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Research Letter

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Effect of RNase E deficiency on translocon protein synthesis in an RNase E-inducible strain of enterohemorrhagic *Escherichia coli* O157:H7

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One sentence summary: Type III secretion system components are negatively regulated by ribonuclease E in enterohemorrhagic *Escherichia coli*.

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) is a food-borne pathogen that assembles a type III secretion system (T3SS) on its surface. The last portion of the T3SS, called the 'translocon', is composed of a filament and a pore complex that is inserted into the membrane of intestinal epithelial cells. The genes encoding the translocon (*espADB*) are part of the *LEE4* operon. Their expression is regulated by a complex post-transcriptional mechanism that involves the processing of *LEE4* mRNA by the essential endoribonuclease RNase E. Here, we report the construction of an EHEC strain (TEA028-*rne*) in which RNase E can be induced by adding IPTG to the culture medium. EHEC cells deficient in RNase E displayed an abnormal morphology and slower growth, in agreement with published observations in *E. coli* K-12. Under those conditions, EspA and EspB were produced at higher concentrations, and protein secretion still occurred. These results indicate that RNase E negatively regulates translocon protein synthesis and demonstrate the utility of *E. coli* strain TEA028-*rne* as a tool for investigating the influence of this ribonuclease on EHEC gene expression *in vitro*.

Keywords: enterohemorrhagic *Escherichia coli*; ribonuclease E; type III secretion system; posttranscriptional regulation; *LEE4* operon, EHEC

INTRODUCTION

The pathogen enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 employs a type III secretion system (T3SS) to attach to intestinal cells and to deliver effector proteins that disrupt cell function (Jarvis and Kaper 1996; Stevens and Frankel 2014). The terminal portion of the T3SS, called the 'translocon', encompasses a long filament that is composed of EspA subunits and ends in a complex of the EspB and EspD proteins (Knutton *et al.* 1998; Luo and Donnenberg 2011). This complex is thought to form a pore in the membrane of eukaryotic cells to allow the passage of effector proteins into the cytoplasm (Ide *et al.* 2001; Shaw *et al.* 2001).

The *espADB* genes are part of the *LEE4* operon, which also contains genes encoding the regulator of secretion SepL (Deng *et al.* 2005, 2015), chaperones CesD2 and L0017 (Neves *et al.* 2003; Su *et al.* 2008), the T3SS needle component EscF (Wilson *et al.* 2001) and the effector protein EspF (Nougayrède and

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Table I. Strains used in this study.

Strain	Description	Source or reference
TEA028	Derivative of EHEC serotype O157:H7 strain EDL933, Δ*galETKM::tetA*	Ho and Waldor (2007)
TEA028-*rne*	Derivative of TEA028 into which the IPTG-inducible, HA- and His6-tagged *rne* gene from *E. coli* RNE102 has been transduced. This strain also produces the $LacI^Q$ repressor from a plasmid	This study
EDL933 Δ*galU*	Derivative of EDL933 in which a kanamycin resistance marker has been inserted into the *galU* gene	This study
EDL933ΔLEE	Derivative of EDL933 in which the LEE pathogenicity island encoding the T3SS has been deleted	Pósfai *et al.* (1997)
RNE102	Derivative of *E. coli* Keio BW25113. The *rne* gene is HA- and His6-tagged and transcribed from a P_{lac} promoter. Thus, IPTG is required for *rne* transcription. An ampicillin resistance marker is located upstream P_{lac}	This study
RNE100	Derivative of *E. coli* Keio BW25113. The *rne* gene is HA- and His6-tagged and transcribed from the *rne* promoter	This study
MC4100	Non-pathogenic *E. coli* K-12 strain used as a positive control for P1 transduction experiments	Casadaban (1976)

Donnenberg 2004). The *LEE4* operon, whose regulation is complex and has only partially been elucidated, is required for bacterial attachment to the recto-anal junction in the bovine reservoir (Naylor *et al.* 2005) and initial adhesion to cultured cells through the EspA filaments (Sharma *et al.* 2016). The *LEE4* transcript undergoes post-transcriptional processing by the endoribonuclease RNase E near the 3′ end of *sepL*, the first gene of *LEE4*, to generate an *espADB* mRNA product that has a monophosphorylated 5′ end and an AU-rich leader region (Lodato and Kaper 2009). These features would ordinarily render *espADB* mRNA highly susceptible to further degradation by RNase E, but the binding of ribosomes to a mini-open reading frame located in the leader region increases transcript stability (Lodato *et al.* 2012). Processing of the primary transcript appears to be important for determining the stoichiometry of the EspADB proteins and the regulator SepL. Recently, it was proposed that RNase E is a positive post-transcriptional regulator of *LEE4* (Connolly, Finlay and Roe 2015). However, the role of RNase E as a regulator of *LEE4* is rather unclear because of its overlapping contributions to the processing and degradation of *LEE4* mRNA and its possible influence on the stability of protein-coding mRNAs or small RNAs (sRNAs) that may regulate *LEE4* gene expression.

In this study, we examined the effect of RNase E on the production of the translocon proteins EspA and EspB. To do so, we constructed an EHEC strain in which RNase E levels can be manipulated by varying the concentration of an inducer in the culture medium.

MATERIALS AND METHODS

Strains and growth conditions

The strains used in this work are described in Table I. Bacteria were grown in Luria-Bertani (LB) medium or Dulbecco's Modified Eagle's Medium (DMEM) low glucose (HyClone, GE Healthcare), which were supplemented with antibiotics and IPTG when required. Antibiotics were added at the following concentrations: ampicillin (100 μg mL^{-1}), tetracycline (6 μg mL^{-1}), chloramphenicol (25 μg mL^{-1}). When grown in DMEM, the strains were inoculated from overnight cultures in LB at a ratio of 1/500 and then incubated statically for 15 h at 37°C. CFU quantification was done on LB agar supplemented with antibiotics and 100 μM IPTG. To generate P1 lysates, R agar and R-top agar were prepared according to Miller (1992). The R agar composition was 1% tryptone, 0.1% yeast extract, 1.2% agar, 0.8% NaCl; $CaCl_2$ and glucose were added after autoclaving (final concentrations of 2 mM and 0.1%, respectively). R-top agar had the same composition as R agar except that it contained 0.8% agar.

Determination of generation times

LB medium was inoculated with overnight cultures of the strains TEA028 and TEA028-*rne* at a ratio of 1/500 and dispensed into a multiwell plate. In the case of strain TEA028-*rne*, the LB medium was supplemented with appropriate antibiotics and with IPTG at various concentrations (500, 100, 1 and 0.1 μM). The multiwell plates were incubated at 37°C with agitation in a BioTek Epoch 2 plate reader, and cell growth was followed by measuring optical density at 600 nm at 15 min intervals. Generation times were calculated as reported by Hall *et al.* (2014), using the software GrowthRates 2.1. Only growth curves with correlation coefficients >0.995 were considered in the growth rate analysis.

Construction of plasmid pLacI^Q and EDL933 *galU::kan* strain

The *lacI* gene was amplified from EHEC genomic DNA by PCR (AccuPrime Taq DNA Polymerase, Thermo Fisher Scientific), using the forward primer f-prlacIq (5′-CCCAAGCTTG**T**GCAAAA CCTTTCGCGGTATG-3′) and the reverse primer r-prlacIq (5′-CGCGGATCCTCACATTAATTGCGTTGCGCTC-3′). In the forward and reverse primers, underlined sequences correspond to *Hind*III and *Bam*HI restriction sites, respectively. The bold '**T**' nucleotide in the forward primer introduced a single nucleotide change in the *lacI* promoter to enhance the expression of the *lacI* gene. The amplified DNA fragment was cloned between the *Hind*III and *Bam*HI restriction sites of plasmid pACYC184*rrnB* (Lodato and Kaper 2009) to generate plasmid pLacI^Q, which was then introduced into the TEA028-*rne* strain. The cloned *lacI*^{Q} gene sequence was confirmed by Sanger sequencing. The EDL933 *galU*::*kan* strain was constructed by the gene doctoring method described by Lee *et al* (2009). The kanamycin resistance gene (*kan*) was amplified with primers f-GalU and r-GalU, using plasmid pDOC-K as the template. The sequences of primers were f-GalU (5′-CCGGAATTC*GCCGTTATCCCCGTTGCGGGATTAGGAACC AGGATGTTGCCGGCGGACCGGTCAATTGGCTGGAG*-3′) and r-GalU (5′-CCGCTCGAG*CCAGGCTTTAAATTCCGTGCCAAGGGTGTTATGAC GAATACCGTAAATATCCTCCTTAGTTCC*-3′). In the f-GalU and r-GalU primers, the underlined sequences are *Eco*RI and *Xho*I restriction sites, respectively, while the sequences in italics are

homologous to the *gal*U gene. The PCR fragment was cloned between the *Eco*RI and *Xho*I sites of plasmid pDOC-C, and the resulting plasmid was then introduced into the EDL933 strain. Next, the strain was transformed with plasmid pACBSCE, which carries the genes encoding the λ-Red proteins to facilitate recombination and the gene for the restriction enzyme I-SceI. The recombinants were able to grow in the presence of kanamycin but not in minimal medium with galactose as the carbon source. PCR, restriction enzyme digests and DNA ligation were done according to the manufacturers' recommendations.

Construction of *Escherichia coli* strain RNE102

The P_{lac}-*rne*Δ337 allele of CJ1832 (Jain, Deana and Belasco 2002) was transferred to *E. coli* strain BW25113 by P1 transduction in the presence of 10 μM IPTG and selection for ampicillin resistance. The *rne* gene of the transductant was then modified further by allelic exchange (Edwards, Keller and Schifferli 1998) so as to add carboxy-terminal HA and hexahistidine tags (GYPYDVPDYAGHHHHHH) to RNase E. The resulting recombinant strain, RNE102, encodes epitope-tagged RNase E under the control of P_{lac}. Its genotype was verified by chromosomal DNA sequencing and immunoblotting.

P1 transduction

The protocol of Ho and Waldor (2007) was followed with minor modifications. Cells from overnight cultures of recipient strains (*E. coli* K-12 strain MC 4100, EHEC TEA028, EHEC *galU*::*kan*) in LB broth (0.5 mL) were pelleted and resuspended in 200 μl of MC buffer (5 mM $MgSO_4$, 50 mM $CaCl_2$). The cells were infected with 50–100 μl of a P1 lysate (~3×10^9 PFU mL^{-1}) and incubated for 15 min at 37°C. LB medium containing 10 mM sodium citrate (2 mL) was added, and the cells were incubated for 1 h at room temperature, pelleted, resuspended in 100 μl of 1M sodium citrate and plated on LB agar supplemented with ampicillin (100 μg mL^{-1}) and IPTG (300 μM).

P1 lysate production

The procedure described by Miller (1992) was followed. Briefly, a 1:100 dilution of an overnight culture of *E. coli* strain RNE102 was prepared in LB medium supplemented with 100 μM IPTG and 5 mM $CaCl_2$. The bacterial subculture was incubated at 37°C without agitation for ~6 h and then for 1 h at 37°C with agitation. An aliquot (1 mL) of the subculture was collected and infected with 100 μl of a P1 stock. After incubation for 20 min at 37°C, 2.5 mL of R-top agar was added and the infected cells were plated on R agar plates and incubated overnight at 37°C. The soft agar layer was scraped and washed with 1 mL of LB medium. The recovered liquid was treated with chloroform (100 μl), vortexed and spun in a centrifuge to eliminate cellular debris. The supernatant containing the P1 lysate was stored at 4°C.

Preparation of protein extracts and western blotting

Cells from bacterial cultures were collected by centrifugation (15 000 × *g* for 5 min), resuspended in water and boiled for 5 min. The cell extracts were mixed with 2.5% SDS and 4 M urea (final concentrations), and then total protein was quantified by the DC Protein Assay kit (Bio-RAD). The cell extracts were mixed with urea to disrupt RNase E aggregates that otherwise are not well resolved by PAGE, and aliquots containing the same amount of total protein were mixed with Laemmli Buffer (Bio-RAD) containing β-mercaptoethanol (5%) and subjected to electrophoresis on an SDS-10% polyacrylamide gel. Proteins were transferred to a PVDF membrane (Immobilon P-Millipore) by a semidry method. The membrane was blocked with chemiluminescence blocker (Bløk, Millipore) or Tropix I-Block for 30 min and then incubated overnight at 4°C with primary antibodies. The blot was washed with PBS containing 0.05% Tween 20 (3 × 5 min) and then incubated for 1–3 h with the secondary antibody. After washing again, the membrane was incubated for 5 min with a chemiluminescent substrate (Clarity Western ECL substrate, Bio-RAD), and then scanned in a C-DiGit Blot Scanner (LiCor). Alternatively, the membrane was incubated with Pierce 1-Step Ultra TMB Blotting Solution (Thermo Scientific). The antibodies used for RNase E detection were anti-6X His tag (GeneTex) and anti-mouse IgG (HRP) (GeneTex), whereas those for GroEL detection were anti-GroEL mouse monoclonal (GeneTex) and anti-mouse IgG (HRP) (GeneTex) antibodies. The EspA and EspB proteins were detected with polyclonal antibodies (Lodato and Kaper 2009) and EasyBlot anti-rabbit IgG (HRP) (GeneTex).

Protein precipitation from supernatants

Supernatants from bacterial cultures were collected after centrifugation (14 000 × *g* for 10 min at 4°C). The proteins they contained were precipitated from equivalent volumes (2.6 mL) of the culture supernatants by addition of deoxycholic acid (2.2% final concentration) and trichloroacetic acid (TCA, 10% final concentration), incubation on ice for 30 min and subsequent centrifugation for 10 min at 15 000 rpm and 4°C. Bovine serum albumin (BSA) was added to each sample prior to TCA addition as a control for the efficiency of precipitation. Pellets were washed with chilled acetone at –20°C for 1 h on ice with occasional vortexing, and the samples were then centrifuged (15 000 × *g* for 10 min at 4°C). The resulting pellets were air-dried, mixed with sample buffer and analyzed by PAGE. Proteins were stained with lumitein (Biotium) following the manufacturer's recommendations.

Statistical analysis

The experiments were each repeated at least twice. Graphs and statistical analysis were performed with GraphPad Prism 7.01 software. Colony forming units and generation times were analyzed by one-way ANOVA corrected for Dunnett's multiple comparisons test. Cell lengths were analyzed by non-parametric ANOVA (Kruskal-Wallis test) corrected for Dunnett's multiple comparisons test.

RESULTS AND DISCUSSION

RNase E is an essential enzyme in *Escherichia coli*. Therefore, to investigate its influence on translocon protein synthesis in EHEC, we used P1 transduction to place the RNase E gene (*rne*) under the control of an IPTG-inducible P_{lac} promoter. Although P1 transduction is a common genetic manipulation in *E. coli* K-12 strains, this method is hampered in EHEC, presumably because the O antigen shields the P1 receptor. Ho and Waldor (2007) reported that chromosomal markers can be moved effectively among EHEC strains that carry deletions of galactose utilization genes (Δ*galETKM* or Δ*galU*). Thus, we decided to use their method to generate an EHEC strain in which RNase E synthesis is inducible. To this end, we produced P1 lysates of the *E. coli* K-12 strain RNE102 (Table I and Materials and Methods section), which carries an *rne* gene under the control

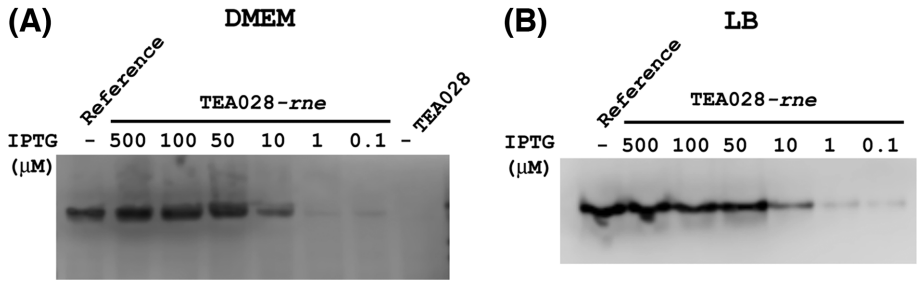

Figure 1. RNase E synthesis in the RNase E-inducible strain TEA028-*rne*. In the EHEC strain TEA028-*rne*, transcription of the *rne* gene is under the control of an IPTG-inducible promoter. Bacteria were grown in (**A**) DMEM or (**B**) LB medium supplemented with various concentrations of IPTG, as indicated. The reference is an *E. coli* K-12 strain in which RNase E is expressed under the control of its own regulatory region (*E. coli* K-12 strain RNE100). In both TEA028-*rne* and the reference strain, RNase E bears a C-terminal hexahistidine (6xHis) tag. Cellular proteins were subjected to SDS-PAGE and western blot analysis with antibodies against the 6xHis tag. The parental strain (TEA028), in which RNase E lacks a 6xHis tag and thus is undetectable with these antibodies, served as a negative control. The immunoblot in panel A was also stained with Ponceau S (not shown) to show that equal amounts of total protein had been loaded in each lane.

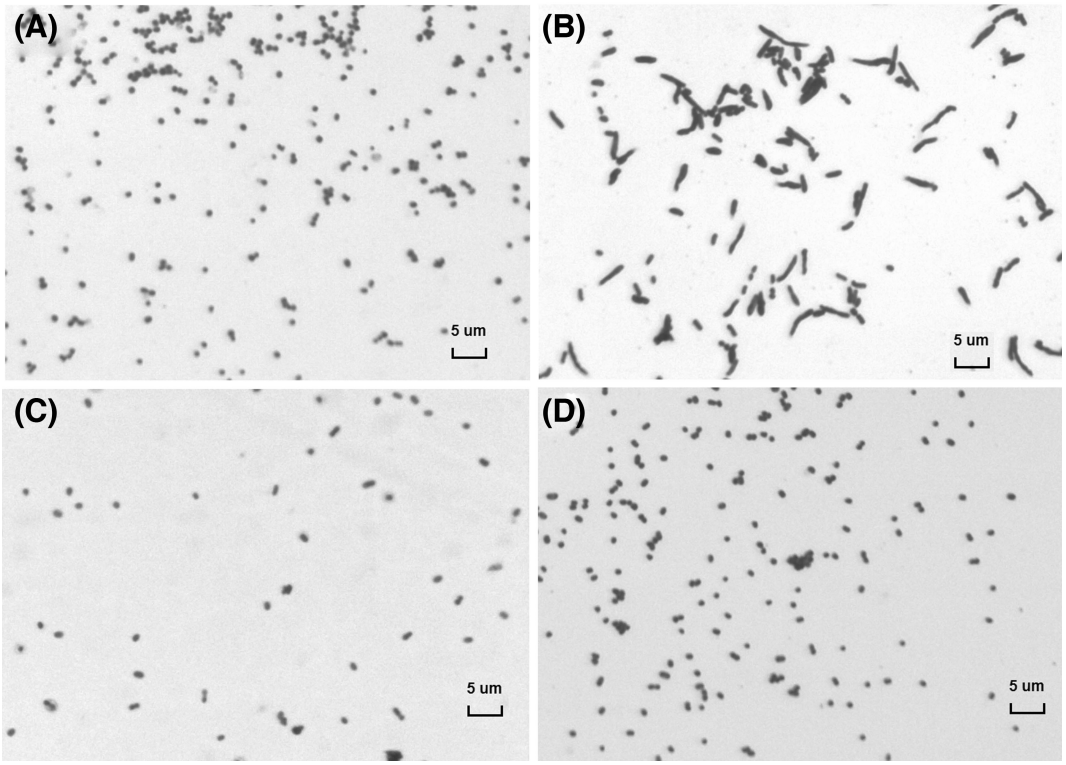

Figure 2. RNase E depletion causes abnormal morphology of EHEC cells. (**A**) TEA028; (**B, C, D**) TEA028-*rne*. Bacteria were grown overnight in LB medium either in the absence of IPTG (A) or in the presence of 0.1 μM (B), 100 μM (C) or 500 μM (D) IPTG. Smears were stained with crystal violet. At the lowest IPTG concentration (0.1 μM), TEA028-*rne* cells grew abnormally as filaments. Magnification = $\times$1000. Bar: 5 μM.

of a P_{lac} promoter and a nearby ampicillin resistance marker. Hence, transcription of the *rne* gene in this strain can be controlled by adjusting the concentration of IPTG in the culture medium, and the proximity of the antibiotic resistance marker facilitates selection after P1 transduction. Two EHEC *gal*$^-$ strains, TEA028 (Δ*galETKM*) and EDL933 Δ*galU* (Table I), were used as recipients for transduction from RNE102. We were unable to transduce the ampicillin resistance marker to the EHEC Δ*galU* strain, and only one ampicillin-resistant colony was obtained by transduction into the TEA028 strain. By contrast, using the same P1 lysates to transduce *E. coli* K-12 strain MC4100 resulted in more than a hundred ampicillin-resistant colonies. Therefore, P1 transduction of EHEC *gal* mutants was possible in our hands but was less efficient than previously reported (Ho and Waldor 2007).

To achieve tighter control of RNase E synthesis in the P1 transductant of TEA028, we introduced a plasmid that overproduces the *lac* repressor (pLacI^Q). Hereafter, we will refer to the resulting plasmid-bearing strain as TEA028-*rne*. As expected, the concentration of RNase E in TEA028-*rne* varied depending on the concentration of IPTG in the culture medium. RNase E was scarce at IPTG concentrations $\leq$1 μM but present at levels that resembled the reference strain at 50–500 μM IPTG (Fig. 1).

We then examined the effect of RNase E levels on cell shape. Cells deficient in RNase E displayed an altered morphology characterized by filamentation (Fig. 2 and Figs S1 and S2, Supporting

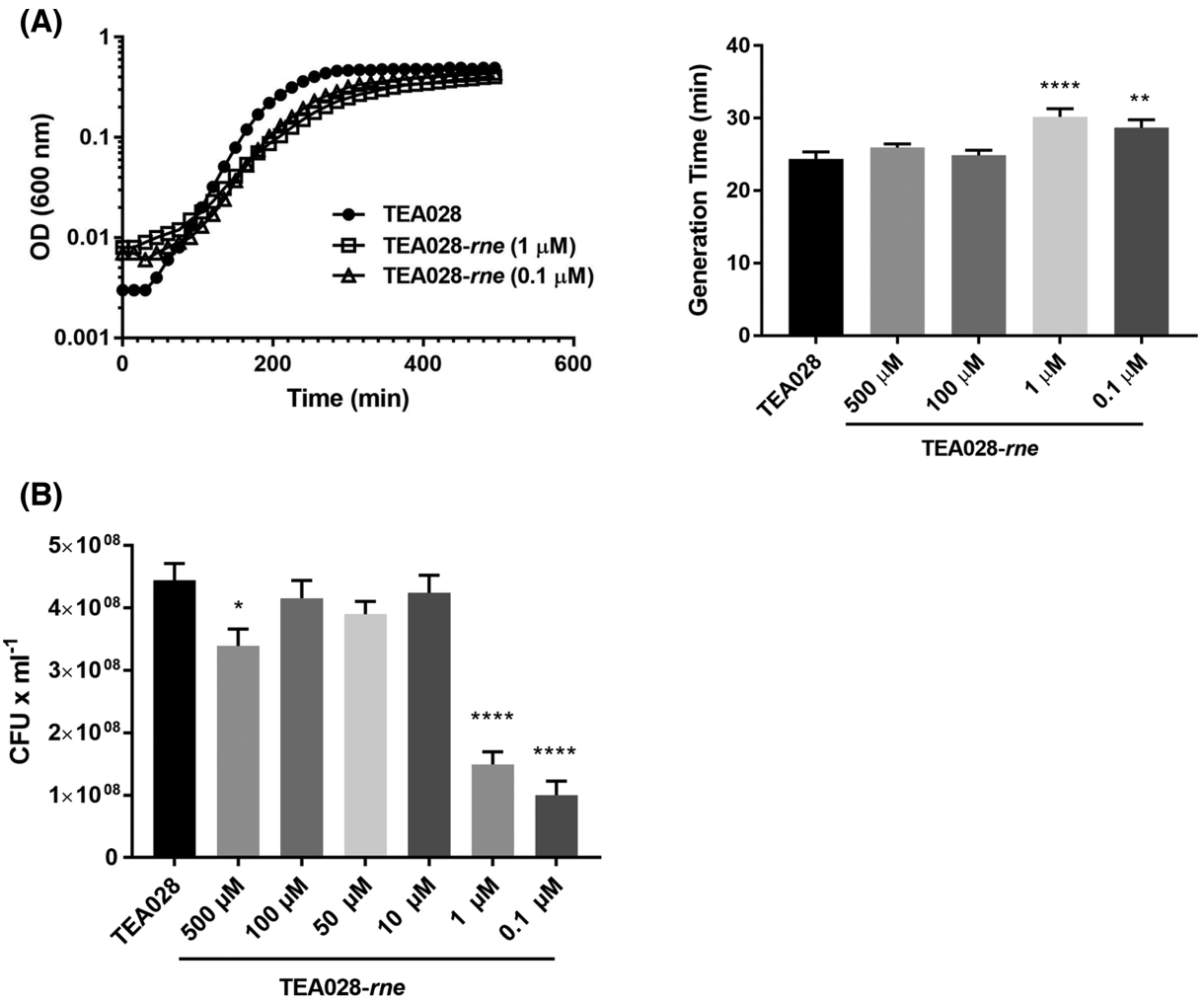

Figure 3. IPTG-dependent growth of the RNase E-inducible strain TEA028-*rne* in various culture media. (**A**) Bacteria were grown in LB media supplemented with IPTG as indicated. Representative growth curves of TEA028 (control) and TEA028-*rne* (grown in the presence of 1 or 0.1 μM IPTG) are shown on the left. Growth curves for TEA028-*rne* at 100 or 500 μM IPTG, which resemble that for TEA028, are omitted for clarity. (**B**) Bacteria were grown in DMEM medium supplemented with IPTG as indicated. Colony forming units (CFU) were measured after overnight growth under static conditions. Means and standard errors are indicated in each graph. In panel A: **adjusted p-value versus TEA028 = 0.0098; ****adjusted p-value versus TEA028 = 0.0001. In panel B: *adjusted p-value versus TEA028 = 0.018; ****adjusted p-value versus TEA028 = 0.0001.

Information). A similar growth defect has been described for *E. coli* K-12 strains at low RNase E concentrations (Goldblum and Apririon 1981; Cam *et al.* 1996; Jain, Deana and Belasco 2002). The formation of filaments has been attributed to the altered decay of *ftsA* and *ftsZ* mRNA, which encode two proteins essential for cell division. The resulting reduction in the ratio of FtsZ/FtsA proteins affects septum formation (Tamura *et al.* 2006).

Depletion of RNase E was observed not only to alter cell morphology but also to slow cell growth in rich media (Fig. 3A). In addition, CFU yields were reduced at low concentrations of IPTG in the defined medium DMEM (Fig. 3B), which is normally used to grow EHEC because it stimulates the expression of virulence genes (Tatsuno *et al.* 2000). The growth of TEA028-*rne* was significantly retarded at IPTG concentrations (0.1 or 1 μM) that cause RNase E to be produced at levels well below its concentration in the reference strain. Diminished cell growth has also been reported for *E. coli* K-12 cells in which RNase E has been depleted (Jain, Deana and Belasco 2002).

Next, we investigated the effect of RNase E levels on the production of two translocon proteins (EspA and EspB) and on protein secretion. A lower than normal concentration of RNase E (0.1 μM IPTG) increased EspB and especially EspA levels in both cell pellets and culture supernatants (Fig. 4A and B). Other proteins could also be detected in the culture supernatants at low RNase E levels (Fig. 4C), indicating that secretion still occurs. By contrast, the cytoplasmic protein GroEL was undetectable in the culture supernatants (Fig. S3, Supporting Information), indicating that the presence of translocon proteins in the supernatants was mainly due to secretion and not to cell lysis. As expected, protein levels were significantly reduced in supernatants of an EHEC strain lacking type III secretion (Fig. S4, Supporting Information). The increase of EspA and EspB levels under conditions of RNase E deficiency implies either that the degradation of *espADB* mRNA is RNase E-dependent or that processing of the primary *LEE4* transcript in the *sepL* coding region negatively affects the production of translocon proteins. Contradicting the latter proposition is the previous observation that production of EspA was not noticeably affected by a deletion of the RNase E processing site in *sepL* (Lodato and Kaper 2009). However, that earlier deletion was made in a plasmid-borne fragment of

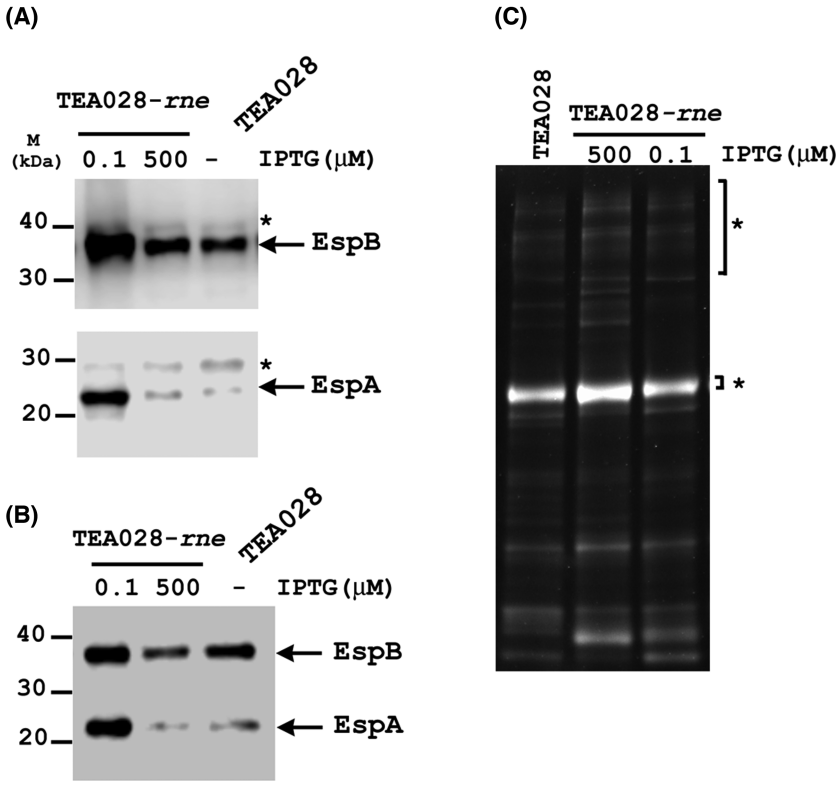

Figure 4. IPTG-dependent production and secretion of EspA and EspB by the RNase E-inducible strain TEA028-*rne*. TEA028-*rne* and its parental strain (TEA028) were grown statically overnight in DMEM supplemented with IPTG at the indicated concentrations. Cellular proteins from (**A**) bacterial pellets or (**B**) supernatants were subjected to SDS-PAGE and western blotting with antisera raised against EspA or EspB. Arrows indicate the EspA and EspB proteins, while asterisks indicate non-specific proteins that cross-reacted with the antisera. In panel **C**, secreted proteins in equivalent volumes of culture supernatants were precipitated and examined by SDS-PAGE and staining with lumitein. Asterisks indicate BSA, which was added to each supernatant as an internal control for the efficiency of protein precipitation.

the *LEE4* operon in which only the *sepL*, *espA* and *espD* genes were present. Ascertaining whether or not *LEE4* mRNA processing affects the production of translocon proteins in the context of the entire operon will require the more difficult approach of deleting the RNase E processing site in the chromosomal *sepL* gene.

Besides affecting mRNA lifetimes directly, RNase E can influence gene expression indirectly by degrading sRNAs that act post-transcriptionally to regulate specific mRNAs (Waters and Storz 2009). In recent years, various sRNAs that regulate virulence have been discovered in EHEC (Bhatt *et al.* 2016; Hücker *et al.* 2017), and some of them have been found to exert either a positive (Gruber and Sperandio 2015) or negative (Gruber and Sperandio 2014) effect on *LEE4* gene expression. However, in most cases the role of RNase E has not yet been defined. In principle, the balance of direct and indirect effects of RNase E on *LEE4* may be important for producing the translocon components in the correct ratio and for their timely and efficient assembly during infection.

It remains to be determined whether cells with low RNase E content are able to attach to eukaryotic cells and form the characteristic pedestals that are the hallmark of EHEC infections (Knutton *et al.* 1989). Of note is that RNase E processes another polycistronic transcript, *LEE5*, which encodes proteins required for the intimate attachment of EHEC to host cells (Gruber and Sperandio 2014). Therefore, processing of EHEC transcripts by RNase E or other nucleases appears to be of broad significance.

While the construction of RNase E-inducible strains has been described for *E. coli* K-12 (Jain, Deana and Belasco 2002), to our knowledge this is the first report of such a modification of the *rne* gene on the chromosome of a Gram-negative pathogen. Few studies have examined the role of RNase E in pathogenesis. In *Yersinia pseudotuberculosis*, overproducing a truncated form of RNase E that lacked the non-catalytic C-terminal domain was found to impair type III secretion and the infection of macrophage-like cells *in vitro* (Yang, Jain and Schesser 2008). In addition, a similar RNase E truncation in *Salmonella enterica* serovar Typhimurium attenuated infection of insect larvae (Viegas *et al.* 2013). The TEA028-*rne* strain described here is expected to prove useful for examining the role of RNase E in the expression of the many EHEC virulence genes *in vitro*, such as those required for Shiga-toxin synthesis or adhesion to eukaryotic cells (Torres, Zhou and Kaper 2005; Mellies and Lorenzen 2014).

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflict of interest. None declared.

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