Influence of RNase E deficiency on the production of stx2-bearing phages and Shiga toxin in an RNase E-inducible strain of enterohaemorrhagic Escherichia coli (EHEC) O157:H7

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Abstract

Purpose. In enterohaemorrhagic Escherichia coli (EHEC), stx1 or stx2 genes encode Shiga toxin (Stx1 or Stx2, respectively) and are carried by prophages. The production and release of both stx phages and toxin occur upon initiation of the phage lytic cycle. Phages can further disseminate stx genes by infecting naïve bacteria in the intestine. Here, the effect of RNase E deficiency on these two virulence traits was investigated.

Methodology. Cultures of the EHEC strains TEA028-rne containing low versus normal RNase E levels or the parental strain (TEA028) were treated with mitomycin C (MMC) to induce the phage lytic cycle. Phages and Stx2 titres were quantified by the double-agar assay and the receptor ELISA technique, respectively.

Results. RNase E deficiency in MMC-treated cells significantly reduced the yield of infectious stx2 phages. Delayed cell lysis and the appearance of encapsidated phage DNA copies suggest a slow onset of the lytic cycle. However, these observations do not entirely explain the decrease of phage yields. Stx1 phages were not detected under normal or deficient RNase E levels. After an initial delay, high levels of toxin were finally produced in MMC-treated cultures.

Conclusion. RNase E scarcity reduces stx2 phage production but not toxin. Normal concentrations of RNase E are likely required for correct phage morphogenesis. Our future work will address the mechanism of RNase E action on phage morphogenesis.

INTRODUCTION

Enterohaemorrhagic Escherichia coli (EHEC) is an enteric pathogen that can cause diarrhoea, bloody diarrhoea and, in some patients, haemolytic uraemic syndrome [1–3]. The most commonly identified serotype in the U.S.A. and other countries is O157:H7 [4–6]. The severity of the disease is due to the production of a potent cytotoxin, Shiga toxin (Stx), which targets endothelial cells in the kidney and, on occasions, the central nervous system [7–9]. Once inside the cells, Stx blocks protein synthesis by cleaving the N-glycosidic bond at a specific adenine in 28S ribosomal RNA [10]. Stx comprises two immunologically distinct varieties, designated Stx1 and Stx2, and several sub-types for each variety [11]. Studies of EHEC infections in animal models [12, 13] and epidemiological studies [14–19] have suggested that strains carrying the stx2 genes cause disease with the worst outcomes. The stx2 genes are located in the late genome region of lambdoid phages that are integrated into the EHEC genome [20]. Under non-inducing conditions, the stx2-bearing phage (stx2 phage) is in a quiescent state (pro-phage) and replicates with the host chromosome. DNA damage caused by exposure to compounds such as mitomycin C (MMC) [21, 22] or oxidative stress [22–24] induces the lytic cycle, leading to phage excision from the host chromosome, DNA replication and the production of infectious phages as well as toxin [25, 26]. Expression of lytic genes proceeds after the phage transcriptional repressor, cI, undergoes self-cleavage, likely mediated through RecA [27]. RecA is an essential mediator of the SOS response, which is triggered by DNA damage [28]. The lytic cycle ends when cells burst and release the toxin as well as stx2 phages, which can then infect other phage-sensitive bacteria. In this regard, both in vitro and in vivo studies have shown that stx phages can infect laboratory and wild-type strains of E. coli [29–33] as well as non-Escherichia faecal isolates [30, 33]. Upon the infection of naïve cells, the phage either initiates the lytic cycle or integrates into the host genome. While the first outcome can result in an amplification of toxin production and
the worsening of disease [34, 35], the second one can contribute to the dissemination of stx2 genes.

Our laboratory is investigating the role of ribonuclease E (RNase E) in EHEC pathogenesis. RNase E has a central role in RNA metabolism, as it degrades mRNAs, cleaves precursor rRNAs and tRNAs, and also degrades or processes small regulatory RNAs (sRNAs) [36]. Few studies have addressed the role of RNase E in virulence. For instance, in Salmonella Typhimurium, a deletion of the noncatalytic C-terminal domain of RNase E attenuates virulence in an insect model of infection [37]. A similar deletion in Yersinia pseudotuberculosis impairs type III secretion and the infection of macrophage-like cells in vitro [38]. As with other pathogens, EHEC employs a type III secretion system (T3SS) to colonize the intestinal epithelium and inject effector proteins into the eukaryotic cell [39, 40]. In EHEC, RNase E processes primary transcripts that encode T3SS proteins, likely to achieve the proper protein stoichiometry for T3SS assembly [41–43]. Beyond these studies, the influence of RNase E on the expression of EHEC’s main virulence determinants is unknown. We previously reported the construction of an EHEC strain (TEA028-rne) in which RNase E concentrations can be manipulated by the addition of a compound (IPTG) to the culture medium [44]. In this study, we examined the effect of RNase E deficiency on the production of stx phages and Shiga toxin 2.

**METHODS**

**Strains and growth conditions**

TEA028 (AgaLTKM) [45] is a derivative of the EHEC strain EDL933 [46]. The strain TEA028-rne carries an IPTG-inducible rne gene and a plasmid that overproduces the lac repressor (pLacI) [44]. The strains were grown in Luria–Bertani (LB) medium, which was supplemented with tetracycline (tet) (6 µg ml⁻¹) for TEA028 and tet (6 µg ml⁻¹), ampicillin (100 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹) and IPTG (100 or 0.1 µM) for TEA028-rne. To induce the production of phages and toxin, the strains were grown in LB medium, supplemented as indicated above, to an OD₆₅₀ of ~0.30–0.35 (Alpha 1106 spectrophotometer, Laxco, Inc.), at which point mitomycin C (1 µg ml⁻¹) was added.

**Plasmid construction**

To generate the plasmid Topo-stx2, a PCR product of 1572 bp encompassing the stx2AB genes was amplified from EHEC gDNA with the primers f-stx2-rec (5'-ccggcacagagcaatgcccct-3') and r-stx2-rec (5'-ccggacccgtgctgaataata-3') using AccuPrime PfX DNA polymerase (Thermo Fisher Scientific). The PCR product was cloned using the Zero Blunt Topo PCR Cloning kit (Invitrogen). By a similar procedure, a PCR product of 1408 bp carrying the stx1AB genes was cloned to generate the plasmid Topo-stx1. The stx1AB genes were amplified using primers f-stx1-rec (5'-aggttatgtagctttatccg-3') and r-stx1-rec (5'-gcacacagctagttat-3'). The identity of the PCR products was confirmed by Sanger sequencing.

**Plaque assays**

Supernatants were collected by centrifugation at 15 000 g for 10 min, treated with one-tenth volume of chloroform (Sigma-Aldrich), and then mixed with an equal volume of 4 M (NH₄)₂SO₄ solution (Sigma-Aldrich) before storage at 4°C [47]. Plaque assays were performed according to Miller [48] using E. coli strain MC1061 [49], provided by the E. coli Genetic Stock Center, as host. Briefly, LB medium containing 5×10⁻³ M CaCl₂ (Sigma-Aldrich) was inoculated (1:100 ratio) with an overnight culture of the MC1061 strain. The culture was incubated statically for 8 h at 37°C, followed by 1 h incubation with shaking. Then, 1 ml of culture was mixed with 2 ml of molten LB soft agar (0.8 % LB plus 0.8 % agar (Thermo Fisher Scientific)) supplemented with 10 mM CaCl₂ and 10 mM MgSO₄ and poured on top of standard LB agar plates. Serial dilutions (10-fold) of supernatants were prepared in Lambda diluent [10 mM Tris-HCl (pH 7.5), 8.1 mM MgSO₄] [50] and then aliquots of 10 µl were applied on the double-layer agar plates. Plaques were noticeable after overnight incubation at 37°C.

**Plaque hybridization**

Detection of plaques carrying the stx2 or stx1 genes was performed by plaque hybridization with specific probes, which were random prime-labelled with digoxigenin-11-dUTP [Dig DNA Labeling and Detection kit (Roche)]. To prepare the probes, stx2 or stx1 PCR products were amplified from plasmids Topo-stx2 or Topo-stx1, respectively, run in a 0.7 % agarose gel, and purified from the gel slice using the GenElute Gel Extraction kit (Sigma-Aldrich). The stx2 or stx1 PCR products were amplified with the primers f-stx2-rec and r-stx2-rec or f-stx1-rec and r-stx1-rec, respectively. DNA concentrations were measured in a BioTek Epoch 2 plate reader. Plaques were lifted to a positively charged nylon membrane (Roche or GE Healthcare), which was then treated sequentially with the following solutions: denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 20 min, neutralizing buffer (1.5 M NaCl, 1.0 M Tris-HCl, pH 7.4) for 5 min and 2× SSC (0.3 M NaCl, 0.03 M Na-citrate, pH 7.0) for 5 min. The membrane was air-dried, 1–2 µl of stx2 or stx1 PCR product (positive controls) was applied close to a membrane edge and the DNA was fixed by UV crosslinking. The next steps (pre-hybridization, hybridization and detection) were performed as described in the Dig DNA Labeling and Detection kit (Roche). Hybridization was conducted at 58°C using 11 ng ml⁻¹ (final concentration) of denatured probe.

**Toxin quantification**

To quantify Stx2, the receptor ELISA technique (RELISA) was modified from previously published methods [35, 51]. A stock of ceramide trihexosides (500 µg ml⁻¹, bottom spot) (Matreya LLC) was diluted 10-fold in methanol. Aliquots of 100 µl were dispensed into wells of Immulon 1B 96-well plates (Thermo Fisher Scientific) and let dry at room temperature. Then, the wells were filled with 100 µl of blocker solution (block-CH, Millipore), incubated for 2 h and washed twice with 100 µl of phosphate-buffered saline (PBS).
(Thermo Fisher Scientific) containing 0.05% Tween 20 (PBST) (Sigma-Aldrich). Appropriate dilutions of culture supernatants in PBST, cell lysates or purified Stx2 (List Biological Laboratories) were dispersed in triplicate into the wells and incubated for 1 h. To prepare cell lysates, an aliquot of culture was mixed with Bacterial Protein Extraction Reagent (B-PER) (Thermo Fisher Scientific) in a 1:1 ratio and incubated for 5 min at 37°C. The plates were then washed three times with PBST, followed by incubation for 2 h with rabbit anti-Stx2 antibodies (1: 5000) (List Biological Laboratories). After three washes with PBST, the wells were filled with 100 µl of HRP-conjugated goat anti-rabbit IgG antibodies (1:10000) (GeneTex) and incubated for 1–1.5 h. The plates were washed twice with PBST and twice with PBS followed by the addition of 100 µl of substrate (Pierce 1-Step Ultra TMB Blotting Solution, Thermo Fisher Scientific). After 15–20 min incubation, the reactions were stopped by the addition of 2 M H2SO4 (100 µl) and absorbance at 450 nm was measured in an Epoch 2 plate reader (BioTek). All incubations and washes were performed at room temperature. Proper dilutions of purified Stx2 (List Biologicals) were used to construct standard curves. Absorbance values were interpolated to the standard curves. Supernatants or whole-cell lysates of EHEC strain EDL933 carrying a deletion of the stx2 genes were used as negative controls.

**Preparation of protein extracts and Western blotting**

Cells were collected by centrifugation (15,000 g for 5 min), resuspended in B-PER reagent and incubated for 5 min at 37°C. The cell extracts were mixed with 4 M urea (final concentration), and total protein was quantified by the DC Protein Assay kit (Bio-Rad) using bovine serum albumin (Sigma-Aldrich) as a standard. Aliquots containing 20 µg of total protein were mixed with Laemmli Buffer (Bio-Rad) containing β-mercaptoethanol (5%) and subjected to electrophoresis on a SDS-10% polyacrylamide gel (TGX, Bio-Rad). Proteins were transferred to a PVDF membrane (Immobilon P, Millipore) by a semidry method. The membrane was blocked with chemiluminescence blocker (block CH, Millipore) for 30 min and then incubated overnight at 4°C with antisera raised against RNase E (1:20,000) [52]. The blot was washed three times for 5 min with PBST and then incubated for 2 h with secondary antibody HRP-conjugated goat anti-rabbit (1:10,000) (GeneTex). After three washes for 5 min with PBST, the membrane was incubated for 5 min with a chemiluminescent substrate (Clarity Western ECL, Bio-Rad). The membrane was scanned in a C-Digit Blot Scanner (LiCor). The antibodies were diluted in a solution of 0.5% of Tropix I-Block reagent (Invitrogen) in PBS containing 0.1% Tween 20.

**Quantification of phage DNA copies**

Phage DNA copies were quantified in culture supernatants by qPCR using the primers f-rqPCR-stx2B (5’-gagtttatgcgggtattg-3’) and r-qPCR-stx2B (5’-tggaaactcaattttaccttagca-3’) to amplify the stx2B gene. The reactions were performed in a CFX Connect real-time detection system (Bio-Rad) employing the iTaq Universal SYBR Green Supermix (Bio-Rad). The cycling protocol was 95°C for 3 min and then 40 cycles of 95°C for 5 s and 60°C for 30 s. A melting curve analysis was performed afterward to confirm the presence of only one amplification product and the absence of primer dimers. The number of DNA copies was calculated by the interpolation of Cq values to the standard curves constructed with the linearized plasmid Topo-stx2. To quantify the phage gDNA that is encapsidated, the supernatants were first treated with Turbo DNase (Thermo Fisher Scientific) for 30 min at 37°C following the manufacturer’s recommendations. Then, the samples were heated for 30 min at 95°C to inactivate DNase and release the encapsidated DNA.

**Statistical analysis**

The experiments were each repeated at least three times. The graphs and statistical analysis were constructed and performed with GraphPad Prism 7.01 software. The plaque-forming units (p.f.u.) counts and phage DNA copies were log-transformed before the statistical analysis. The data were analysed by two-way ANOVA and Dunnett’s multiple comparisons test, or by t-test corrected with the Holm–

**Fig. 1.** IPTG-dependent production of RNase E in the EHEC strain TEA028-rne. In the strain TEA028-rne, an IPTG-inducible promoter controls transcription of the RNase E encoding gene (rne), while in the parental strain TEA028 (control) the rne gene is transcribed from its own natural promoter. Bacterial cultures were grown in LB medium supplemented with 100 or 0.1 µM IPTG for the TEA028-rne strain. Cells were collected at an OD600 of 0.30–0.35, and cell extracts were obtained as described in the Methods section. Cellular proteins from two independent cultures of TEA028 (lanes 1–2), TEA028-rne grown at 100 µM IPTG (lanes 3–4) or TEA028-rne grown at 0.1 µM IPTG (lanes 5–6) were subjected to SDS-PAGE. Left panel, RNase E was detected by Western blot analysis using antisera raised against RNase E. Right panel, the immunoblot on the left was stained with Ponceau S to show the total protein loaded in each lane.
Sidak method when the number of biological replicates was different among groups.

RESULTS
The EHEC strain TEA028-rne grown at 0.1 µM IPTG is RNase E-deficient
In the strains TEA028 (parental) or TEA028-rne, transcription of the gene encoding RNase E (rne) is under the control of its natural promoter or under the control of a Plac promoter, respectively [44]. TEA028-rne cells grown at 100 µM IPTG and the parental strain produced similar quantities of RNase E (Fig. 1). In contrast, the strain TEA028-rne grown at 0.1 µM IPTG under produced RNase E (Fig. 1).

Growth kinetics of EHEC cells containing normal versus low RNase E levels in mitomycin C-treated cultures
We next examined the growth profile of cultures containing low versus normal RNase E concentrations in the presence or absence of sub-inhibitory concentrations of MMC. Cultures of TEA028 (control) and TEA028-rne at 100 or 0.1 µM IPTG were grown to an OD<sub>600</sub> of ~0.30–0.35 (time 0), at which point an aliquot of each culture was treated with MMC and growth was followed by the measurement of turbidity at various time intervals. As expected, MMC caused a drastic decline in turbidity in the control or TEA028-rne at 100 µM IPTG, indicating cell lysis due to the release of phages (Fig. 2a, b). Similarly, there was a decline in the turbidity of MMC-treated cultures of TEA028-rne at 0.1 µM IPTG, but at a seemingly slower rate than for the control or TEA028-rne at 100 µM IPTG (Fig. 2c). This result is made more evident by plotting the difference of turbidity readings between MMC-treated and non-treated cultures for each time point (Fig. 2d). Cultures of TEA028-rne at 0.1 µM IPTG lysed more slowly than those of the control or TEA028-rne at 100 µM after the addition of MMC, suggesting that RNase E deficiency might impair phage production.

Phage production by EHEC cells containing normal versus low RNase E levels
Next, we compared the kinetics of infectious phage production between cells containing normal versus low RNase E concentrations in supernatants of MMC-treated cultures. At various time points after the addition of MMC, the control and TEA028-rne at 100 µM IPTG produced phage titres that were significantly higher than in RNase E-deficient cultures (Fig. 3a). At later time points, reduced phage yields are still significant (data not shown). Moreover, hybridization with a probe specific for the stx2 genes indicated that

![Fig. 2. Growth of TEA028 and TEA028-rne strains in cultures treated or non-treated with mitomycin C (MMC). The EHEC parental strain TEA028 (a) and the RNase E-inducible strain TEA028-rne (b, c) were grown to an OD<sub>600</sub> of ~0.30–0.35 (time 0) in LB medium supplemented with IPTG, as indicated. The cultures were then split and an aliquot was treated with MMC (1 µg ml<sup>-1</sup>) to induce the phage lytic cycle. Thereafter, samples were collected at 1 h time intervals to measure the OD<sub>600</sub>. (d) For each time point, the OD<sub>600</sub> of the MMC-treated cultures was subtracted from the OD<sub>600</sub> of non-treated aliquots. The means and standard errors are indicated in each graph. A lack of error bars indicates that the standard error is smaller than the plot symbol. In panel (d): *, adjusted P-value versus TEA028≤0.0003; #, adjusted P-value versus TEA028=0.001.](image-url)
cultures deficient in RNase E yielded significantly fewer stx2-bearing phages (stx2 phages) (Fig. 3b). By contrast, we did not detect plaques produced by stx1 phages in our experimental conditions, which is in agreement with previously reported results for the EDL933 strain [53]. A previous report on E. coli K-12 indicated that RNase E inactivation precludes normal initiation of the SOS response following treatment with MMC [54]. Therefore, we hypothesized that a defect in the SOS response under RNase E scarcity would affect phage DNA replication. In agreement with this hypothesis, the number of phage DNA copies was significantly lower in RNase E-deficient cultures at 3 and 4 h after MMC treatment (Fig. 4). Surprisingly, at later time points, the number of DNA copies was similar in the supernatants of cells containing low versus normal RNase E concentrations. This result suggests that RNase E scarcity affects additional steps in phage morphogenesis other than a delay in phage replication. Not only does initiation of the lytic cycle occur in the presence of inducing agents such as MMC, but a fraction of stx prophages undergo spontaneous induction even in the absence of an inducer [55]. In this case, a subpopulation of EHEC produces phages spontaneously without a conspicuous change in turbidity. Hence, we wondered whether spontaneous phage production would also be reduced under RNase E scarcity. Phage titres were determined in the supernatants of cells with normal versus deficient RNase E levels at various time intervals after the cultures reached an OD_{600} of ~0.30–0.35. Fig. 5 shows that phages were detected in all supernatants, but higher titres were detected in cultures with normal RNase E levels at two time points.

**Toxin production by EHEC cells containing normal versus low RNase E levels**

Next, we compared the kinetics of toxin production between the control and TEA028-rne at 100 or 0.1 µM IPTG in MMC-treated cultures. In supernatants, the toxin concentrations increased with time for all conditions, even though this occurred at a slower rate in cultures of TEA028-rne deficient RNase E levels at various time intervals after the cultures reached an OD_{600} of ~0.30–0.35. At hour 2, the toxin level was below the limit of detection (~1 ng ml^{-1} in our assay) in cultures with a low RNase E concentration, but was quantifiable in supernatants of the control or TEA028-rne at 0.1 µM IPTG (Fig. 6a). At hour 2, the toxin level was below the limit of detection (~1 ng ml^{-1} in our assay) in cultures with a low RNase E concentration, but was quantifiable in supernatants of the control or TEA028-rne at 0.1 µM IPTG. At later times (hours 5 and 6), RNase E-deficient cells released as much toxin as cells with normal levels of RNase E (Fig. 6a). In whole-cell lysates, the toxin reached high concentrations in all cultures, and only at hour 2 was the toxin concentration reduced under RNase E deficiency (Fig. 6b). Taken together, these results suggest that slower lysis of RNase E-deficient cells delayed the release of toxin.

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**Fig. 3.** Production of phages by TEA028 and TEA028-rne strains treated with mitomycin C (MMC). The EHEC parental strain TEA028 and the RNase E-inducible strain TEA028-rne were grown to an OD_{600} of ~0.30–0.35 (time 0) in LB medium supplemented with IPTG, as indicated. The cultures were then split and an aliquot of each culture was treated with MMC (1 µg ml^{-1}) to induce phage production. (a) Plaque-forming units (p.f.u.) in supernatants of samples collected at 1 h time intervals after MMC addition. (b) stx2-bearing PFU at 5 h and 6 h after MMC addition. The means and standard errors are indicated in each graph. In panel (a): *, adjusted P-value versus TEA028=0.0001. In panel (b): *, adjusted P-value versus TEA028=0.0006; #, adjusted P-value versus TEA028=0.0020.

**Fig. 4.** Encapsidated phage DNA produced by cells containing normal versus deficient RNase E concentrations. The EHEC parental strain TEA028 and the RNase E-inducible strain TEA028-rne were grown to an OD_{600} of ~0.30–0.35 in LB medium supplemented with IPTG, as indicated. At this point, MMC (1 µg ml^{-1}) was added to induce the phage lytic cycle. Thereafter, supernatants were collected and processed as described in the Methods section. Phage DNA was amplified by qPCR with specific primers for the stx2 gene. Quantification was performed by the interpolation of Cq values in standard curves. The means and standard errors are indicated in each graph. *, adjusted P-value versus TEA028=0.0009; #, adjusted P-value versus TEA028=0.027.
into the medium. However, low levels of RNase E did not impair toxin production. Regarding the spontaneous production of toxin, Fig. 7 shows that toxin levels were reduced in supernatants of RNase E-deficient cultures, but not in whole culture lysates. The negative effect of RNase E scarcity on spontaneous phage production could reduce cell lysis, and thus release of toxin into the medium.

**DISCUSSION**

Here we report that EHEC cells deficient in RNase E produced significantly fewer infectious stx2 phages. In MMC-treated *E. coli* K-12, RNase E deletion or inactivation of temperature-sensitive RNase E abrogates the normal initiation of the SOS response [54]. The mechanisms by which RNase E modulates the SOS response have not been elucidated yet, but it seems that the effect is not due to a global defect in protein synthesis [54]. After the addition of MMC to RNase E-deficient cells, the cells lysed slowly (Fig. 2) and the phage DNA copies were reduced up to 4 h (Fig. 4). These results are consistent with the delayed induction of the prophage lytic cycle, possibly because of SOS abrogation by RNase E scarcity. A delay in phage DNA replication, either in the presence of an inducer agent or spontaneously, could be due to reduced concentrations of RecA and/or Cl. Spontaneous induction occurs at higher frequency in stx prophages than in the related non-stx encoding counterparts, despite them sharing a similar mechanism to maintain lysogeny or to initiate lytic cycle [55]. Livny and Friedman [55] proposed that stochastic fluctuations of repressor levels are unlikely to play a role in spontaneous induction, which rather occurs when activated RecA reaches a critical level. In contrast, lower amounts of Cl repressor was proposed, at least as a partial explanation, for stx phages spontaneous induction [56].

Besides the delay in lytic cycle onset, RNase E scarcity could influence stx2 phage morphogenesis by affecting the half-lives of mRNAs, the processing of polystronic mRNAs or the stability of sRNAs. In an *E. coli* K-12 strain the absence of RNase E was revealed to have a complex effect on the transcriptome [57]. In this case, many mRNAs increased their steady-state levels, as expected from the loss of a ribonuclease, but many others showed reduced abundance compared with the control. Further, annotated sRNAs varied their concentrations under those experimental conditions. RNase E scarcity could alter the steady-state levels of transcripts from structural and/or regulatory proteins disrupting protein stoichiometry with a negative effect on phage yields. Analysis of the transcriptome of MMC-treated cells containing low versus normal RNase E levels can reveal changes in phage transcripts that are essential for the production of infectious particles. A recent study identified nine genes that encode essential structural proteins for stx2 phage morphogenesis, such as the main capsid protein and the tail fibre protein [58], but how those genes are regulated is presently unknown.

There was an initial delay in toxin appearance in RNase E-deficient cultures under inducing conditions. However,
RNase E scarcity did not impair toxin production (Fig. 6), in contrast to what was observed for infectious phase yields. As far as we know, the influence of ribonucleases such as RNase E on the degradation of the stx2AB mRNA has not yet been studied. The lack of effect on toxin production could be due to direct and indirect effects of RNase E depletion on multiple mRNAs and small regulatory RNAs. RNase E comprises an N-terminal catalytic domain and a C-terminal domain that is dispensable for growth and is involved in multiple protein–protein interactions [36, 59]. Therefore, it would be of interest to determine whether a deletion of the C-terminal domain affects phage or toxin production. Ribonucleases have been proposed as potential and novel targets for antimicrobial therapy [60], but the influence of those enzymes on the expression of virulence traits in EHEC or other Shiga toxin-producing E. coli has not been extensively investigated. The work presented here is an initial contribution to this topic and provides the foundation for addressing the role of RNase E in the morphogenesis of stx phages.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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Fig. 7. Spontaneous production of Shiga toxin 2 by TEA028 and TEA028-rne strains. The EHEC parental strain TEA028 or the RNase E-inducible strain TEA028-rne were cultured in LB medium supplemented with IPTG, as indicated. The cultures were grown to an OD600 of ~0.30–0.35 (time 0), and thereafter supernatants (a) or whole cultures (b) were collected at 5 and 6 h. The means and standard errors are indicated in each graph. In panel (a): *, adjusted P-value versus TEA028=0.0066; **, adjusted P-value versus TEA028=0.0067.
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