Functional analysis of EspB from enterohaemorrhagic *Escherichia coli*

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In enterohaemorrhagic *Escherichia coli* (EHEC), the type III secretion protein EspB is translocated into the host cells and plays an important role in adherence, pore formation and effector translocation during infection. The secretion domain of EspB has been mapped previously. To define the other functional determinants of EspB, several plasmids encoding different fragments of EspB were created and analysed to see which of them lost the functions of the full-length molecule. One finding was that residues 118–190 of EspB were required for both efficient translocation of EspB and interaction of EspB with EspA. Additionally, the segment consisting of residues 217–312 was necessary for bacterial adherence. Furthermore, a predicted transmembrane domain (residues 99–118) was found to be critical for EHEC to cause red blood cell haemolysis, presumably by forming pores in the cell membrane. The same segment was also important for actin accumulation induced beneath the bacterial-attachment site. Taken together, these data indicate that the EspB protein (312 residues in total) has functions associated with its different regions. These regions may interact with each other or with other components of the type III system to orchestrate the intricate actions of EHEC during infection.

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INTRODUCTION

Escherichia coli O157:H7 is the major pathogen that causes a serious illness known as enterohaemorrhagic diarrhoea and haemolytic uraemic syndrome (Nataro & Kaper, 1998). Several virulence factors have been reported to contribute to the pathogenesis of this microbe. One of the factors is a type III secretion system (TTSS) that induces the attaching and effacement lesion (A/E lesion) of enterocytes. The TTSS is encoded by the locus of enterocyte effacement (LEE) island, which contains a total of 41 genes. Homologous LEE islands have been found in Gram-negative pathogens, such as enteropathogenic *E. coli* (EPEC) and *Citrobacter rodentium* (McDaniel & Kaper, 1997; Schauer & Falkow, 1993), but not reported to exist in other, non-pathogenic *E. coli*.

Most of the LEE genes are organized into five major operons: *LEE1*, *LEE2*, *LEE3*, *LEE4* and *LEE5*. EspA (Kenny *et al.*, 1996), EspB (Donnenberg *et al.*, 1993) and EspD (Donnenberg *et al.*, 1993; Lai *et al.*, 1997) are encoded by *LEE4* and secreted by the TTSS; deletion of these three genes abolished the translocation of effector proteins, such as Tir (Kenny *et al.*, 1997), EspF (Crane *et al.*, 2001) and MAP (Kenny & Jepson, 2000). EspA is believed to form a hollow, filamentous structure on the bacterial surface to deliver the

effector proteins (Knutton *et al.*, 1998) and it may also play a role in adherence of enterohaemorrhagic *E. coli* (EHEC) to the host cell (Cleary *et al.*, 2004). EspD has been found to target the host-cell membrane (Wachter *et al.*, 1999), whereas EspB is translocated to both membrane and cytoplasm (Wolff *et al.*, 1998). Both EspB and EspD are believed to be involved in pore formation on the membranes of the infected cells (Ide *et al.*, 2001) and have been classified as translocators (Roe *et al.*, 2003). EspB has also been reported to interact with EspA (Hartland *et al.*, 2000) and EspD (Ide *et al.*, 2001), and complexes formed by these three proteins may participate in the initial step of bacterial adherence (Nougayrède *et al.*, 2003). On the host membrane, the N-terminal region of EspB has been found to bind directly to the intracellular α -catenin and to result in the recruitment of α -catenin underneath the bacterial-adherence site (Kodama *et al.*, 2002). Recently, it has also been demonstrated that host-cell α_1 -antitrypsin interacts with EspB and EspD, and this interaction has been implicated to block the formation of the EspB/EspD translocation pore (Knappstein *et al.*, 2004).

In our previous study on the functional domain of EHEC EspB, one essential region (residues 1–118) and two auxiliary segments (residues 118–190 and 191–282) were defined for EspB secretion (Chiu *et al.*, 2003). When appropriate conditions are supplied, such as the provision of M9 medium for cell cultivation, EspB and other TTSS-secreted proteins can be found in abundance in the medium.

Abbreviations: A/E lesion, attaching and effacement lesion; EHEC, enterohaemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; LEE, locus of enterocyte effacement; RBC, red blood cell; TTSS, type III secretion system.

In this study, we further dissected the EspB molecule and determined the regions required for its own translocation and its interaction with EspA. As bacteria lacking *espB* lose adherence ability (Donnenberg *et al.*, 1993), we also examined whether there is a relationship between the EspB structure and bacterial adherence. We report here the results from a series of deletion analyses.

METHODS

Bacterial strains and cell culture. EHEC strain 43888, which does not produce either Shiga-like toxin I or II and lacks *stx* genes, was used in this study; this strain and its mutant with *espB* deleted (*E. coli* 43888 Δ B) have been described previously (Chiu *et al.*, 2003). The *tir* deletion mutant (*E. coli* 43888 Δ T) was constructed similarly. In brief, bacterial chromosomal DNA was used as the template for PCR amplification. Primers Lee-22239 (5'-CAGAAAG-TTGATGAGCAGTGC GGC-3') and NTir-171R (5'-TATCAATCAG-CCATAGAATTCC-3') were used to amplify a fragment covering a 5' flanking region and a small 5' sequence of *tir*. The obtained PCR fragment was digested with *Bam*HI, which made a single cut near the 5' end of the amplified sequence. A second PCR using primers NTir-F (5'-TTATGCCTATTGGTAACCTTGG-3') and NTir-R (*Pst*I) (5'-CAGCTGCAGACGAAACGATGGGATCCCG-3') was performed to generate a fragment covering a 3' segment of *tir*; the nucleotide sequences recognized by restriction enzymes are underlined. This PCR product was digested with *Bam*HI and *Eco*RV. Then, these two PCR products were three-way-ligated into *Bam*HI-restricted PinPoint Xa-3 (Promega) to result in PinPoint Xa-3- Δ Tir. To construct pKO3- Δ Tir, PinPoint Xa-3- Δ Tir was digested with *Not*I/*Nru*I and the fragment containing the *tir*-flanking regions was ligated into *Not*I/*Sma*I-digested pKO3 (kindly provided by G. M. Church, Harvard University, MA, USA). The resulting plasmid was electroporated into *E. coli* 43888. Bacteria with homologous recombination were then selected and confirmed with an expected deletion at *tir* as described previously (Chiu *et al.*, 2003).

Bacteria were regularly grown in LB medium (Difco). To activate the type III secretion, bacteria were transferred into M9 medium. Media were supplemented with ampicillin (100 μ g ml⁻¹) or chloramphenicol (34 μ g ml⁻¹) when necessary. HeLa cells were maintained in Dubelcco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum and cultured at 37 °C in the presence of 5% CO₂.

General recombinant DNA techniques. Unless otherwise stated, restriction endonucleases, DNA-modifying enzymes and polymerases were purchased from New England BioLabs. DNA-manipulation procedures were followed either as described by Sambrook & Russell (2001) or as recommended by the manufacturers. DNA was purified from a mini-column (Qiagen) and sequenced automatically by a contract service (Mission Biotech).

Plasmids. To create plasmid pBP₃₁₂D, pB₃₁₂D (Chiu *et al.*, 2003) was amplified with primers PQE60-2R (5'-TGACTGCAGGGTTAA-TTCTCCTC-3') and PEspB-9 (5'-TAAGTCGAGATGAATACTA-TTGATAAT-3'). After amplification, the inverted PCR product was digested with *Pst*I and self-ligated by using T4 DNA ligase. To create plasmids pB1-312, pB1-282, pB1-250, pB1-220 and pB1-190, pBP₃₁₂D was used as the template and PCR-amplified with primer pairs PB939RS (5'-CGCGGATCCTTACCCAGCTAAGCGACCCG-3')/PEspB-9, PB846RS (5'-CGCGGATCCTTAATCATCCTGCGTTCT-GCG-3')/PEspB-9, PB750RS (5'-GCGGGATCCTTAAGTCGATTT-GACGACTC-3')/PEspB-9, PB660RS (5'-GCGGGATCCTTACAG-TTTACTACGGAATTCAA-3')/PEspB-9 and PB570RS (5'-CGC-GGATCCTTAACATCATCTGCAACGCC-3')/PEspB-9, respectively.

After amplification, the PCR products were digested with *Pst*I/*Bam*HI and ligated with the vector derived from pBP₃₁₂D digested with the same enzymes. Plasmids pBA191-253, pBACC, pBA118-190 and pBATM were created by an inverted PCR method (Vandeyar *et al.*, 1988; Weiner *et al.*, 1993) using pB312 as template. The primer pairs used were PB570R (5'-TTAAACATCATCTGC-AACGCC-3')/PB760 (5'-AATGAACAACGTGCGAAG-3'), PB558R (5'-AACGCCAGATGCACGGCT)/PB649 (5'-GTAGATAAACTGA-CCAATACC-3'), PEspB-14R (5'-TGCTGCAAAAGAACCTAA-3')/PEspB-19 (5'-GCGAAAGCCACTGAC-3') and PB294R (5'-AGC-GGTTGCCGCGGCTTT-3')/PB355 (5'-AACACGCGGCTAAAG-GG-3'), respectively. The PCR products were phosphorylated by T4 DNA kinase and then self-ligated to produce the designated constructs. After so doing, these plasmid-encoded EspB constructs had authentic EspB N termini and carried no tag at the C termini of the molecules.

To create pEspA-His, the entire *espA* sequence was PCR-amplified from the chromosomal DNA of *E. coli* 43888; the primers used were PEspA-1 and PEspA-3R. These had the nucleotide sequences 5'-CTAACCATGGATACATCAAATGCA-3' and 5'-CGCAGATCT-TTACCAAGGGATATTGC-3', respectively. After PCR amplification, the obtained DNA fragment was digested with *Nco*I/*Bam*HI and cloned into *Nco*I/*Bam*HI-restricted pQE60 (Qiagen); the resulting pEspA-His expressed a recombinant EspA tagged with a hexahistidine epitope at the C terminus (i.e. EspA-His₆).

Fractionation of cell components after bacterial infection. A previously described method (Gauthier *et al.*, 2000) was followed with a slight modification. In brief, HeLa cells in 100 mm plates cultured to 80% confluence were washed with PBS. Overnight-grown bacteria were diluted 1:100 (v/v) in DMEM and added to the HeLa cells for 6 h. After infection, the cells were washed twice with PBS and scraped off the plates into a cold imidazole buffer (3 mM, pH 7.4) containing 250 mM sucrose, 0.5 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride and 1 μ M pepstatin. The mixture was passed through a 22-gauge needle eight times and the unbroken cells with the bacteria were pelleted down by centrifugation twice at 3500 g for 15 min. To collect the disrupted membrane fraction of the cells, the recovered supernatant was further centrifuged at 20 000 g for 45 min. Cytosolic proteins in the supernatant were precipitated by adding 1/9 vol. trichloroacetic acid and further incubated at 4 °C for 1 h. Proteins in the precipitates were then collected by centrifugation. Proteins from each sample were dissolved in SDS sample buffer and separated by SDS-PAGE (12% gel), followed by analysis using Western blotting.

Immunoblotting. Western blotting analysis of antigens was performed as described previously (Hsu *et al.*, 2000). The anti-EspB antibody has been described previously (Chuang *et al.*, 2001) and anti-EspA was prepared by immunizing rabbits with gel-purified EspA from the secreted proteins of EHEC. Anti-OmpA was a mouse mAb prepared previously (Yu *et al.*, 2000). Mouse mAbs against syndecan (Santa Cruz) and actin (Chemicon) were obtained commercially. To detect the primary antibodies bound to the antigens on the nitrocellulose membrane, horseradish peroxidase-conjugated goat secondary antibodies (Sigma) were used. The membranes were finally developed by a chemiluminescence reagent (Hsu *et al.*, 2000).

Affinity purification with an Ni²⁺ column. Ni²⁺-NTA agarose beads (Qiagen) were packed into columns and charged with Ni²⁺ by passing through five bed volumes of 100 mM NiSO₄, and finally equilibrated in Tris-buffered saline (TBS). *E. coli* lysates derived from bacteria harbouring pEspA-His, pQE60, pB1-312, pB1-282, pB1-190, pBA118-190 or pBACC were prepared in TBS and clarified by centrifugation. The lysate containing EspB or its truncated form was mixed with an equal volume of that containing

EspA-His₆. The mixture was inverted gently at 4 °C for 1 h and then passed through the TBS-equilibrated Ni²⁺ column. After washing with TBS containing 150 mM imidazole, the proteins retained in the columns were eluted with 500 mM imidazole in TBS and analysed by Western blotting.

Adherence assay. Analysis of the adherence of EHEC to cells was modified from the method described by Gansheroff *et al.* (1999). In brief, 2×10^5 HeLa cells were plated in 12-well plates. After incubation overnight, the cells were washed with PBS and maintained in DMEM without any additives. Overnight-grown bacteria were diluted 1:100 (v/v) and added to the cells, and infection was maintained for 6 h. Thereafter, the cells were harvested and lysed in PBS containing 10% (w/v) saponin solution (5 mM Tris/HCl, pH 7.4, 0.4 mM NaVO₄, 0.1 mM PMSF) at 4 °C for 10 min. The lysates were then diluted serially in LB medium and plated on LB agar plates supplemented with ampicillin (100 µg ml⁻¹). After 16 h incubation at 37 °C, c.f.u. were scored and the relative adherence efficiencies were calculated.

Red blood cell (RBC) haemolysis assay. A previously described method (Warawa *et al.*, 1999) was followed. In brief, human RBCs, type B, were washed with PBS three times and suspended in PBS to a final concentration of 3% (v/v). The RBCs were then plated on poly-lysine-coated 12-well plates (700 µl per well) for 20 min at 37 °C. After two washes with PBS, the cells were kept in DMEM without phenol red. Overnight-grown bacteria were diluted 1:100 (v/v) to the RBC culture. After 6 h incubation at 37 °C in the presence of 5% CO₂, the culture medium was collected and centrifuged. The released haemoglobin was evaluated by measuring OD₅₄₃ of the supernatant with an ELISA reader (TECAN RainBow). Experiments were carried out in triplicate and the degrees of RBC haemolysis were calculated accordingly (Warawa *et al.*, 1999).

Immunofluorescence staining. HeLa cells were infected with bacteria as described above except for cultivation on glass coverslips in six-well plates. After infection, the cells were washed gently with cold PBS twice and fixed with 4% (v/v) paraformaldehyde for 20 min. The cells were then permeated with 0.5% Triton X-100 in PBS and blocked with 3% BSA in PBS (Lin *et al.*, 1999). To stain the bacteria, rabbit anti-O157 antibody (Difco) was used and the bound primary antibodies were in turn detected by FITC-labelled anti-rabbit immunoglobulin (Jackson Laboratory). To stain the actin filaments, cells were treated with tetramethylrhodamine isothiocyanate (TRITC)-labelled phalloidin at 5 µg ml⁻¹ (Jackson Laboratory) in PBS containing 1% (w/v) BSA. The stained cells on coverslips were examined by using a fluorescence microscope (Olympus BX51) and pictures were taken with a CCD camera (Photometrics CoolSNAP).

RESULTS

The segment of EspB critical for translocation

EspB is a type III-secreted protein that translocates into the membrane and cytoplasm of bacteria-infected cells (Wolff *et al.*, 1998). Our previous study demonstrated that the C-terminal region (residues 283–312) of EspB is completely dispensable for its secretion (Chiu *et al.*, 2003). To study whether the translocation capacities of various constructs of EspB are in parallel with that of secretion, we examined the membrane and cytosolic fractions of the infected host cells for the presence of the molecules. As the N-terminal 118 aa segment of EspB is essential for secretion

Fig. 1. Secretion competence of EspB constructs. (a) Schematic illustration of the EspB truncation constructs. In EspB, a hydrophobic region predicted to form a transmembrane structure is hatched, whereas a region predicted to form a coiled-coil structure is labelled by a filled box. (b) EspB constructs present in bacterial lysates. Expression plasmids, based on pQE60, were transformed separately into *E. coli* 43888 ΔB, and the transformants were cultured in M9 medium, harvested and lysed directly in SDS sample buffer. EspB in the bacterial lysates was detected with Western blotting using anti-EspB antibody. (c) EspB secreted in the medium. Proteins in the medium spent for sample preparations in (b) were concentrated by trichloroacetic acid (Chiu *et al.*, 2003) and analysed with Western blotting as in (b). Open circles indicate EspB molecules with expected sizes from the expression plasmid.

(Chiu *et al.*, 2003), it was preserved in almost all of the constructs (Fig. 1a) except for pBΔTM, in which residues 99–118, predicted to form a transmembrane domain (http://www.ch.embnet.org/software/TMPRED_form.html), were deleted. Previously, a coiled-coil domain of EspD was found to be functionally important for EPEC infectivity (Daniell *et al.*, 2001). Thus, a construct named pBΔCC was included in the tests and, within this construct, residues 187–216 of EspB, predicted to form a coiled-coil structure, were deleted. All of the constructs summarized in Fig. 1(a) carry no tag at either end of the molecules. They have been

examined for proper expression in bacterial lysates (Fig. 1b) and demonstrated to retain secretion capacity (Fig. 1c).

To address the translocation property of mutated EspB, bacteria must interact well with host cells so that a failure of translocation could not simply arise from a lack of bacterium–host contact. Therefore, the expression plasmids were transformed into wild-type *E. coli* 43888. By doing so, detection of the authentic EspB in the host-cell lysates affirms a proper bacterium–cell interaction. Experimentally, the infected cells with the attached bacteria were prepared as a total lysate (Fig. 2a) in SDS gel-sample buffer. In a parallel investigation, bacteria were removed together with unbroken cells by low-speed centrifugation after cells were scraped off. The remains were then fractionated into cell-membrane (Fig. 2b) and cytosolic (Fig. 2c) fractions. These preparations were analysed by Western blotting using anti-EspB antibodies. The authentic EspB encoded by the chromosomal *espB* was detected in all samples (Fig. 2a) and the truncated EspB molecules, as seen with sizes smaller than that of the authentic molecule, were also detected.

The infected cells were fractionated into a membrane fraction and a cytosolic part after washing off the bacteria attached. When the membrane fraction of the infected cells was examined, all but pB1–190 and p Δ 118–190 gave products that could be detected by anti-EspB (Fig. 2b, lanes 2–9). As neither bacterial outer-membrane protein A (OmpA) nor the cytosolic molecule actin was detected in the sampled cellular-membrane fraction (Fig. 2d), the possibility of bacterial or cytosolic contamination in this fraction was excluded. Thus, a positive detection of EspB on the membrane fractions represented a proper translocation of EspB. The same set of EspB constructs seen in the membrane fraction was also detected in the cytosolic fraction (Fig. 2c), a result consistent with the translocation results observed with the membrane fraction.

The products derived from pB1–190 and p Δ 118–190 were not found in the membrane fractions of the infected cells; nor were they seen in the cytosol (lanes 5 and 8, Fig. 2b, c). The failure to detect these proteins could not be attributed to a low level of expression, as pB1–250, expressed equally well as pB1–190 and poorer than p Δ 118–190, was translocated successfully. These facts indicate that the loss of translocation ability with these constructs was because regions critical for translocation had been deleted or altered. Intriguingly, these two constructs are secreted and have been demonstrated repeatedly in the spent medium (Chiu *et al.*, 2003; Fig. 1c). Thus, at least for EspB, secretion does not warrant successful translocation and, taken together with the observation that the construct encoded by pB1–220 was translocated (lane 4, Fig. 2b and c), the EspB region spanning residues 118–220 must be critical for EspB translocation. Additional products with sizes smaller than expected (Fig. 2a) were seen with some of the total lysates. They were present in a relatively small amount and may represent minor degradation products.

Fig. 2. Translocation capacity of EspB constructs. Expression plasmids shown in Fig. 1(a) were transformed into *E. coli* 43888, and the corresponding bacteria were then used to infect HeLa cells. The infected HeLa cells were prepared as (a) a total lysate (TL) derived from both bacteria and HeLa cells, or fractionated into (b) host-cell membrane (CM) fraction and (c) cytosolic (CS) fraction. EspB molecules present in different samples were detected by Western blotting using anti-EspB antibody. Open circles indicate the plasmid-expressed EspB, whilst arrows label the authentic EspB encoded by the bacterial chromosome; open boxes highlight the expected migration positions of products if translocated. Note: the EspB construct derived from p Δ TM was barely separable from the authentic EspB in lane 9. (d) Control Western blotting analysis of the cell content fractionated above. Protein components marked by antibodies are: OmpA, a bacterial outer-membrane protein; syndecan, a host-cell membrane protein; actin, a host-cell cytosolic protein.

Interaction of EspB with EspA

EspB has been reported to interact with EspA when secreted into the medium (Hartland *et al.*, 2000) and deletion of *espA* in EPEC has been found to abolish the translocation of EspB (Knutton *et al.*, 1998). For these reasons, we investigated whether EspB defective in translocation also

fails to interact with EspA. Proteins from pB1-190 and pBΔ118-190 were tested, along with three translocatable controls, in an affinity column-retention assay (Fig. 3). In the assay, EspA tagged with a C-terminal His₆ epitope was retained in a nickel-ion column and tested for the co-eluted EspB molecules. The results (Fig. 3b) indicated that the full-length EspB, encoded by pB1-312, and those derived from pB1-282 and pBΔCC were co-eluted from the column with EspA (Fig. 3c). In contrast, the products resulting from pB1-190 and pBΔ118-190 were not eluted, a fact suggesting that constructs that do not translocate have also lost the ability to interact with EspA.

Region of EspB important for adherence of EHEC to host cells

In EPEC, deletion of *espB* affects the adherence of bacteria to the host cell (Donnenberg *et al.*, 1993), but not the length of EspA filaments or the translocation of EspD (Delahay *et al.*, 1999; Hartland *et al.*, 2000; Knutton *et al.*, 1998; Wachter *et al.*, 1999). Therefore, we used *E. coli* 43888 ΔB to investigate which EspB constructs could restore adherence of bacteria to the host cells.

Fig. 3. Interaction of truncated EspB with EspA. (a) Analysis of the EspB constructs contained in the bacterial lysates. (b) Analysis of EspB that interacts with EspA-His₆ bound on an Ni²⁺-NTA column. After washing the column with TBS containing 150 mM imidazole, the proteins retained were eluted with the same buffer containing 500 mM imidazole, and EspB molecules in the eluants were detected by Western blotting with anti-EspB antibodies. (c) The EspA-His₆ in the same eluants as in (b) was analysed with anti-EspA antibodies. Arrows indicate the specific products detected.

EspB-expressing plasmids were transformed into *E. coli* 43888 ΔB and recovery of the lost bacterial adherence was examined. Fig. 4 shows that *E. coli* 43888 ΔB, when complemented with the full-length EspB expressed from pB1-312, had restored bacterial adherence to a level similar to that of the wild-type strain transformed with a vector control (i.e. pQE60). Constructs with the C-terminal region of EspB deleted, such as those from pB1-282, pB1-250, pB1-220 and pB1-190, did not recover bacterial adherence. Furthermore, the construct from pBΔ191-253, with a deletion of residues 191-253 of EspB but retaining the C-terminal 59 aa, did not restore the adherence of *E. coli* 43888 ΔB (Fig. 4). In contrast, truncation at residues 118-190 (in pBΔ118-190), which abolished both translocation and interaction with EspA, apparently did not damage EspB's bacterial-adherence property. Deletion of the hypothetical coiled-coil domain (residues 187-216) of EspB or the possible transmembrane domain (residues 99-118) alone also did not affect bacterial adherence (Fig. 4, pBΔCC and

Fig. 4. Ability of various EspB constructs to restore the adherence activity lost in *E. coli* 43888 ΔB, an *espB*-deleted strain of EHEC. HeLa cells were infected with plasmid-transformed bacteria for 6 h. Then, the cells were washed, harvested in PBS and lysed with PBS containing saponin. The cell lysates were diluted serially and plated on LB plates supplemented with ampicillin (100 μg ml⁻¹). After overnight incubation, c.f.u. were scored and presented as a percentage of those observed with the wild-type EHEC (Wt). Note: *E. coli* 43888 ΔT (ΔTir) was included as a control, showing that this adherence assay was dependent on EspB rather than on Tir. Experiments were performed in triplicate. Significant differences compared with the wild-type strain 43888 after using Student's *t*-test ($P < 0.01$) are marked with an asterisk.

pBATM). Therefore, the region of EspB in residues 216–312 that locates C-terminally to the hypothetical coiled-coil domain of EspB (see summary in Fig. 7) must contribute to bacterial adherence, and this region of EspB obviously differs from that required for the functions of translocation and EspA interaction seen above.

Tir is a type III effector protein, translocated into the host cells to function as a receptor for the bacterial membrane protein intimin. Tir–intimin interaction has been reported to be important for the intimate attachment of bacteria to the host cells (Kenny *et al.*, 1997). However, deletion of *espB* also blocks the translocation of Tir (Kenny & Finlay, 1995) and, thus, it is important to clarify the role that Tir plays in this experiment. For this purpose, an isogenic strain with Tir deleted was included in the assays. Results in Fig. 4 showed that *E. coli* 43888 Δ Tir did not lose this adherence activity and, apparently, EspB was the major determinant monitored in this system. Therefore, this assay (Gansheroff *et al.*, 1999) may represent a measurement of bacterium–cell adherence before intimin binds to the translocated Tir.

Regions of EspB critical for causing RBC haemolysis

EspB, together with EspD, is thought to form pores at the membranes of the infected cells (Ide *et al.*, 2001; Kenny *et al.*, 1997; Warawa *et al.*, 1999). In EPEC, this activity has been assayed by using haemolysis of human RBCs, and deletion of either *espA*, *espD* or *espB* attenuates the haemolytic activity of EPEC (Ide *et al.*, 2001; Shaw *et al.*, 2001; Warawa *et al.*, 1999). To examine the domain of EHEC EspB possibly involved in pore formation, RBCs were incubated with the bacteria used in Fig. 4, and haemoglobin release was measured after 6 h incubation. Fig. 5 shows that *E. coli* 43888 Δ B transformed with pQE60 lost the haemolytic activity found with the wild-type EHEC. The constructs of EspB derived from pB1–282, pB1–250, pB1–220, pB1–190 and pB Δ 191–253 failed to show the complementation activity seen with the full-length EspB. Therefore, truncated EspB that failed in the adherence assay also failed to show haemolysis in RBCs (compare Figs 4 and 5). Thus, the results with the adherence and haemolysis assays seemed to be in parallel, except for the results with pBATM, in which deletion of residues 99–118 apparently did not affect adherence, but did abolish RBC-haemolysis activity. This observation suggests that residues 99–118 of EspB may be involved in pore formation, and this notion is consistent with a transmembrane domain predicted for these residues. Thus, it is proposed that EspB may mediate adherence through a structure that involves the C-terminal region and, after adherence, the transmembrane domain of EspB may further participate in pore formation. In a control with *E. coli* 43888 Δ Tir, transformation with pQE60 decreased the haemolysis only to a small degree (Fig. 5), a result similar to that reported previously (Warawa *et al.*, 1999).

Fig. 5. Haemolytic activity of *E. coli* 43888 Δ B after complementation with various EspB constructs. Human RBCs in 12-well plates were incubated in DMEM without phenol red. Overnight-grown bacteria were added to the RBC culture in triplicate and incubated for 6 h. The whole culture was harvested and centrifuged, and OD₅₄₃ of the supernatant was measured with an ELISA reader. The results are shown as a percentage of the readings obtained with the wild-type 43888 strain; asterisks indicate significant differences at $P < 0.01$ compared with the wild-type strain 43888.

Regions of EspB critical for actin-accumulation activity

Deletion of *espB* may abolish the membrane-pore formation and could theoretically affect translocation of the type III effectors (Foubister *et al.*, 1994), including Tir, which is necessary for actin accumulation underneath the bacterial-attachment site (Kenny *et al.*, 1997). To test this possibility, cells infected with the plasmid-transformed *E. coli* 43888 Δ B were stained for condensed actin by using TRITC-labelled phalloidin (Fig. 6). Strain 43888 Δ B transformed with a vector control (i.e. pQE60) showed no condensed actin accumulation beneath the bacteria, whereas the similarly treated wild-type bacteria clearly showed this phenotype (Fig. 6a and b). As expected, the actin-accumulation property was restored when *E. coli* 43888 Δ B was transformed with pB1–312 (Fig. 6c). In contrast, when the same bacteria were transformed separately with pB1–282, pB1–250, pB1–220 and pB Δ 191–253, which failed to make strains adhere to the host cells, it is clear from Fig. 6(d–g) that there is no actin condensation beneath the sites where bacteria can be seen. Furthermore, the transformant derived from pBATM, which adhered well to the cells but failed to produce lysis of RBCs, also yielded a negative result for actin

Fig. 6. Accumulation of actin beneath *E. coli* 43888 ΔB after complementation with EspB constructs. HeLa cells were plated on glass coverslips and cultured in DMEM containing 10% (v/v) fetal bovine serum. After infection for 6 h, the cells were washed and fixed with 4% (w/v) paraformaldehyde. The fixed cells were then permeated with 0.5% (v/v) Triton X-100. Bacteria adhering to the cells were detected with rabbit anti-O157 antibodies followed by staining with FITC-conjugated goat anti-rabbit antibodies. To stain the condensed actin, the cells were treated with TRITC-labelled phalloidin. Samples were observed by using a fluorescence microscope and the images were taken with a CCD camera; the plasmid carried by infected bacteria in each panel is shown in parentheses.

condensation (Fig. 6j). On the other hand, strain 43888 ΔB transformed with pBACC or pBΔ118-190, which

showed haemolytic activity against RBCs, retained actin-accumulation activity (Fig. 6h and i). Therefore, to assist in

the translocation of Tir, as reflected by actin accumulation, the EspB constructs of bacteria must be able to adhere to the host membrane and to form the pores.

DISCUSSION

The LEE comprises 41 ORFs that are involved in the A/E lesion. Systematic deletion of individual genes has recently been performed with *C. rodentium* (Deng *et al.*, 2004) and the results indicated that bacterial virulence was reduced to a certain degree when any of these genes was deleted. The implication, then, is the existence of a gene-regulation network and the presence of numerous interplays between the gene products. Deletion of EspB completely abolishes bacterial adhesion, actin rearrangement, translocation of Tir and, thus, bacterial virulence. Therefore, EspB may play multiple roles during the process of host–bacterium interaction. To unveil the functional complexity built into the structure of the EspB molecule, we have dissected the molecule and generated different constructs of EspB. By complementation of an EspB-deleted mutant, the general structure–function relationship of EspB was established; the results are summarized in Fig. 7.

As shown previously, the N-terminal 118 aa region of EspB is critical, but not sufficient, for EspB secretion (Chiu *et al.*, 2003). To serve as a translocator, EspB must retain secretion competence. Therefore, any construct that lacks the N-terminal 118 aa is not secreted at all and could not possibly function for other purposes.

Translocation of EspB into the host cells apparently requires the same region of EspB that is also critical to interact with

EspA (Fig. 7a). Thus, these two functions are currently assigned to the same domain. Interaction of EspA and EspB has been reported (Hartland *et al.*, 2000) and here we have further demonstrated that residues 118–190 in the middle region of EspB are needed for this interaction. Less clear is the function of EspB residues 187–216, which are predicted to form a coiled-coil structure. In comparison of pB1–190 with pB1–220 (Fig. 7a), residues 191–216 seem to be crucial for EspB translocation. However, in pBΔ191–253, the deletion includes residues 191–216 of EspB, but does not abolish EspB translocation activity. As many factors, such as conformational changes, could account for this inconsistency, we currently prefer a conservative way and assign no function for this region (Fig. 7b).

Adherence of bacteria to HeLa cells apparently involves EspB, particularly the C-terminal region, and we have narrowed this down to the region spanning from residue 216 to the C terminus (Fig. 7a). This region is also needed for pore formation on the membrane, as measured by the RBC-haemolysis experiment. The latter activity of causing RBC lysis appeared to be associated with an extra domain in residues 99–118 that has a high degree of hydrophobicity, as evidenced by the data from pBATM. Intriguingly, both the C-terminal region and the hydrophobic residues important for RBC haemolysis matched with the regions critical for the formation of the condensed actin structure underneath the sites where bacteria are attached (Fig. 7a). Actin accumulation has been correlated with successful translocation of Tir, a function that results from the correct formation of membrane pores. Therefore, residues 99–118 and 216–312 in EspB must play an essential role to function effectively as a translocator.

Fig. 7. Summary of the structure–function relationship of EspB. (a) Summarized results observed from Figs 1–6. Abbreviations used for the phenotypes/properties described in the text are: Sec, secretion (Chiu *et al.*, 2003); Translo., translocation of EspB; EspA, interaction of EspB with EspA; Adhe., adherence of bacteria to host cells; Haemo., RBC haemolysis; Actin, focused actin accumulation. Dotted lines are used simply for easy association of the relative relationship. NT, Not tested. (b) Schematic illustration of the functional-analysis results in EspB. TM and CC label the predicted regions forming transmembrane and coiled-coil structures, respectively. It should be noted that secretion is the principal requirement for the other functions to be achieved.

The functional domains of EspB described above seem to be discernible, and they are grouped accordingly. It is worth noting that, without the EspA-interacting region, the truncated EspB molecule (from pBA118–190) remains active in helping bacterial adherence (Fig. 7a). Similarly, EspB losing the pore-forming activity (from pBATM) did not lose its bacterial-adherence activity. On the other hand, EspB mutants that lost the activity of assisting bacterial adherence all gave a negative result in the pore-formation assay, an observation suggesting that the pore-formation activity of EspB relies on its active function of adherence. It is then proposed that EspB's assistance in bacterial adherence is an action prior to the formation of membrane pores. On the other hand, bacterial adherence and EspB translocation/EspB–EspA interaction seem to be independent events, as no clear association could be made (Fig. 7a).

In our experimental results shown in Fig. 4, we measured the bacteria attached to the cells after 6 h incubation and calculated the relative activity after comparison with the parental strain. To ensure that the measured values did not result from the rate differences of bacterial growth, parallel experiments were performed with individual bacteria, to follow their growth curves. The fact that no apparent differences were found thus excludes this possibility. However, adherence activity normally monitors the ratio of bacteria attached to the number of bacteria inoculated. Our values of the relative adhering activity of different strains should then reflect more than a simple association at the early stage. Away from a direct input of EspB, an indirect effect of EspB on other adherence-assisting molecules, such as EspA (Cleary *et al.*, 2004) and EspD, may have been measured collectively.

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