

RNA isolation and mRNA quantification. Culture samples (3, 5, or 6 mL) were collected at 4 °C and added in a 10:1 ratio to a mixture of 95% ethanol and 5% saturated phenol. After centrifugation, the cell pellets were stored at −70 °C. To extract total RNA, the cell pellets were resuspended in 300 µL of lysis buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.5 mg/mL lysozyme), 20 µL of 20% SDS, and 20 µL of water. The lysate was incubated at 64 °C for 2 min, and then 1.2 mL of TRI Reagent solution (Invitrogen) was added. Next, total RNA was isolated with the Direct-zol RNA Miniprep kit (Zymo Research). On-column DNA digestion was performed during the RNA extraction following the manufacturer’s directions. RNA was eluted in water, and the concentration determined with a NanoDrop 2000c spectrophotometer. To digest remaining DNA contamination, an aliquot of each RNA sample was treated with TURBO DNase (Ambion) in a reaction mixture containing SUPERase-In RNase Inhibitor (Thermo Scientific). The total RNA was then repurified using saturated sodium acetate and ethanol.

The reactions (10 µL) were performed in a CFX Connect real-time detection system (Bio-Rad) using the iTaq Universal SYBR Green Supermix (Bio-Rad). The cycling protocol was 95 °C for 3 min and then 40 cycles at 95 °C for 5 s and 60 °C for 30 s. A melting curve analysis was completed afterwards to confirm the presence of only one product of amplification and the absence of primer dimers. The initial concentration of molecules (N0) was calculated using the open-source software LinReg71,72. Then, the N0 of stx2B, recA, Q, and rrsH genes were amplified by qPCR from appropriate dilutions of cDNA using the primers described in Supplementary Table S2. The reactions (10 µL) were performed in a CFX Connect real-time detection system (Bio-Rad) using the iTaq Universal SYBR Green Supermix (Bio-Rad). The cycling protocol was 95 °C for 3 min and then 40 cycles at 95 °C for 5 s and 60 °C for 30 s. A melting curve analysis was completed afterwards to confirm the presence of only one product of amplification and the absence of primer dimers. The initial concentration of molecules (N0) was calculated using the open-source software LinReg71,72. Then, the N0 of stx2B, recA, or Q cDNA was normalized to the N0 of the rrsH cDNA for each experiment. For each gene, the fold change was calculated as the ratio of the normalized N0 at each time point after MMC addition to the normalized N0 before the MMC addition (time 0). Fold changes were log-transformed before statistical analysis.

Statistical analysis. The results of this study are derived from at least three biological replicates. The statistical analysis was performed with the software GraphPad Prism 7.0. Groups were compared using one-way or two-way analysis of variance and Dunnett’s or Sidaik’s multiple comparison test, or a t-test when two groups were compared. Measurements of toxin, protein concentrations, and qPCR were performed in three technical replicates.

Data availability
Data are available from the author upon request.

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References
1. Joseph, A., Cointe, A., Mariani Kurkdjian, P., Rafat, C. & Hertig, A. Shiga toxin-associated hemolytic uremic syndrome: a narrative review. Toxins (Basel) 12, 67. https://doi.org/10.3390/toxins12020067 (2020).
2. O’Brien, A. O., Lively, T. A., Chen, M. E., Rothman, S. W. & Formal, S. B. Escherichia coli O157:H7 strains associated with haemorrhagic colitis in the United States produce a Shigella dysenteriae 1 (SHIGA) like cytotoxin. Lancet 1, 702 (1983).
3. Karmali, M. A., Steele, B. T., Petric, M. & Lim, C. Sporadic cases of haemolytic-uraemic syndrome associated with facel cytotoxin and cytotoxin-producing Escherichia coli in stools. Lancet 1, 619–620 (1983).
6. Endo, Y. et al. Site of action of a Vero toxin (VT2) from Escherichia coli O157:H7 and of Shiga toxin on eukaryotic ribosomes. *Eur. J. Biochem.* 171, 45–50 (1988).
7. Melton-Celsa, A. R. Shiga toxin (Stx) classification, structure, and function. *Microbiol. Spectr.* https://doi.org/10.1128/microbiolspec.EHEC-0004-2013 (2014).
8. Tesh, V. L. et al. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect. Immun.* 61, 3392–3402 (1993).
9. Donohue-Rolfe, A., Kondova, L., Oswald, S., Hutto, D. & Tzipori, S. *Escherichia coli* O157:H7 strains that express Shiga toxin (Stx) 2 alone are more neurotropic for gnotobiotic piglets than are isotypes producing only Stx1 or both Stx1 and Stx2. *J. Infect. Dis.* 181, 1825–1829 (2000).
10. Friedrich, A. W. et al. *Escherichia coli* harboring Shiga toxin 2 gene variants: Frequency and association with clinical symptoms. *J. Infect. Dis.* 185, 74–84 (2002).
11. Werber, D. et al. Strong association between Shiga toxin-producing *Escherichia coli* O157 and virulence genes stx1 and eae as possible explanation for predominance of serogroup O157 in patients with haemolytic uraemic syndrome. *Eur. J. Clin. Microbiol. Infect. Dis.* 22, 726–730 (2003).
12. Werber, D. & Scheutz, F. The importance of integrating genetic strain information for managing cases of Shiga toxin-producing *E. coli* infection. *Epidemiol. Infect.* 147, e264. https://doi.org/10.1017/S0950268819001602 (2019).
13. Plunkett, G. 3rd., Rose, D. J., Durfee, T. J. & Blattner, F. R. Sequence of Shiga toxin 2 phage 933W from *Escherichia coli* K12. *J. Bacteriol.* 181, 1767–1778 (1999).
14. Casjens, S. R. & Hendrix, R. W. Bacteriophage lambda: Early pioneer and still relevant. *Virology* 47, 289–298 (2009).
15. Mühldorfer, I. Identification of additional genes belonging to the *LeXa* regulon in *Escherichia coli*. *Mol. Microbiol.* 35, 1560–1572 (2000).
16. Ronen, M., Rosenberg, R., Shraiman, B. I. & Alon, U. Assigning numbers to the arrows: Parameterizing a gene regulation network by using accurate expression kinetics. *Proc. Natl. Acad. Sci. U. S. A.* 99, 15555–15560 (2002).
17. Koudelka, A. P., Hufnagel, L. A. & Koudelka, G. B. Purification and characterization of the repressor of the Shiga toxin-encoding bacteriophage binding, gene regulation, and autocleavage. *J. Bacteriol.* 186, 7659–7669 (2004).
18. Wagner, P. L., Acheson, D. W. & Waldor, M. K. Isogenic lysogens of diverse shiga toxin 2-encoding bacteriophages produce markedly different amounts of shiga toxin. *Infect. Immun.* 67, 6710–6714 (1999).
19. Lai, J. M., Lai, M., Wegryn, G. & Wegryn, A. Differential efficiency of induction of various lambda prophages responsible for production of Shiga toxin in response to different induction agents. *Microb. Pathog.* 47, 289–298 (2009).
20. De Henestrosa, A. R. F. & Obrig, T. Role of Shiga/Vero toxins in pathogenesis. *Microbiol. Spectr.* https://doi.org/10.1128/microbiolspec.EHEC-0005-2013 (2014).
21. Obata, F. & Obrig, T. Role of Shiga/VerO toxins in pathogenesis. *Microbiol. Spectr.* https://doi.org/10.1128/microbiolspec.EHEC-0005-2013 (2014).
22. Scott, S. & Pohlandt, F. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect. Immun.* 61, 3392–3402 (1993).
23. McGannon, C. M., Fuller, C. A. & Weiss, A. A. Different classes of antibiotics differentially influence Shiga toxin production. *Antimicrob. Agents Chemother.* 57, 3790–3798 (2010).
24. Silhavy, T. J., Berman, K. L. & Enns, H. L. *Bacillus subtilis and other Gram-Positive Bacteria: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1984).
25. Blattner, F. R. et al. The complete genome sequence of *Escherichia coli* K-12. *Science* 277, 1453–1462 (1997).
26. Pallen, M. J., Gough, J., Dimopoulos, R. & Parkinson, I. H. How we predict the function of protein-coding genes in bacteria. *Nat. Rev. Microbiol.* 10, 249–257 (2012).
27. Blair, C. G., Blattner, F. R., Metz, S. E., Caruthers, H. B. & McKenney, L. M. Sequencing the *Escherichia coli* K-12 genome. *Science* 260, 1453–1462 (1993).
28. Black, M., Black, J. & Black, B. *Escherichia coli* O:157–H7, a human pathogen: Pathogenesis and genetic variability. *FEMS Microbiol. Rev.* 24, 471–486 (2000).
29. Nierman, W. C. et al. The complete genome sequence of *Escherichia coli* O157:H7. *Science* 277, 1453–1462 (1997).
30. Sadowski, G. D., Blattner, F. R., Murray, R. W. & Taylor-Gray, F. A. Isolation and sequencing of the complete DNA sequence of *Escherichia coli* K-12. *Science* 277, 1453–1462 (1997).
31. Pallen, M. J., Gough, J., Dimopoulos, R. & Parkinson, I. H. How we predict the function of protein-coding genes in bacteria. *Nat. Rev. Microbiol.* 10, 249–257 (2012).
32. Schmitt, S. et al. The complete genome sequence of *Escherichia coli* O157:H7. *Science* 277, 1453–1462 (1997).
33. Blattner, F. R. et al. The complete genome sequence of *Escherichia coli* K-12. *Science* 277, 1453–1462 (1997).
34. Scott, S. & Pohlandt, F. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect. Immun.* 61, 3392–3402 (1993).
35. McGannon, C. M., Fuller, C. A. & Weiss, A. A. Different classes of antibiotics differentially influence Shiga toxin production. *Antimicrob. Agents Chemother.* 54, 3790–3798 (2010).
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mRNA metabolism in Escherichia coli. Genes Cells 6, 403–410 (2001).

Lee, J., Lee, D. H., Jeon, C. O. & Lee, K. RNase G controls tpA mRNA abundance in response to oxygen availability in Escherichia coli. J. Microbiol. 57, 910–917 (2019).

Lee, M. et al. The coordinated action of RNase III and RNase G controls enolase expression in response to oxygen availability in Escherichia coli. Sci. Rep. 9, 17257. https://doi.org/10.1038/s41598-019-35883-y (2019).

Bernardini, A. & Martínez, J. I. Genome-wide analysis shows that RNase G plays a global role in the stability of mRNAs in Stenotrophomonas maltophilia. Sci. Rep. 7, 16016. https://doi.org/10.1038/s41598-017-16091-0 (2017).

Lodato, P. B., Thuraisamy, T., Richards, J. & Belasco, J. G. Effect of RNase E deficiency on toxin protein synthesis in an RNase E-inducible strain of enterohemorrhagic Escherichia coli O157:H7. FEMS Microbiol. Lett. 364, fnx131. https://doi.org/10.1093/femsec/fnx131 (2017).

Thuraisamy, T. & Lodato, P. B. Influence of RNase E deficiency on the production of stx2-bearing phages and Shiga toxin in an RNase E-inducible strain of enterohemorrhagic Escherichia coli (EHEC) O157:H7. J. Med. Microbiol. 67, 724–732 (2018).

Deana, A. & Belasco, J. G. The function of RNase G in Escherichia coli is constrained by its amino and carboxyl termini. Mol. Microbiol. 51, 1205–1217 (2004).

Ringquist, S. et al. Translation initiation in Escherichia coli: Sequences within the ribosome-binding site. Mol. Microbiol. 6, 1219–1229 (1992).

Stead, M. B. et al. Analysis of Escherichia coli RNase E and RNase III activity in vivo using tiling microarrays. Nucl. Acids Res. 39, 3188–3203 (2011).

Gruber, C. C. & Sperandio, V. Posttranscriptional control of microbe-induced rearrangement of host cell actin. MBio 5, e01025-13. https://doi.org/10.1128/mBio.01025-13 (2014).

Gamage, S. D., Patton, A. K., Hanson, J. F. & Weiss, A. A. Diversity and host range of Shiga toxin-encoding phage. J. Infect. 72, 133–141 (2003).

Balasubramanian, S., Osburne, M. S., BrinJones, H., Tai, A. K. & Leong, J. M. Prophage induction, but not production of phage particles, is required for lethal disease in a microbe-replete murine model of enterohemorrhagic E. coli infection. PLoS Pathog. 15, e1007494. https://doi.org/10.1371/journal.ppat.1007494 (2019).

Rasko, D. A. et al. Origins of the E. coli strain causing an outbreak of hemolytic-uremic syndrome in Germany. N. Engl. J. Med. 365, 709–717 (2011).

Trachtman, H. et al. Effect of an oral Shiga toxin-binding agent on diarrhea-associated hemolytic uremic syndrome in children: A randomized controlled trial. JAMA 290, 1337–1344 (2003).

Mukherjee, J. et al. Human Stx2-specific monoclonal antibodies prevent systemic complications of Escherichia coli O157:H7 infection. Infect. Immun. 70, 612–619 (2002).

Shoaran, A. S. et al. Human antibody against shiga toxin 2 administered to piglets after the onset of diarrhea due to Escherichia coli O157:H7 prevents fatal systemic complications. Infect. Immun. 73, 4607–4613 (2005).

Moody, R. A. et al. Efficacy of urtoxazumab (TMA-15 humanized monoclonal antibody specific for Shiga toxin 2) against post-diarrheal neurological sequelae caused by Escherichia coli O157:H7 infection in the neonatal gnobiotic piglet model. Toxins (Basel) 9, 49. https://doi.org/10.3990/toxins9020049 (2017).

Dowling, T. C. et al. Phase 1 safety and pharmacokinetic study of chimeric murine-human monoclonal antibody c alpha Stx2 administered intravenously to healthy adult volunteers. Antimicrob. Agents Chemother. 49, 1808–1812 (2005).

Bitzan, M. et al. Safety and pharmacokinetics of chimeric anti-Shiga toxin 1 and anti-Shiga toxin 2 monoclonal antibodies in healthy volunteers. Antimicrob. Agents Chemother. 53, 3081–3087 (2009).

López, E. L. et al. Safety and pharmacokinetics of urtoxazumab, a humanized monoclonal antibody, against Shiga-like toxin 2 in healthy adults and in pediatric patients infected with Shiga-like toxin-producing Escherichia coli. Antimicrob. Agents Chemother. 54, 239–243 (2010).

Kakoullis, L., Papachristodoulou, E., Chra, P. & Panos, G. Shiga toxin-induced haemolytic uremic syndrome and the role of antibiotics: A global overview. J. Infect. 79, 75–94 (2019).

Kime, L. et al. The first small-molecule inhibitors of members of the ribonuclease E family. Sci. Rep. 5, 8028. https://doi.org/10.1038/srep08028 (2015).

Mardle, C. E. et al. Identification and analysis of novel small molecule inhibitors of RNase E: Implications for antibacterial targeting and regulation of RNase E. Biochem. Biophys. Rep. 23, 100773. https://doi.org/10.1016/j.bbrep.2020.100773 (2020).

Lee, D. J. et al. Gene doctoring: A method for recombining in laboratory and pathogenic Escherichia coli strains. BMC Microbiol. 9, 252. https://doi.org/10.1186/1471-2180-9-252 (2009).

Cambray, G. et al. Measurement and modeling of intrinsic transcription terminators. Nucl. Acids Res. 41, 5139–5148 (2013).

Ramakers, C., Ruiter, J. M., Deprez, R. H. & Mooiman, A. F. Assumption-free analysis of quantitative real-time polymerase chain reaction (qPCR) data. Neurosci. Lett. 339, 62–66 (2003).

Ruiter, J. M. et al. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. Nucl. Acids Res. 37, e45. https://doi.org/10.1093/nar/gkp045 (2009).

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Competing interests
The author declares no competing interests.
