Gene *l0017* encodes a second chaperone for EspA of enterohaemorrhagic *Escherichia coli* O157:H7

Marcia Shu-Wei Su, Hsi-Chun Kao, Ching-Nan Lin and Wan-Jr Syu

Correspondence
Wan-Jr Syu
wjsyu@ym.edu.tw

Institute of Microbiology and Immunology, National Yang-Ming University, Beitou 112, Taipei, Taiwan

Escherichia coli O157:H7 tightly associates with host cells through the formation of a pedestal structure in which cell cytoskeleton rearrangement has been observed. These pathogenic properties have been attributed to an island, known as the locus of enterocyte effacement (LEE), located on the bacterial chromosome. Gene *l0017* is one of the LEE genes that has been less well characterized. To understand further the function of the gene, an *l0017*-deleted mutant was created. The mutant lost type III protein secretion (TTS) capacity. In terms of intracellular components, there was a substantial decrease in the level of EspA, but no apparent effect on Tir and EspB was observed. Fractionation of the bacterial proteins indicated that *l0017* was part of the inner-membrane fraction. This association with the membrane is consistent with the hypothesis that *l0017* may act as one of the TTS components. In addition, *l0017* was found to affect regulation of EspA at a post-transcriptional level. The presence of *l0017* readily stabilized EspA and the interaction between *l0017* and EspA was demonstrated by their co-purification as well as by a bacterial two-hybrid system. Therefore, *l0017* is a chaperone, the second chaperone identified in this system after CesAB, and escorts EspA, a protein with a great tendency to polymerize.

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INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is an aetiological pathogen that causes diseases that range from bloody diarrhoea to haemolytic uraemic syndrome to acute renal failure (Nataro & Kaper, 1998). One of the pathogenic mechanisms used by the bacterium is to cause attaching and effacing (A/E) lesions on the intestine, a histopathological change that results from bacteria attaching to enterocytes, destroying the host microvilli and causing the formation of a pedestal-like structure (Frankel *et al.*, 1998). The major virulence factors involved in A/E lesions reside on a locus of enterocyte effacement (LEE), a pathogenicity island on the chromosome that has also been found in human enteropathogenic *E. coli* (EPEC), rabbit EPEC and a mouse pathogen called *Citrobacter rodentium*. Among these LEEs, there are many genes in common (McDaniel *et al.*, 1995) and the sequences of the various LEEs show a high degree of homology and gene similarity (Deng *et al.*, 2001).

The LEE in EHEC consists of 41 ORFs; these encode the type III secretion (TTS) apparatus (Esc and Sep proteins) (Pallen *et al.*, 2005), effectors (EspF, EspG, EspH, EspI,

MAP and Tir) (Crane *et al.*, 2001; Elliott *et al.*, 2001, 2002; Lai *et al.*, 1997; Mundy *et al.*, 2004), translocators (EspA, EspB and EspD) (Chiu *et al.*, 2003; Kenny *et al.*, 1996; Kresse *et al.*, 1999), chaperones (e.g. CesAB, CesD, CesD2, CesF and CesT) (Creasey *et al.*, 2003a, c; Wainwright & Kaper, 1998), regulators (Ler, GrlR, GrlA and Mpc) (Deng *et al.*, 2004; Mellies *et al.*, 1999; Tsai *et al.*, 2006) and adhesin (intimin) (Donnenberg *et al.*, 1993; Jerse & Kaper, 1991; Kenny *et al.*, 1997b). While some of them have been thoroughly studied, others remain less well characterized, and gene *l0017* of EHEC is one of the latter.

The TTS system secretes translocators and effectors across the inner and outer membranes of the bacteria after appropriate induction. To achieve this function, the system consists of a basal apparatus with proteins distributed over the inner membrane, periplasm and outer membrane (Roe *et al.*, 2003). While the effectors and the translocators are all secreted by the TTS system, subtle differences have been found in their secretion. Overall, 19 LEE genes, when individually deleted, affect secretion of both translocators and effectors, while four others preferentially affect the secretion of translocators (Deng *et al.*, 2004). The counterpart of *l0017* in *C. rodentium*, *orf29*, is among the 19 genes required for all TTS. This ORF encodes a protein of 92 aa of which 86 % are identical between *C. rodentium* and EHEC (Deng *et al.*, 2001). In a yeast two-hybrid

Abbreviations: A/E lesion, attaching and effacement lesion; EHEC, enterohaemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; LEE, locus of enterocyte effacement; TTS, type III secretion.

screening, the product of *orf29* has a positive interaction with that of *orf2* (10053 in EHEC). Furthermore, *orf2* is homologous to *Pseudomonas aeruginosa* *pscE* and *Yersinia pestis* *yscE*, and the latter binds to YscG (Pallen *et al.*, 2005). By correlation, *orf29* is speculated to play the YscG-like role. YscG shares 47 % sequence identity with PscG and has a chaperone-like activity (Day *et al.*, 2000; Quinaud *et al.*, 2005). However, no homology has been found between YscG and the *orf29* product (Pallen *et al.*, 2005). In this study, we have experimentally confirmed the effect of *l0017* by deletion and determined the location and biochemical properties of L0017. Intriguingly, L0017 was found to have a chaperone-like function that interacts with and stabilizes EspA.

METHODS

Bacterial growth and molecular biology manipulations. EHEC (ATCC strain 43888) lacking Shiga toxin genes was used as a parental wild-type (WT) strain for creating deletion mutants. Bacteria were regularly cultured in Luria-Bertani (LB) broth. To activate the TTS of EHEC, minimal M9 medium or Dulbecco's Modified Eagle Medium (DMEM) at 37 °C with 5 % CO₂ was used. Selection media were supplemented with ampicillin (100 µg ml⁻¹) or chloramphenicol (34 µg ml⁻¹) when required. Oligonucleotides were purchased from either Prisma Biotech or MDBio. Restriction enzymes (New England Biolabs), T4 DNA ligase (TaKaRa) and Taq DNA polymerase (Protech) were used during cloning. DNA sequencing was routinely carried out after PCR cloning and this was done by a contract service (Mission Biotech).

Generation and verification of the deletion mutants. To delete *l0017* from the EHEC chromosome, a previously used method of homologous recombination without inserting a selectable marker was employed (Link *et al.*, 1997; Tsai *et al.*, 2006). First, a 5' fragment flanking *l0017* was amplified from the chromosomal DNA of the parental WT strain (WT) using primers L17-33909F (5'-GCTGAGATCTTGCAGAC-3') and L17-34978R(*Xba*I) (5'-TGCTCTAGACCGCCACACAGTATCTTATT-3'). The PCR product was ligated into the pGEM-T Easy vector (Promega) to create pGEMT-1/2-L17. A plasmid, pGEMT-3/4-L17, containing an *l0017*-3' flanking fragment, was similarly generated using primers L17-35188F(*Xba*I) (5'-TGCTCTAGAGGTAGTGGCTGGGTACGAGGATTT-3') and L17-36194R(*Sal*I) (5'-GTCGACGACTTTTAAGCTCTGTGCGC-3'). From the above plasmids, the two *l0017*-flanking fragments were cut and ligated to take advantage of the engineered *Xba*I and *Sal*I sites within the PCR primers; by so doing, pGEMT-A/B-L17 was obtained. This plasmid was subsequently digested with *Not*I and *Sal*I and the fragment encompassing the two *l0017*-flanking segments was gel-purified and ligated with *Not*I/*Sal*I-restricted pKO3 (Link *et al.*, 1997), to generate pKO3-d17. Thereafter, pKO3-d17 was transformed into the EHEC WT strain to create an *l0017*-deleted mutant strain, which was named ΔL17. To confirm the construction of the *l0017*-deleted strain, PCR was performed using two primer pairs (Fig. 1). Amplification using the primers L19-34637F(*Xba*I) (5'-TGCTCTAGACGGAATTTGGTTCGT-3') and L19-35660R(*Sal*I) (5'-GTCGACGCGGGCTTAAACCTAAAGC-3') should give a fragment of 1035 bp from the WT and of 800 bp from ΔL17. A second primer pair L17 F (5'-ATGGTTAATGATATTTCTGC-3') and C1R (5'-CCACTCGAGTTAAATCCTCGTACCCAGCC-3') was also used that should amplify a DNA fragment of 280 bp from the WT and a 50 bp fragment from ΔL17. In both cases the putative deletion strain ΔL17 produced the latter fragment sizes, confirming deletion of *l0017*.

Fig. 1. Schematic representations of the *LEE4* operon in the parental (WT) EHEC strain and in the *l0017*-deleted mutant (ΔL17). Homologous recombination was used to generate ΔL17; the remaining nucleotide sequence of *l0017* with amino acids encoded is shown. Primers used for PCR amplification and deletion confirmation are also shown with the relative positions marked.

Generation of the *l0036* deletion mutant (ΔL36) has been described previously (Tsai *et al.*, 2006).

Plasmid construction. The entire *l0017* DNA fragment was amplified by PCR using primers pUC(-)FB (5'-ACTAGTGGATCC-AGAATT-3') and pUC(-)RK (5'-ATGGTACCGCCGCCACTCAT-3') from pUC-T-L17, which contains the full-length *l0017*. The PCR product was cut with *Bam*HI/*Kpn*I and ligated into pQE-30 (Qiagen), which had been digested with the same enzymes, to create pQH-L17. This construct was designed to express full-length L0017 protein with a RGS-His×6-tag attached to the N terminus. The DNA in the construct was verified by sequencing. To clone *espA* into pQE-60 (Qiagen), primers ESPA-1 (5'-CTAACCATTGGATACATCAAAATGCA-3') and ESPA-3R (5'-CGCAGATCTTTTACCAAGGGATATTGC-3') were used to amplify *espA* from EHEC genomic DNA. The PCR product was then digested with *Nco*I and *Bgl*II, and cloned into *Nco*I/*Bgl*II-restricted pQE-60 to give pQ-EspA. Plasmid pB₃₁₂, expressing *espB*, was also derived from pQE-60 and has been described previously (Chiu *et al.*, 2003); it was renamed pQ-EspB for this paper.

Transcriptional fusion and translation fusion reporter constructs. The EspA-LacZ translational fusion construct pKMespAL and the transcriptional fusion construct pKMespAX were generated using pKM005 that contains a promoterless *lacZ* reporter gene (Masui *et al.*, 1983; Lee & Cerami, 1987). The *espA* fragment was PCR-amplified from pQ-EspA using the primers XT5 (5'-GCTCTAGACCCGAAAAGTGCCACCTG-3') and ESPARB (5'-TTGGATCCTTACCAAGGGATATTGC-3'). The PCR product was restricted with *Xba*I/*Bam*HI and ligated into pKM005 that had been digested with the same enzymes; the result was pKMespAL. Similarly, *espA* was PCR-amplified using the primers XT5 and ESPARX (5'-GCTCTAGATTATTACCAAGGGATATTGC-3'), and the product was treated with *Xba*I and then ligated into *Xba*I-treated, calf intestinal phosphatase-digested pKM005 to generate pKMespAX.

pEspA-His-L0017 was constructed by amplification of *espA* from pQ-EspA using primers *Xho*I-EspA-F (5'-CCGCTCGAGAAATCATAAAAAAT-3') and *Xho*I-EspA-R (5'-CCGCTCGAGTTTACCAAGGG-3'). The PCR product was inserted into *Xho*I-treated pQE-30 after

digestion with *Xho*I. The resulting plasmid, pT5-EspA, was next restricted with *Bam*HI and *Kpn*I, and ligated with a *Bam*HI-*Kpn*I fragment obtained from pQH-L17 that encodes RGS-His $\times$ 6-tagged L0017. The plasmid thus obtained simultaneously encodes tag-free EspA and RGS-His $\times$ 6-tagged L0017. By making the above construction, expression of each gene in either transcriptional fusion or translational fusion is driven by an exogenous T5 promoter.

Measurement of β -galactosidase activity. The method (Miller, 1972) used ONPG as the substrate. Basically, bacterial transformants were cultivated at 30 °C, and three representative colonies were picked and measured in triplicate for enzyme activity. Each value was individually calibrated with respect to bacterial growth density (Miller, 1972).

Adherence activity of bacteria to HeLa cells. A previously described method (Chiu & Syu, 2005) was followed with a slight modification. HeLa cells were seeded in 6-well plates (2×10^5 cells per well) in DMEM supplemented with 10% fetal calf serum (FCS) and ampicillin ($100 \mu\text{g ml}^{-1}$) for 40 h. Prior to infection, the wells were washed with DMEM without FCS. The monolayer of cells was infected with the various EHEC strains, which had been grown overnight and then diluted 1:100. The infections were then allowed to proceed for 6 h. After discarding the supernatants, the wells were washed with phosphate-buffered saline (PBS) five times at 4 °C. Then the HeLa cells were scraped into new microcentrifuge tubes and pelleted down by centrifugation at 1800 g for 5 min at 4 °C. Finally, the HeLa cells were lysed and the total number of bacteria associated with the cells in a well was counted on LB-ampicillin agar plates.

Actin fluorescence staining of HeLa cells. Immunofluorescence microscopy analysis was carried out as described by Kresse *et al.* (1999).

Immunoblotting. EHEC-secreted proteins and bacterial total protein lysates were prepared and analysed as described previously (Chiu & Syu, 2005; Kenny *et al.*, 1997a). Anti-L0017 was prepared by immunizing mice with purified recombinant proteins from *E. coli* JM109. Anti-EspA, anti-EspB and anti-Tir have been described previously (Tsai *et al.*, 2006). Immunoblots were developed with Renaissance Western Blot Chemiluminescence Reagent Plus (NEN) and the images were captured using X-ray film (Fuji).

Bacterial cell fractionation. To separate bacterial proteins into fractions, the method of Neves *et al.* (2003) was slightly modified. EHEC was inoculated into M9 medium (200 ml) and grown for 6 h at 37 °C in the presence of 5% CO₂. The bacteria were then pelleted by centrifugation at 4 °C. After removing the supernatants, the pellets were weighed and resuspended by adding 50 ml osmotic shock buffer A (20% sucrose, 20 mM Tris, pH 8.0) g⁻¹. EDTA, at a final concentration of 1 mM, was subsequently added and this was followed by gentle shaking at 4 °C for 10 min. The cells were then centrifuged at 8000 g for 20 min at 4 °C and then the supernatants were removed. The pellets were resuspended by adding 50 ml osmotic shock buffer B (5 mM MgSO₄) g⁻¹, which was followed by gentle shaking at 4 °C for 10 min. The supernatants were collected by centrifugation as described above. The resulting supernatants were combined for individual cultures and the periplasmic proteins thus obtained were concentrated using a Centricon (Millipore). The bacterial pellets were resuspended in 16 ml lysis buffer (100 mM Tris/HCl, pH 7.5, 1.0 mM PMSF, 0.5 μl aprotinin ml⁻¹) and the bacteria were disrupted by five passages through a French pressure press. After centrifugation at 14 000 g for 20 min at 4 °C, the collected supernatants were spun in an ultracentrifuge at 80 000 g for 30 min at 4 °C. The resulting supernatants were then concentrated using a Centricon and saved as the cytosolic fraction. The pellets with the membrane fractions were further processed into outer- and

inner-membrane fractions by washing once with lysis buffer and resuspension in 1.6 ml sarkosyl buffer (10 mM Tris/HCl, pH 8, containing 100 mM NaCl, 1.0 mM PMSF, 0.5 μl aprotinin ml⁻¹ and 0.5% *N*-lauroylsarcosine). The suspensions were incubated at 4 °C for 4 h to obtain suitable dissolution of the inner-membrane proteins. After another ultracentrifugation step, the supernatants containing the inner-membrane proteins were obtained and subsequently concentrated using a Centricon. Finally, the remaining pellets were dissolved in SDS sample buffer and these samples were defined as the outer-membrane fraction. To inspect how well the fractionation was performed, proteins known to be enriched within each compartment were examined by Western blotting. CesD2 has been reported as a chaperone for EspD (Neves *et al.*, 2003) and this protein is found in both the inner-membrane and the cytosolic fraction; the protein was detected with mouse anti-CesD2 polyclonal antibodies (M. S.-W. Su & W.-J. Syu, unpublished data). The outer-membrane protein OmpC was detected by a mouse polyclonal anti-OmpC. The periplasmic maltose-binding protein (MBP) was detected using a mouse mAb (SC1D7) (Hsu *et al.*, 1997).

Co-purification of EspA and L0017. Affinity binding of a His $\times$ 6-tagged protein to a Ni²⁺-NTA agarose column (Qiagen) was carried out as described previously (Chiu & Syu, 2005) except for the buffers. In this case, the buffer used for equilibration and lysis was 50 mM NaH₂PO₄, pH 8.0, containing 300 mM NaCl, 10 mM imidazole and 1 mM PMSF, and the washing buffer consisted of 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl and 50 mM imidazole. Finally, the elution buffer was identical to that used for washing except that imidazole was added at 250 mM.

Bacterial two-hybrid assay. The system (Stratagene) was used as described previously (Tsai *et al.*, 2006). In essence, pBT, which encodes the λ CI protein, was used to produce a bait protein fused with λ CI, and pTRG was used to generate a target protein fused to the C terminus of an N-terminal domain of the RNA polymerase α subunit. Interactions of the bait and target proteins in the system would then yield a high level of β -galactosidase expression, the activity of which was measured as described above.

RESULTS

Effect of *l0017* deletion on cell adhesion and actin rearrangement

To address the role of *l0017* in the EHEC TTS, the entire gene, except for the region encoding the first 15 N-terminal residues was deleted by homologous recombination (Fig. 1). The resultant mutant strain (Δ L17) was confirmed by PCR using two sets of primer pairs that gave amplified PCR fragments of the expected sizes. Direct sequencing of an 800 bp PCR fragment, amplified using primers L19-34637F(*Xba*I) and L19-35660R(*Sal*I), indicated a precise excision at the intended junction (Fig. 1). This mutant was then tested for adherence activity with HeLa cells. Δ L17 appeared to have lost most of its adherence activity, retaining only 4% residual adherence when compared to the parental strain transformed with the control vector pQE-30. After complementation with L0017 expression from pQH-L17, this mutant's adherence activity was restored to 94% of the control. When Δ L17 was used to infect HeLa cells that were subsequently examined by immunofluorescence staining, no cytoskeleton

rearrangement and actin aggregation were observed (data not shown).

Localization of L0017 in the inner-membrane fraction

To elucidate the potential function of *l0017*, it is of value to establish the location to which L0017 moves after ribosomal synthesis. To do so, WT bacteria were activated to express LEE proteins in M9 medium, and total bacterial lysate was fractionated. No fraction gave a positive L0017 signal when analysed by Western blotting using a specific antibody raised against recombinant L0017. We reasoned that the intrinsically expressed L0017 might be at such a low level that it was beyond the sensitivity of the Western blot (Fig. 2a). To circumvent this difficulty, plasmid pQH-L17 was transformed into the EHEC WT strain and protein fractionation was carried out again. Proteins from the individual fractions were likewise analysed by Western blotting, except that an additional antibody, anti-His $\times$ 6 tag, was used (compare Fig. 2a and b). Fig. 2(b) shows that L0017 was concentrated in the inner-membrane fraction (lane IM). The identification of L0017 in the inner-membrane fraction is supported by positive detection using both anti-L0017 and anti-His $\times$ 6. The fractionation was

itself successful as shown by the correct detection of the fraction marker proteins OmpC, MBP and CesD2.

Effect of deleting *l0017* on representative TTS proteins

The presence of L0017 in the inner membrane supports the notion that L0017 is a component of the TTS apparatus (Deng *et al.*, 2004). To further explore the effect of deleting this gene, the synthesis and secretion of representative LEE proteins were examined. Like the *C. rodentium* Δ orf29 strain (Deng *et al.*, 2004), no Tir, EspA and EspB were detected in the spent media produced by Δ L17 (Fig. 3b, lane 2), unless Δ L17 was further transformed with pQH-L17 (Fig. 3b, lane 3). It is worth noting that overexpressed L0017 was not found in the concentrated media and was only found in the bacterial lysate (Fig. 3a). In the bacterial lysate, it was also notable that the deletion of *l0017* affected the intracellular level of EspA, but not that of Tir or EspB (Fig. 3a, lane 2). Again, the decrease in the level of EspA in Δ L17 could be restored by complementation by L0017 expression ectopically (Fig. 3a, lane 3).

Effect of deleting *l0017* on ectopically expressed EspA

To exclude the possibility that the transcription of *LEE4* was modulated by L0017, the expression levels of EspA and EspB driven by the same exogenous T5 promoter based upon pQE-60 were examined (Fig. 4a, lanes 3–6). While comparable levels of EspB were detected in the WT and Δ L17 strains (lanes 5 and 6), the levels of EspA in the two strains differed greatly (lanes 3 and 4). Therefore, the absence of L0017 indisputably affects the intracellular level of EspA, regardless of their associated promoters. To carry out controls, we examined expression in two additional mutants of EHEC. In *C. rodentium*, it has been observed that deletion of *orf16* (equivalent to EHEC *l0032*) affects the secretion of translocators, but favours that of effectors. Therefore, we created an *l0032* deletion mutant (Δ L32) (J. C.-W. Lio & W.-J. Syu, unpublished results), and repeated the transformation and analyses for EspA and EspB as described above. The results of Fig. 4(b) show that there is no apparent difference in the intracellular levels of these proteins between the WT and Δ L32 strains (EspA, lanes 1 and 2; EspB, lanes 3 and 4). The observed protein levels in the second mutant, Δ L36 (Fig. 4c), were similar to those observed in the WT and Δ L32 strains. Therefore, the low expression level of EspA in Δ L17 is a unique property. It is worth noting that Δ L36 (Δ orf12 in *C. rodentium*) differs from Δ L32 by a complete loss of secretion in both translocators and effectors, as reported by Deng *et al.* (2004) and confirmed in Fig. 4(d). It is therefore conceivable that the intracellular level of EspA in a LEE mutant is independent of bacterial TTS capacity.

To substantiate the notion that L0017 affects EspA expression through a transcriptional event, rather than

Fig. 2. Biochemical localization of L0017. EHEC was transformed with or without plasmid pQH-L17 (expressing *l0017*) and the bacteria were cultured to activate the LEE island. The cultures were separated into total bacteria (T) and spent media (S), the latter being subsequently concentrated. After disruption in a French pressure press, the bacteria were fractionated into cytoplasm (C), inner-membrane (IM), outer-membrane (OM) and periplasm (P) fractions. Samples were analysed by Western blotting with antibodies against representative proteins enriched in individual fractions: OmpC (40 kDa), outer-membrane porin C; MBP (43 kDa), periplasmic maltose-binding protein; CesD2 (16 kDa), inner-membrane chaperone for EspD (Neves *et al.*, 2003). Anti-L0017 is a polyclonal antibody raised in mice against RGS-His $\times$ 6-tagged L0017, whereas commercial anti-His $\times$ 6 mAb reacts only with the His $\times$ 6 tag.

Fig. 3. Western blotting analysis of representative EHEC secreted proteins. (a) Proteins in the cell lysate. Total proteins were prepared from bacteria cultured for 5 h in M9 medium at 37 °C in the presence of 5% CO₂. Proteins were examined by immunoblotting using specific antibodies. pQH-L17 encodes recombinant L0017 N-terminally tagged with His $\times$ 6. Note the absence of detectable EspA (dashed box). (b) Proteins secreted into M9 medium. The spent media of EHEC cultures in (a) were concentrated and studied similarly by Western blotting. None of the proteins were detected in the Δ L17 culture supernatant unless the deletion mutant was transformed with pQH-L17.

through control of the *espA* mRNA level, reporter assays were carried out. *lacZ* was fused downstream from *espA* in two ways, as shown in Fig. 5. First, a transcriptional fusion was made in pKMespAX where *lacZ* with intact translational initiation elements was inserted immediately downstream of the *espA* stop codon (Fig. 5a). By doing

so, the T5 promoter controls the transcription of an mRNA encoding both *espA* and *lacZ*. Since EspA and β -galactosidase are independently translated but share a common mRNA, any factors from the different host cells that hinder transcription or accelerate the degradation of the mRNA are likely to affect β -galactosidase activity.

Fig. 4. Effect of gene deletion on EspA expression in EHEC. (a)–(c) Bacteria were transformed with an EspA-expressing plasmid or an EspB-expressing plasmid. The expression levels of the His $\times$ 6-tagged proteins in the total bacterial proteins were monitored by Western blotting using anti-His $\times$ 6. Detection of OmpC was used to assure comparable protein loadings in each lane. Note, Δ L32 is a deletion mutant control with a similar construction to Δ L17 except that *I*0032 within the LEE was deleted; as a consequence, the secretion of translocators is impaired, but not that of effectors (Deng *et al.*, 2004). Δ L36 is a similarly constructed but distinct mutant with a deletion at *I*0036 (Tsai *et al.*, 2006); it abolishes secretion of both translocators and effectors, but does not affect the intracellular levels of these proteins. (d) Western blotting with different antibodies to detect the corresponding proteins in the bacterial lysate and spent medium, to support the characteristics of Δ L36 described above.

Fig. 5. Reporter assay to study the effect of L0017 on EspA expression. (a) Reporter gene *lacZ* preceded by *espA* in the same operon in a transcriptional fusion. In pKMespAX, a T5 promoter-driven mRNA encodes EspA and β -galactosidase that both have independent translation initiation and stop codons. Presumably, any effect of a factor on the nucleotide sequence ahead of *lacZ* would be detected through β -galactosidase activity. (b) EspA fused to β -galactosidase in a single ORF. In pKMespAL, the coding sequence of *lacZ* was directly fused to the 3' end of *espA* (without a stop codon) so that a fusion protein EspA- β -galactosidase is produced. Any external effects on translation or the stability of the fusion protein would be observed by changes in reporter activity. (c) Comparison of reporter activities when assayed in congenic bacteria. Black bars, WT; grey bars, Δ L17 pKM005, control vector used for creating pKMespAX and pKMespAL.

Second, a fusion of *lacZ* in-frame with the 3' end of *espA* before the stop codon was constructed in pKMespAL; in this case β -galactosidase translation directly follows that of EspA (Fig. 5b) because β -galactosidase with an N-terminal EspA fusion is produced when *espA-lacZ* is transcribed, and this is subsequently translated. Any effect of the host cells (with or without L0017) on the translation process or

on the degradation of the fusion protein will be reflected in the level of β -galactosidase activity.

After the two constructs were separately transformed into bacteria, the β -galactosidase activity of the bacteria was measured (Fig. 5c). The enzyme activity was seen to show a 6.8- and 8.1-fold increase in the WT and Δ L17 strains, respectively, when the transformants containing pKMespAX were compared to those carrying the control plasmid (pKM005). On the other hand, when the β -galactosidase activities were compared with those from the pKMespAL transformants, an intriguing difference was observed. There was a 9.6-fold increase in enzyme activity from the pKMespAL-transformed WT strain compared to that from the pKM005-transformed WT, whereas there was an opposite effect (a 22-fold reduction) in the case of pKMespAL-transformed Δ L17 compared to Δ L17 carrying pKM005. The 1.2-fold difference found between the transcriptional fusion data obtained with the WT and Δ L17 strains is likely to be due to experimental variation. In contrast, the 210-fold difference seen between the two translational fusion (pKMespAL) results strongly suggests that L0017 regulates at the protein level after the transcription.

There are a number of possible reasons for the post-transcriptional effects of the L0017 protein. These include the stability of the translated protein, which is the easiest scenario to test. To do this, pQ-EspA was transformed separately into the WT and Δ L17 strains. After inducing LEE expression for 5 h, ribosomal translation was blocked with chloramphenicol (at a final concentration of 200 μ g ml⁻¹). The existing intracellular EspA was then detected over a period of time by Western blotting. Fig. 6(a) shows that the level of EspA in Δ L17 declined quickly within the first hour. By the second hour, EspA is barely detectable. On the other hand, in the WT strain, EspA was relatively stable during the first 2 h, and substantial amounts of EspA remained detectable after inoculation for 16 h (Fig. 6b). To exclude the possibility that the decreased stability of EspA in Δ L17 is simply due to degradation of EspA arising from the loss of bacterial secretion capability, we performed a similar experiment in the control Δ L36 mutant. Fig. 6(c) shows that EspA was comparably detected in Δ L36 over the same period of time as seen in the WT strain (Fig. 6d). Therefore, it would seem that the presence of L0017 stabilizes intracellular EspA in EHEC.

Interaction of EspA and L0017

To determine whether the stabilization of EspA by L0017 is brought about through direct interaction, pEspA-His-L0017 was used to express RGS-His $\times$ 6-tagged L0017 together with a tag-free EspA. Affinity chromatography using a nickel ion column was carried out to purify RGS-His $\times$ 6-L0017 and the protein from the column was used to test whether EspA co-purified with the His-tagged protein. Fig. 7(a) shows that EspA was abundantly detected in the eluted fractions when the bacterial proteins loaded

Fig. 6. Stability of EspA in bacteria. Bacteria were transformed to express EspA from pQ-EspA. After cultivation in M9 medium for 5 h, bacterial protein synthesis was arrested by chloramphenicol and culturing was continued for a period of time before harvesting. Harvested bacteria were dissolved in SDS sample buffer and processed for Western blotting using anti-His $\times$ 6 tag antibody. Detection of OmpC was carried out to assure comparable protein loading. In the different experiment panels, the parental WT strain was similarly treated and run in parallel for comparison.

into the column were prepared from bacteria harbouring pEspA-His-L0017. Remarkably, it was seen that EspA was most prominently detected in fractions 2–4 with intensities directly proportional to the amount of His $\times$ 6-L0017 detected, a fact that unequivocally reveals an interaction between EspA and L0017. In contrast, in a control where EspA alone was expressed from pT5-EspA, EspA was detected only in the pass-through and washing fractions, but not in the eluant. Therefore, these biochemical data support that the interaction between EspA and L0017 does occur when they are expressed in the same bacterium. To corroborate this interaction, a bacterial two-hybrid assay was performed (Fig. 7b, inset). When L0017 was cloned as bait, a positive signal was found after pairing with the target of EspA. The interaction seemed to be as strong as L0017 paired with L0053, a previously reported binding protein in the yeast two-hybrid system (Creasey *et al.*, 2003b).

DISCUSSION

L0017 is a small protein of 92 aa with a predicted pI of 5.5. We have shown that EHEC with an *l0017* deletion has a phenotype similar to that of *C. rodentium* with an *orf29* deletion (Deng *et al.*, 2004). Secretion of translocators (exemplified by EspB) and effectors (exemplified by Tir) were disrupted and the bacterial activities of cell adherence and actin accumulation disappeared. In addition, we located L0017 in the inner-membrane fraction of EHEC,

a biochemical property that is consistent with the proposed role of the protein as a TTS component (Deng *et al.*, 2004).

In examining whether L0017 has any additional functions, we have found for the first time that a lack of an inner-membrane component from EHEC resulted in a decrease in the intra-bacterial level of EspA, but not of EspB or Tir. Mechanistically, we have ruled out the possibility that L0017 regulates the EspA level at the corresponding promoter. The first line of evidence was obtained by placing *espA* under the control of an exogenous promoter, such as the T5 promoter, and we found that Δ L17 produced a low level of intracellular EspA when compared with its congenic counterpart (Fig. 4). Second, by using a reporter gene transcriptionally fused downstream to *espA*, the reporter activity of the two strains demonstrated no apparent difference (Fig. 5c). Therefore, it would seem that L0017 regulates EspA expression at a level other than transcription.

When a translational fusion assay was carried out to monitor the EspA- β -galactosidase fusion protein (Fig. 5b), a 210-fold difference was found between Δ L17 and its parental strain (Fig. 5c). This difference was consistent with the direct detection of the EspA protein seen in Fig. 4. Therefore, these results indicate that for EHEC to keep a constant and high level of EspA in the cytoplasm, the presence of L0017 is required, perhaps to retain an effective TTS system. The amount of L0017 needed is far less than that of EspA, as revealed by the fact that the level of

(a) pEspA-His-L0017

(b) pT5-EspA

Fig. 7. Interaction of EspA and L0017. (a) Analysis of co-eluted EspA and L0017 in fractions obtained by affinity chromatography using a nickel-NTA column. Bacterial lysate from pT5-EspA-His-L0017-transformed *E. coli* JM109 was loaded onto the column and the flow-through fraction was collected. After washing with 50 mM imidazole, the retained proteins were eluted with 250 mM imidazole. Proteins from these fractions were detected for EspA and L0017 by Western blotting using anti-EspA and anti-Hisx6, respectively. (b) Analysis of EspA in chromatography fractions as in (a) except that the bacterial lysate was prepared from JM109 harbouring pT5-EspA. EspA interacting with L0017 was alternatively assayed with a bacterial two-hybrid system (inset). Positive interactions of the cloned target and bait proteins were reported by an increase in β -galactosidase activity. A reported interaction between L0017 and L0053 (Creasey *et al.*, 2003b) and that between the dimerization domain of yeast transcriptional activator Gal4 (LGF2) and a domain derived from a mutant form of GAL11 (Stratagene) served as positive controls. Vectors only or vector with no gene cloned as a bait or target served as negative controls.

intrinsic L0017 was very hard to detect in the WT strain. However, when L0017 is missing, newly synthesized EspA is degraded quickly and the intracellular level of EspA drops aberrantly.

EspA constitutes the major part of the filamentous surface appendages of pathogenic *E. coli* (Ebel *et al.*, 1998) and it alone is sufficient to form filamentous structures in the absence of other LEE proteins *in vitro* (Delahay *et al.*, 1999; Yip *et al.*, 2005). How this protein, which has a strong tendency to polymerize, reaches the extracellular surface, remains a puzzle. In a previous model proposed by Yip *et al.* (2005), the newly synthesized EspA is maintained partially unfolded, with two extensive coiled coils preserved after binding to its intracellular chaperone, CesAB. Since it is known that the CesAB chaperone is not secreted and thus can only act to maintain the monomeric status of EspA monomeric inside the bacterium (Yip *et al.*, 2005), a component of the TTS system must have an adaptor that allows consecutive transit of EspA. From our data above, it can be seen that once the TTS is handicapped by a lack of L0017, intracellular EspA destabilizes. A bridging protein on the membrane apparently is needed to prevent EspA degradation. A study with a yeast two-hybrid system did not detect EspA interacting with a membrane component

of the TTS system, except for the known CesAB chaperone (Creasey *et al.*, 2003b). However, such an interaction within the bacterium cannot be completely ruled out by a genetic system that functions in yeast. A prominent undetected example is the interaction of CesD2 and EspD (Creasey *et al.*, 2003b; Neves *et al.*, 2003). Inner-membrane association and the ability to stabilize EspA have made L0017 a compelling candidate for handing over the unfolded EspA. This is supported by the co-expression experiment, and we have demonstrated that EspA and L0017 indeed do interact with each other.

Two chaperones (CesD in the cytosol and CesD2 in the inner-membrane fraction) have been found with EspD, which has a high tendency to aggregate (Daniell *et al.*, 2001). However, so far, CesAB is the only known chaperone reported to both stabilize and directly interact with EspA. The characteristic whereby L0017 interacts with EspA, stabilizes EspA expression and is localized on the inner membrane are analogous to those seen with CesD2 and EspD (Neves *et al.*, 2003). Therefore, L0017 should probably be categorized as one of the TTS chaperones and named CesA2, as the second EspA chaperone. This uniqueness makes CesA2 distinct from the inner-membrane-associated SepD-SepL that may

form a molecular switch to ensure the secretion of translocators prior to effectors, but not to affect their stability (Deng *et al.*, 2005; O'Connell *et al.*, 2004). A speculation is that EspA chaperoned by CesAB and CesA2 may transit outwards via some kind of contact with the SepD-SepL complex. If so, it remains to be explored whether and how EspA interacts with these proteins, including those indirect binders such as L0053, to result in transient complexes.

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