Small RNA Esr41 inversely regulates expression of LEE and flagellar genes in enterohaemorrhagic Escherichia coli

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Abstract
Enterohaemorrhagic Escherichia coli (EHEC) is a life-threatening human pathogen worldwide. The locus of enterocyte effacement (LEE) in EHEC encodes a type three secretion system and effector proteins, all of which are essential for bacterial adherence to host cells. When LEE expression is activated, flagellar gene expression is down-regulated because bacterial flagella induce the immune responses of host cells at the infection stage. Therefore, this inverse regulation is also important for EHEC infection. We report here that a small regulatory RNA (sRNA), Esr41, mediates LEE repression and flagellar gene activation. Multiple copies of esr41 abolished LEE expression by down-regulating the expression of ler and pch, which encode positive regulators of LEE. This regulation led to reduced EHEC adhesion to host cells. Translational gene-reporter fusion experiments revealed that Esr41 regulates ler expression at a post-transcriptional level, and pch transcription, probably via an unknown target of Esr41. Esr41-mediated ler and pch repression was not observed in cells lacking hfq, which encodes an RNA-binding protein essential for most sRNA functions, indicating that Esr41 acts in an Hfq-dependent manner. We previously reported an increase in cell motility induced by Esr41. This motility enhancement was also observed in EHEC lacking ler, showing that Esr41-mediated enhancement of cell motility is in a ler-independent manner. In addition, Esr41 activated the expression of flagellar Class 3 genes by indirectly inducing the transcription of fliA, which encodes the sigma factor for flagellar synthesis. These results suggest that Esr41 plays important roles in the inverse regulation of LEE and flagellar gene expression.

INTRODUCTION
Enterohaemorrhagic Escherichia coli (EHEC) infections cause diarrhoea and haemorrhagic colitis, and, in serious cases, the patients develop a haemolytic uraemic syndrome [1]. The adherence of EHEC to intestinal epithelial cells, which induces attaching and effacing lesions, is required for full virulence [2]. The type three secretion system (T3SS) and effector proteins that are secreted through T3SS play essential roles in this adherence and are encoded by the locus of enterocyte-effacement (LEE) [3]. The expression of LEE, which contains at least six operons (LEE1–5 and grlRA operon), is controlled at the transcriptional and post-transcriptional levels by several factors [3–5]. Ler, encoded by the first gene of the LEE1 operon, is a central activator of the expression of LEE2–5 operons and other LEE genes [6–9]. The transcription of LEE1, including ler, is directly and indirectly regulated by various factors, such as ClpXP, DksA, Fis, GrlA, H-NS, IHF, LrhA, Pch and QseA [7, 10–15]. Pch, encoded in EHEC by three paralogous genes (pchA, pchB and pchC), is one of the activators of LEE1 expression, and therefore is required for the adhesion of EHEC to HEp-2 cells [16].

The ability to produce and rotate flagella allows cells to swim, and the genes of flagellar components and their regulation factors comprise a large and complex regulon, called the flagellar regulon [17]. Extracellular flagella are a disadvantage during the establishment of infection because strong antigenic properties of flagellin, the major component of flagellum, induce pro-inflammatory responses, such as activation of Toll-like receptor 5 and IL-8 secretion by epithelial cells [18, 19]. Thus, EHEC may need to suppress the flagellar synthesis at the infection stage to avoid the immune responses of the host cell. Therefore, inverse regulation of LEE and flagellar synthesis is crucial for successful EHEC infection of the host. GrlA is one of the factors involved in this inverse regulation and functions both as a positive

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Abbreviations: DMEM, Dulbecco’s modified Eagle’s medium; EHEC, enterohaemorrhagic Escherichia coli; FRT, FLP recognition target; LEE, locus of enterocyte effacement; sRNA, small regulatory RNA; T3SS, type three secretion system; qPCR, quantitative real-time PCR.

Supplementary material is available with the online version of this article.
transcriptional regulator of the LEE1 operon, including ler, and as a negative transcriptional regulator of the flagellar regulon [20].

Small regulatory RNAs (sRNAs) are widely conserved in many bacteria [21–24] and are induced by a variety of stress conditions to regulate the expression of their target genes at the post-transcriptional level [25–28]. The molecular mechanism of sRNA regulation involves base-pairing between the sRNA and the target mRNA, with the RNA-binding protein Hfq often playing a critical role in this interaction in the Enterobacteriaceae [29, 30]. In most Gram-negative bacteria, the disruption of hfq usually results in reduced final cell density, slow growth and increased sensitivity to a variety of stresses [31]. Furthermore, Hfq plays an important role in the full virulence of many pathogenic bacteria; i.e., the ability of hfq deletion mutants to infect and colonize host cells is impaired [23, 32–40]. In contrast to many pathogenic bacteria, disruption of hfq in the EHEC strain EDL933 leads to increased production of proteins comprising T3SS and efficient colonization of HeLa cells [3, 5]. Moreover, the expression of genes encoding T3SS components increases in hfq deletion strains of several EHECs, enteropathogenic E. coli, Vibrio cholerae, Vibrio parahaemolyticus and Pseudomonas aeruginosa, all of which possess pathogenicity islands that encode T3SSs [3, 5, 9]. These observations raise the possibility that an Hfq-binding sRNA controls T3SS synthesis in these bacteria. In fact, some sRNAs affecting T3SS synthesis have been identified in EHEC; for example, DsrA and sRNA350 indirectly promote ler transcription, and GlmY, GlmZ, sRNA56 and sRNA103 regulate the expression of LEE4 and/or LEE5 operons [41–43]. Furthermore, a cis-encoded sRNA, Arl, that down-regulates ler expression has been reported [44].

Here, we show that Esr41 sRNA, which we previously identified as an enhancer of cell motility [45], also acts as a negative regulator of LEE expression, thereby inhibiting efficient adhesion of EHEC to HEp-2 cells. This negative regulation of LEE is an outcome of decreased pch and ler expression, orchestrated by Esr41 in an Hfq-dependent manner. Moreover, the enhancement of cell motility by Esr41 is independent of this LEE regulation, and results from an increase in the expression of the flagellar regulon by Esr41-induced transcriptional activation of filA, a gene encoding the flagellum-specific sigma factor. Such Esr41-mediated regulation might contribute to the inverse control of LEE and flagellar gene expression.

**METHODS**

**Bacterial strains, media and plasmids**

The *E. coli* strains used in this study are listed in Table 1. SKI-5142 is a Lac-negative derivative of EHEC O157: H7 Sakai [14] and was used as a wild-type EHEC strain. SKI-5142Δhfg::kan-S and SKI-5142Δhfg::kan-L, which form small colonies and wild-type sized colonies, respectively, were constructed from SKI-5142 using a one-step gene inactivation protocol [46] with the appropriate primers (Table S2, available in the online version of this article). SKI-5142Δhfg were constructed from SKI-5142Δhfg::kan by elimination of the kan gene using the FRT (FLP recognition target) expression plasmid pCP20 [46]. MG1655Δlac is a lacZ deletion derivative of MG1655 [45] and was used as a wild-type *E. coli* K-12 strain. NS1202 was constructed from MG1655 by P1 transduction with P1 phage lysates prepared from TM587 [47], and by selecting for chloramphenicol resistance, followed by elimination of the cat gene using the FRT expression plasmid pCP20. NS1203 was then constructed by P1 transduction of NS1202 with a P1 phage lysate prepared from W3110Δlac [45] and by selecting for chloramphenicol resistance.

*E. coli* strains were grown aerobically at 37 °C in LB medium or Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with ampicillin (100 µg ml⁻¹), chloramphenicol (15 µg ml⁻¹) or kanamycin (30 µg ml⁻¹).

**Table 1. *E. coli* strains used in this study**

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<tr>
<th>Strain name</th>
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Plasmids used in this study are listed in Table S1. Details of plasmid construction are presented in the Supplementary Methods.

RNA isolation and northern blot analysis

Total RNA for sRNA analysis was prepared by the acid guanidinium thiocyanate–phenol–chloroform method using ISOGEN reagent (Nippon Gene), according to the manufacturer’s protocol, from *E. coli* cells grown at 37 °C in LB or DMEM media. Total RNA for mRNA analysis was prepared by the SDS-hot phenol method, as described previously [48]. To detect Esr41 and 5S rRNA, total RNA was separated on a 10% polyacrylamide gel containing 7 M urea. To detect *ler*-3×*FLAG* mRNA, total RNA was separated on a 1.2% agarose gel containing 3.6% formaldehyde. The separated RNAs were blotted onto a Hybond N+ membrane (GE Healthcare). The RNAs were visualized using DIG reagents and kits for non-radioactive nucleic acid labelling and detection (Roche) according to the manufacturer’s protocol. Band intensities were analysed with ImageQuant TL software (GE Healthcare). The following DIG-labelled RNA probes were prepared by *in vitro* transcription using DIG RNA labelling mix (Roche) and a DNA template PCR-amplified from the appropriate template with the appropriate primers (Table S2): *ler* RNA probe, corresponding to the anti-sense of an incomplete *ler* coding region (+1 to +252 relative to the start codon); *dps* RNA probe, corresponding to the anti-sense of an incomplete *dps* coding region (+1 to +237); *ompA* RNA probe, corresponding to the anti-sense of an incomplete *ompA* coding region (+1 to +320); and *sodB* RNA probe, corresponding to the anti-sense of an incomplete *sodB* coding region (+1 to +231).

The following DIG-labelled oligonucleotide probes were prepared by terminal transferase reaction (NEB) using DIG-11-ddUTP: Oligo-41, corresponding to the *esr41* anti-sense sequence; MS2tag probe, corresponding to the MS2 tag anti-sense sequence; and 5S rRNA probe, corresponding to the *rrnH* anti-sense sequence. The sequences of oligonucleotide probes are given in Table S2.

Analysis of proteins from culture supernatants and whole-cell lysates

*E. coli* cells were grown at 37 °C with shaking until they reached an OD$_{600}$ of 0.8. To analyse the secreted protein from culture supernatants, proteins in cell-free culture supernatants were precipitated with 10% trichloroacetic acid and suspended in 1× SDS loading buffer (56.25 mM Tris-HCl, pH 6.8, 2.4% SDS, 13.5% glycerol, 5% β-mercaptoethanol and 0.015% bromophenol blue). The protein samples were analysed by SDS-PAGE and Coomassie brilliant blue staining [49]. To analyse whole-cell lysates, 0.75 ml of cell cultures was pelleted by centrifugation and cell pellets were suspended in 1× SDS loading buffer. The protein samples were separated using SDS-PAGE and analysed by western blotting, as described previously [16, 49]. Western blot analysis was performed using monoclonal anti-RpoA 4RA2 antibodies (Santa Cruz Biotechnology), polyclonal anti-EspB antibodies [16], polyclonal anti-Hfq antibodies [50] and polyclonal anti-FLAG antibodies (Sigma). The bound antibodies were detected using ECL Western Blotting detection reagents (GE Healthcare), as described previously [16]. Band intensities were analysed with ImageQuant TL software (GE Healthcare). All assays were repeated at least three times.

β-Galactosidase activity assay

β-Galactosidase activity was assayed as described previously [49]. *E. coli* cells grown at 37 °C were harvested at an OD$_{600}$ of 0.8. All assays were performed at least in duplicate and were repeated at least three times.

Gel mobility shift assay

Esr41 and Esr41-4U were synthesized by *in vitro* transcription using a CUGA7 *in vitro* transcription kit (Nippon Gene) and DNA fragments containing the T7 promoter and transcribed sequences corresponding to Esr41 and Esr41-4U, respectively. DNA fragments for Esr41 and Esr41-4U were amplified from pRS-Esr41 by PCR with the appropriate primers (Table S2). The *in vitro* transcripts were purified as described previously [51]. The 5′ termini of Esr41 transcripts were labelled with [γ-32P]ATP by T4 polynucleotide kinase and then purified on Centri-Sep spin columns (Princeton Separations) to remove unincorporated nucleotides. The gel mobility shift assay was performed with a 32P-labelled Esr41 transcript and purified Hfq-His$_6$ [52], as described previously [51].

Quantitative real-time PCR

For quantitative real-time PCR (qPCR), EHEC cells harbouring pRS-Esr41 and pRS414 and grown in DMEM at 37 °C were harvested at an OD$_{600}$ of 0.8, and total RNA was prepared using an RNaseasy Mini Kit (Qiagen), according to the manufacturer’s protocol. Next, cDNA was prepared from total RNA by reverse transcription using random hexamer primer and ReverTra Ace (TOYOBO), according to the manufacturer’s instructions. Expression of the target genes was determined by qPCR using the primers listed in Table S2 with Power SYBR green PCR Master Mix (Applied Biosystems) and a 7500 Real-Time PCR System (Applied Biosystems). Transcript abundance was normalized to 16S rRNA. The qPCRs were carried out using RNA isolated from at least three independent cultures.

HEp-2 adhesion assay

The HEp-2 adhesion assay was performed as described previously [20]. The assay was performed at least three times, in duplicate.

Affinity purification of MS2-tagged RNA

Affinity purification and purification of MS2 coat protein fused to maltose-binding protein (MS2-MBP) were performed as described [53], with minor modifications. Details are presented in the Supplementary Methods.
RESULTS

Esr41 displays typical characteristics of an Hfq-binding sRNA

We previously reported the identification of a novel sRNA, Esr41, encoded by an intergenic region of the EHEC O157:H7 Sakai-specific sequence that is not present in non-pathogenic E. coli K-12, noting that Esr41 possesses sequence and structural features required for the binding of Hfq [45]. Because the amounts of Hfq-binding sRNAs are reduced in most hfq deletion mutants of Gram-negative bacteria, we examined the effect of hfq deletion on Esr41 transcript levels. Growth defects were observed in EHEC strains lacking hfq, which form small colonies on LB agar plates; however, these small colonies often generate wild-type-sized colonies that are probably suppressor mutants [5]. Thus, we performed northern blot analysis with RNA samples obtained from EHEC, SKI-5142 and two isogenic hfq deletion mutants derived from small or wild-type-sized colonies. This experiment revealed that the amount of Esr41 transcript was considerably lower in both hfq deletion mutants than in the wild-type strain (Fig. 1a). This indicated that Hfq is essential for abundance of the Esr41 transcript.

A previous study showed that Esr41 binds to Hfq in vivo [54]. We directly examined Hfq binding to Esr41 in vitro. The binding of Esr41 prepared by in vitro transcription to a purified Hfq-His6, was evaluated by a gel mobility shift assay. Hfq and a radioactively labelled Esr41 transcript were co-incubated, and Esr41-Hfq complex formation was observed as slow-migrating bands in native polyacrylamide gels (Fig. 1b, lanes 1 and 2). This result indicated that Esr41 directly binds Hfq. Next, we examined the effect of an excess of unlabelled Esr41 variants on Esr41-Hfq complex formation. When an excess of unlabelled wild-type Esr41 was incubated with labelled Esr41 and Hfq, the formation of labelled Esr41-Hfq complex decreased, as anticipated (Fig. 1b, lanes 3 and 4). Hfq binds the U-rich 3’-termini of RNA molecules in vitro [55], and the polyU tail of Rho-independent sRNA transcriptional terminators in vivo [56]. Thus, to test whether Hfq was able to bind the polyU tail of Esr41, we constructed an Esr41-4U mutant in which the 3’ terminal seven U residues and subsequent A and U residues in wild-type Esr41 (Fig. S1a) were replaced by four U residues. Adding an equivalent amount of Esr41-4U in a competition experiment had no effect on the formation of a labelled Esr41-Hfq complex (Fig. 1b, lanes 5 and 6), indicating that Esr41-4U lost its Hfq-binding activity. Consistent with previous reports, these results verified that Hfq binds the polyU tail of Esr41 (Fig. S1a).

Because Hfq contributes to the stabilization of most sRNAs, we evaluated the stability of Esr41 in E. coli K-12 strain, MG1655Δlac, and an isogenic hfq deletion mutant. Cells harbouring prs-Esr41, a multicopy plasmid carrying the esr41 gene, were grown to the log phase, and rifampicin was added to prevent further initiation of transcription. RNA samples were obtained at various time points after the addition of rifampicin and were analysed by northern blotting (Fig. 1c). In the absence of Hfq, the half-life of Esr41 RNA was strikingly shorter than that in the presence of Hfq, clearly demonstrating that Hfq stabilized Esr41. We concluded from these results that Esr41 possesses features typical of Hfq-dependent sRNAs.

Esr41 decreases the accumulation of secreted Esp proteins, thereby repressing efficient adhesion of EHEC to the host cells

We previously demonstrated that harbouring a multicopy plasmid carrying the esr41 gene increases EHEC cell motility and the expression of fliC, a flagellin-encoding gene [45]. Because LEE and flagellar expression are inversely regulated [20], we examined the effect of a multicopy plasmid carrying esr41 on LEE expression. Northern blot analysis revealed that Esr41 RNA levels were ~80-fold higher in SKI-5142 harbouring prs-Esr41 than in a strain harbouring the pRS414 control vector (Fig. 2a). Proteins from equivalent amounts of culture supernatants of SKI-5142 cells carrying prs-Esr41 or pRS414 control vector were analysed by SDS-PAGE. The accumulation of LEE-encoded secreted Esp proteins such as EspA, B and D was abolished in cells carrying prs-Esr41, while the amount of flagellin (FliC) was increased upon Esr41 overproduction in accordance with our previous report [45] (Fig. 2b). To address the effect of esr41 deletion on the accumulation of secreted proteins, the same experiments were performed in an SKI-5142Δesr41 background. Deletion of the esr41 gene did not affect the accumulation of secreted proteins (Fig. 2b). Western blot analysis of EspB, an LEE-encoded secreted protein, using whole-cell lysates verified that Esr41 overproduction suppressed espB expression (Fig. 2c), whereas deletion of the esr41 gene did not affect EspB levels (data not shown).

The repression of secreted Esp protein accumulation by Esr41 overproduction was expected to inhibit T3SS formation, thereby reducing efficient adhesion of EHEC to HEp-2 cells. We subsequently examined whether Esr41 overproduction affected the adhesion of EHEC to HEp-2 cells. As shown in Fig. 2(d), SKI-5142 harbouring pRS414 control vector adhered to HEp-2 cells and formed micro-colonies. By contrast, SKI-5142 harbouring prs-Esr41 exhibited markedly reduced adhesion to HEp-2 cells, as anticipated, indicating that Esr41 inhibited EHEC adhesion to HEp-2 cells.

Esr41 decreases the expression of LEE-encoded and LEE-regulating factors

Some secreted proteins related to cell adhesion are encoded by LEE [2], and the transcription of LEE operons is activated by Ler, a protein encoded by the first gene of the LEE1 operon. Therefore, we examined the expression of six genes from operons LEE1-5 and grlR, whose transcription is also controlled by Ler, by qPCR. Expression of the rpoB gene was used as a control. The relative abundance of LEE1-5 and grlR transcripts in SKI-5142 cells harbouring plasmid prs-Esr41 was decreased by about 10- to 100-fold compared
with cells harbouring the control vector, whereas the abundance of the rpoB transcript did not change appreciably (Fig. 3). This finding was consistent with the observation that the levels of LEE-encoded secreted proteins were reduced upon Esr41 overexpression, as shown in Fig. 2(b). It also indicated that the reduction of the LEE2-5 and grlR operon transcript abundances stems from Esr41-mediated repression of ler, because Ler acts as an activator of LEE2-5 and grlR operon transcription [6, 57, 58]. Our qPCR analysis of paralogous pchABC genes that encode a positive regulator of LEE1 (ler) revealed that, relative to cells bearing pRS414, pchABC transcript levels decreased in a strain harbouring pRS-Esr41, but to a lesser degree than LEE transcript levels (Fig. 3). These results suggested that Esr41 down-regulated the expression of LEE-regulating factors, which in turn markedly reduced the expression of all genes in LEE operons.

**Esr41 represses the expression of ler at a post-transcriptional level in an Hfq-dependent manner**

Because the overproduction of Esr41 resulted in decreased LEE expression, we predicted that Esr41 targets ler and/or pchA, the main pch gene for LEE expression [15, 16], and thus represses the expression of their target genes. To examine this prediction and determine whether Esr41 affected gene expression on a transcriptional or post-transcriptional level, we constructed translational pchA- and ler-lacZ fusions, under control of either their native promoters or that of the constitutive bla promoter, on the following low-copy-number plasmids (Fig. 4a): plasmid pMW-pchA19aa,
Fig. 2. Repression of LEE-encoded secretion proteins by Esr41. (a) The effect of multiple copies of *esr41* on the expression of *esr41*. SKI-5142 cells harbouring pRS414 (pRS) or pRS-Esr41 (pEsr41) were grown in DMEM. Total RNA was isolated, and 0.5 and 0.1 µg RNA was analysed by northern blotting using Oligo-41 and 5S rRNA probes, respectively. (b) The effect of multiple copies of *esr41* on the accumulation of secreted proteins from culture supernatants. Protein samples prepared from culture supernatants and derived from equal amounts of DMEM-grown SKI-5142 (WT) or SKI-5142 Δesr41 (Δesr41) cells harbouring pRS414 (pRS) or pRS-Esr41 (pEsr41) were analysed on a 5–20% (w/v) gradient polyacrylamide gel and visualized by Coomassie Brilliant Blue staining. Closed and open arrowheads indicate putative Esp proteins and a putative flagellin (FliC) band, respectively. (c) The effect of multiple copies of *esr41* on EspB expression in whole-cell lysates. Whole-cell lysates from equal amounts of DMEM-grown SKI-5142 (WT) or SKI-5142 Δesr41 (Δesr41) cells harbouring pRS414 (pRS) or pRS-Esr41 (pEsr41) were separated on a 5–20% (w/v) gradient polyacrylamide gel and analysed by western blotting using anti-EspB and anti-RpoA antibodies. (d) The effect of multiple copies of *esr41* on the adhesion of EHEC to HEp-2 cells. SKI-5142 (WT) cells harbouring pRS414 (pRS) or pRS-Esr41 (pEsr41) were inoculated into a medium containing HEp-2 cells and co-incubated for 6 h. After the incubation, the cells were washed and observed using a phase contrast light microscope. Arrowheads indicate micro-colonies.
We also examined the effect of hfq deletion on the Esr41-mediated repression of ler and pchA. β-Galactosidase activities of both Pmaj-ler'-lacZ and Pmaj-pchA'-lacZ fusions were not affected by Esr41 in hfq deletion strains of EHEC and/or *E. coli* K-12 (Fig. 4, b, c). In addition, Esr41 transcript levels in the hfq deletion mutant harbouring pRS-Esr41 were lower than in the wild-type strain harbouring pRS-Esr41 (Fig. S2b). These results suggested that Hfq is essential for post-transcriptional ler repression and transcriptional pchA repression by Esr41. The activities of Pmaj-ler'-lacZ in the hfq deletion mutants harbouring pRS414 were higher than that in the wild-type strains harbouring pRS414, showing that Hfq represses the expression of ler even without Esr41. This result agrees with a previous report that, in EHEC, hfq deletion induces the expression of ler [5], and suggested that ler expression may be regulated by Hfq alone and/or Hfq along with an sRNA present in both EHEC and *E. coli* K-12.

**Esr41, ler mRNA and Hfq can form a ternary complex in vivo**

A stable ternary complex of sRNA, target mRNA and Hfq is formed during translational regulation mediated by several sRNAs in *in vitro* [59–61] and also, in one case, *in vivo* [62]. Therefore, we examined the *in vivo* formation of a ternary complex composed of Esr41, ler mRNA and Hfq. To address this, we affinity-purified MS2-tagged RNA. Using the same approach, Said et al. [53] and Desnoyers and Massé [62] demonstrated that Hfq co-purifies with MS2-tagged sRNA. We constructed a ler mRNA-producing plasmid pMWbla-LF1, carrying the bla promoter followed by a ler-3's-FLAG gene, and two types of MS2-tagged RNA-producing plasmids: pRS-M2-ESr41, producing MS2-Esr41 RNA with the MS2 tag fused to the 5'-end of Esr41; and pRS-MS2terT2, producing MS2terT2 RNA with the MS2 tag fused to the 5'-end of the rrrB T2 terminator. To confirm the repression of ler-3's-FLAG by Esr41, MS2-Esr41 and MS2terT2, whole-cell lysates and total RNAs were prepared from a DMEM-grown MG1655Δlac strain harbouring pMWbla-LF1 in combination with either pRS-Esr41, pRS-MS2-Esr41, pRS-MS2terT2 or pRS414 control vector, and analysed by western and northern blotting, as appropriate. Western blot analysis using anti-FLAG antibodies revealed that the amount of Ler-3's-FLAG construct in cells harbouring pRS-Esr41 and pRS-MS2-Esr41 was ~5.5-fold lower than in cells harbouring the control vector (Fig. 5a). This was consistent with the data from the β-galactosidase activity assays shown in Fig. 4(b, c). Moreover, MS2-Esr41 repressed ler expression to a similar extent as non-tagged Esr41, and MS2terT2 did not affect ler repression, demonstrating that the ler-repressive activity of MS2-Esr41 was comparable to that of Esr41 and that the MS2 tag had little effect on ler repression. Northern blot analysis using a ler-3's-FLAG mRNA-detecting probe indicated that the amount of ler-3's-FLAG mRNA in cells harbouring pRS-MS2-Esr41 was ~2.5-fold lower than in pRS414-harbouring cells (Fig. 5b). These results suggested that both translational inhibition and target mRNA destabilization probably occur during Esr41-mediated ler repression.

Using the same strains as above, we affinity-purified MS2-tagged RNA, and analysed the total RNA and protein samples before (input) and after (output) affinity purification by
northern and western blotting, as appropriate. In the input fractions, the amount of ler-3/FLAG mRNA was lower in the strain expressing Esr41 or MS2-Esr41 than in that expressing MS2terT2 (Fig. 5c), in agreement with the results shown in Fig. 5(b). MS2-tagged RNAs, MS2-Esr41 and MS2terT2, but not the untagged Esr41, were detected in the output fractions (Fig. 5c). Importantly, Hfq and ler-3/FLAG mRNA co-purified with MS2-Esr41 (Fig. 5c).

Next, we examined the specificity of co-purification of ler-3/FLAG mRNA with MS2-Esr41 by northern blot analysis using probes of dps mRNA known to have some affinity to Hfq, and ompA and sodB mRNAs known to be targets of MicA and RyhB sRNA, respectively [24, 63, 64]. The three mRNAs, which are not predicted to be Esr41 targets, were detected in the output fraction of MS2-Esr41; however, the co-purification of ler-3/FLAG mRNA with MS2-Esr41 was more efficient than that of the other three mRNAs (Fig. S3), showing that the large population of Esr41-Hfq complex interacts with ler-3/FLAG mRNA. These results implied that Esr41, ler-3/FLAG mRNA and Hfq form a ternary complex in vivo, and that the formation of the ternary complex is important for Esr41-mediated ler repression, although it remained unclear whether Esr41 binds to ler mRNA directly through base-pairing.

Esr41 enhances the expression of the flagellar regulon in a ler repression-independent manner, leading to an increase in motility

Esr41 enhances EHEC cell motility and the expression of fliC, a gene encoding a major flagellar component [45].
Furthermore, the expression of LE and the flagellar regulon is inversely regulated by the GrlR-GrlA system in EHEC [20]. This led us to hypothesize that Esr41-mediated ler repression leads to the enhancement of cell motility. To explore this possibility, we examined the effect of ler deletion on Esr41-mediated enhancement of cell motility. As shown in Fig. 6(a), an Esr41-mediated increase of cell motility was observed in a ler deletion mutant as well as the wild-type strain, indicating that Esr41 stimulates cell motility in a ler-independent manner.

In E. coli and Salmonella species, the flagellar regulon includes three hierarchical classes (Class 1, 2 and 3). Class 1 contains flhD and flhC, which are essential for the transcription of Class 2 genes. Class 2 contains genes for the formation of the hook basal body and the FliA-sigma factor, which is responsible for the transcription of Class 3 genes. Class 3 contains genes for components of the cell distal structure of flagellum and flagellar function (rotation and chemotaxis) [17, 65–67]. Because Esr41 promotes cell motility, we examined the effect of a multicopy plasmid carrying the esr41 gene on the expression of the flagellar regulon. The relative expression of a Class 1 gene (flhD), Class 2 genes (fliA, flgA, flhB and fliE) and Class 3 genes (flhC and motA) was measured by qPCR and normalized to 16S rRNA levels. The abundance of flhD transcript in SKI-5142 harbouring pRS-Esr41 was 1.8-fold higher than in cells harbouring the control vector (Fig. 6b). The abundances of fliA, flhC and motA transcripts in a strain harbouring pRS-Esr41 relative to those in a strain harbouring pRS414 were even higher, while the relative abundances of Class 2 transcripts apart from fliA were comparable to that of flhD (Fig. 6b). These results suggest that Esr41-mediated activation of the flagellar regulon, especially fliA and Class 3 genes, leads to enhanced cell motility.

To investigate the target gene involved in Esr41-induced enhancement of cell motility, we constructed translational fliD' and fliA'-lacZ fusions (Fig. 4a): plasmid pMWbla-flhD, carrying fliD'-lacZ under control of the bla promoter (Pbla-fliD'-lacZ); plasmid pMWbla-fliA, carrying fliA'-lacZ under control of the bla promoter (Pbla-fliA'-lacZ); and plasmid pMW-fliA, carrying fliA'-lacZ under control of the fliA native promoter (PfliA-fliA'-lacZ). β-Galactosidase activities from an SKI-5142 strain harbouring pRS-Esr41 or pRS414 and transformed with one of the plasmids described above were measured. The activities of both Pbla-flhD'-lacZ and Pbla-fliA'-lacZ were little affected by Esr41, while that of PfliA-fliA'-lacZ was greatly increased, ~76-fold (Fig. 6c). Additionally, no Esr41-mediated increase of PfliA-fliA'-lacZ activity was seen in the hfq deletion mutant (Fig. 6c). These results indicated that Esr41 may indirectly up-regulate the transcription of fliA, but not the translation of flhDC and fliA, via Esr41 post-transcriptionally regulating an unknown target in an Hfq-dependent manner. This expectation was supported by the observation that the transcriptional activity of the PfliA-lacZ fusion was increased by Esr41 (Fig. 6c). These results suggested that Esr41-mediated activation of fliA transcription leads to increased expression of Class 3 genes, and thereby enhanced cell motility.

**DISCUSSION**

In this study, we identified a new function of Esr41 sRNA: the repression of LE expression that leads to a reduction
in efficient EHEC adhesion to HEp-2 cells (Figs 2 and 3). We also showed that the down-regulation of LEE expression results from post-transcriptional repression of ler by Esr41 (Fig. 4b). This ler repression is dependent on Hfq (Fig. 4b, c), indicating that Esr41 acts together with Hfq in the regulation of ler expression. This finding agrees with...
previous observations that Hfq is involved in ler regulation at a post-transcriptional level [5]. Northern blot analysis using the ler-3×FLAG construct revealed that Esr41 induced ler-3×FLAG mRNA destabilization (Fig. 5b). This is consistent with previous findings that most sRNAs induce translational inhibition and subsequent degradation of target mRNAs [68–71]. In addition, Esr41 bound to Hfq in vitro and in vivo, and the 3’-polyU tail of Esr41 was essential for this binding (Figs 1b, 5c and S1a), resulting in stabilization of Esr41 (Fig. 1c). These features of Esr41 and its mode of action are quite similar to those of most Hfq-binding sRNAs, suggesting that the base-pairing between Esr41 and LEE1 mRNA (that contains ler) is important for ler repression by Esr41, as well as for the regulation of target gene expression by most Hfq-binding sRNAs. Indeed, the observations that MS2-tagged Esr41, ler-3×FLAG mRNA and Hfq formed a ternary complex in vivo (Fig. 5c), and that a stretch of 16 nt of Esr41 is partially complementary to the ler translational initiation region of LEE1 mRNA (Fig. S1), seems to support this hypothesis. However, the base-pairing region has yet to be experimentally defined (data not shown).

We showed that Esr41 negatively and positively controls the expression of pch and flia, respectively, at a transcriptional level through unknown target(s) of Esr41 (Figs 4b and 6c), and these regulations are also dependent on Hfq (Figs 4b, c and 6c). The suppression of LEE expression is probably induced by an additive effect of both pch and ler repression by Esr41. Esr41-mediated transcriptional repression of pchA suggests that Esr41 targets other gene(s) that encode(s) regulator(s) of pch gene expression and that those target genes are present in both EHEC and E. coli K-12, as transcriptional repression of pchA by Esr41 was also observed in E. coli K-12 (Fig. 4c). Esr41 certainly activates the expression of flagellar regulon Class 3 genes by promoting flia transcription, resulting in enhanced cell motility (Fig. 6).

Similar to pch regulation, Esr41 seems to up-regulate the flia transcription via a transcriptional regulator whose encoding gene is targeted by Esr41. Although FlhDC is the major transcriptional activator responsible for expression of flagellar regulon Class 2 genes, including flia, Esr41 does not target flhD because of little effect of Esr41 overproduction on Pfla-flhD-lacZ expression (Fig. 6c). Moreover, because YdiV and FliT are known to act as the post-translational regulators of FlhDC [72, 73], their genes are thought to be candidates for Esr41 targets. Note that we cannot exclude the possibility that Esr41 directly controls pch and flia transcription.

Based on this study, we propose a possible regulatory model of Esr41, as depicted in Fig. 7. The Esr41-mediated repression of LEE expression is mainly attributed to the negative regulation of ler expression by Esr41 at a post-transcriptional level, and is partially due to the transcriptional repression of pch genes via an unknown factor whose gene is targeted by Esr41. In addition, Esr41 indirectly stimulates flia transcription, resulting in increased cell motility. Esr41 represses the expression of chiuA (which encodes a haem receptor), cirA (which encodes the receptor for iron binding) and bfr (which encodes a bacterioferritin) through base-pairing [74]; however, it is unknown whether these regulations are involved in the LEE and flagellar regulation by Esr41.

Overexpression of esr41 from a high-copy-number plasmid, pRS-Esr41, represses LEE expression and enhances cell motility, while esr41 deletion did not affect LEE expression and cell motility under our growth conditions (Fig. 2b) [45]. Esr41 expressed from a low-copy-number plasmid had no apparent effect on LEE expression because the levels of Esr41 were lower than from the high-copy-number plasmid (data not shown). There are two possible explanations for the lack of an appreciable phenotype of the esr41 deletion mutant. First, the conditions under which Esr41 is abundant are not known. Thus, if the esr41 deletion mutant had been grown under stress conditions that induce esr41 expression to the same level as in the strain harbouring pRS-Esr41, the effect of esr41 deletion on the phenotype may have been more pronounced. This possibility is supported by the observation that the deletion of an sRNA gene generally affects the phenotype of a mutant only under the sRNA-inducing conditions [25, 75, 76]. The second possibility is the existence of an sRNA(s) with functions redundant to Esr41 in EHEC, serving as backup(s) for Esr41-mediated regulation, although no Esr41 parologue has been identified in the EHEC genome.

In many pathogenic bacteria, the expression of virulence and flagellar genes is inversely regulated by a variety of environmental signals and factors as a response to these signals, and this cross-regulation is important for efficient infection of host cells [77–81]. GrlA is a key factor of EHEC virulence. It acts as a negative regulator of flagellar expression by repressing flhDC transcription and as a positive regulator

\[
\text{Pch} \rightarrow \text{Ler} \rightarrow \text{LEE2-5 operons} \\
\text{Esr41} \rightarrow \text{FlgA} \rightarrow \text{FliC} \rightarrow \text{MotA} \\
\text{FlhD4C2} \rightarrow \text{etc.} \rightarrow \text{etc.} \\
\]

Fig. 7. A model for the Esr41-mediated regulation of LEE and the flagellar regulon in EHEC. Esr41 represses the expression of pch genes and ler at transcriptional and post-transcriptional levels, respectively, resulting in strong repression of LEE expression. Esr41 also activates the expression of flia at the transcriptional level, leading to enhanced cell motility in a Ler-independent manner. The dashed lines indicate additional possible indirect regulation of pchABC and the flagellar regulon by Esr41.
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Conflicts of interest
The authors declare that there are no conflicts of interest.

References
74. Waters SA, McAteer SP, Kudla G, Pang I, Deshpande NP et al. Small RNA interactome of pathogenic E. coli revealed through crosslinking of RNase E. EMBO J 2017;36.

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