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Characterization of Two Virulence Proteins Secreted by Rabbit Enteropathogenic *Escherichia coli*, EspA and EspB, Whose Maximal Expression Is Sensitive to Host Body Temperature

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Enteropathogenic *Escherichia coli* (EPEC) and rabbit EPEC (RDEC-1) cause unique histopathological features on intestinal mucosa, including attaching/effacing (A/E) lesions. Due to the human specificity of EPEC, RDEC-1 has been used as an animal model to study EPEC pathogenesis. At least two of the previously identified EPEC-secreted proteins, EspA and EspB, are required for triggering host epithelial signal transduction pathways, intimate adherence, and A/E lesions. However, the functions of these secreted proteins and their roles in pathogenesis have not been characterized. To investigate the function of EspA and EspB in RDEC-1, the *espA* and *espB* genes were cloned and their sequences were compared to that of EPEC O127. The EspA proteins showed high similarity (88.5% identity), while EspB was heterogeneous in internal regions (69.8% identity). However, RDEC-1 EspB was identical to that of enterohemorrhagic *E. coli* serotype O26. Mutations in RDEC-1 *espA* and *espB* revealed that the corresponding RDEC-1 gene products are essential for triggering of host signal transduction pathways and invasion into HeLa cells. Complementation with plasmids containing EPEC *espA* or/and *espB* genes into RDEC-1 mutant strains demonstrated that they were functionally interchangeable, although the EPEC proteins mediated higher levels of invasion. Furthermore, maximal expression of RDEC-1 and EPEC-secreted proteins occurred at their respective host body temperatures, which may contribute to the lack of EPEC infectivity in rabbits.

Enteropathogenic *Escherichia coli* (EPEC) causes diarrhea in infants and young children (8). Although EPEC does not appear to produce enterotoxins, this pathogen causes a characteristic histopathological lesion on intestinal cell surfaces termed an attaching/effacing (A/E) lesion (27). This lesion is comprised of localized degeneration of host cell microvilli and formation of pseudopod-like structures (30) containing actin and tyrosine-phosphorylated proteins beneath the adherent bacteria (12, 22, 29). Initial localized adherence to epithelial cells is mediated by a plasmid-encoded bundle-forming pilus (BFP) (6, 16, 35, 37). This first step is followed by triggering of epithelial signal transduction pathways involving generation of inositol triphosphates, intracellular calcium fluxes, and induction of tyrosine phosphorylation of a 90-kDa membrane protein (Hp90) (2, 3, 10, 15, 29). Tyrosine-phosphorylated Hp90 binds to a 94-kDa bacterial outer membrane protein, intimin (29, 30), encoded by *eaeA* (19). These events, including tyrosine phosphorylation of Hp90, are mediated by at least two EPEC-secreted proteins, EspA (21) and EspB (14). Both secreted proteins are also required for invasion into epithelial cells in vitro (21).

EPEC secretes at least five proteins, of 110 kDa (EspC), 40 kDa, 39 kDa, 37 kDa (EspB), and 25 kDa (EspA), into culture media under certain conditions (20). These proteins, except EspC, are secreted by a type III secretion system that is encoded by the *sep* machinery (18). This *sep* locus is homologous to similar type III secretion systems found in other enteric pathogens, including *Yersinia*, *Shigella*, and *Salmonella* species (18). A 35-kb EPEC locus, termed the locus of enterocyte effacement (25), encodes the *sep* cluster, *eaeA*, *espA*, and *eaeB* (*espB*).

Rabbit EPEC (RDEC-1) has been used as an EPEC infection model, since EPEC does not infect rodents. RDEC-1 causes diarrhea in weanling rabbits and also causes histopathological features similar to those caused by EPEC, including attaching/effacing lesions, on the intestinal mucosa (38). Shiga-like toxin I-producing RDEC-1 (RDEC-H19A) has already been established as an animal model for human enterohemorrhagic *E. coli* (EHEC) (34). RDEC-1 does not form localized adherence due to differences in adhesins. Instead of BFP in EPEC, RDEC-1 has a plasmid-encoded fimbrial adhesin termed AF/R1 (40), which mediates specific adherence to Peyer's patches (5, 32). Although this adhesin enhances virulence, it is not essential for disease (39). RDEC-1 *eaeA* encodes an intimin that is similar to EPEC (1). However, secreted proteins have not been characterized in RDEC-1. In this report, we cloned and sequenced two RDEC-1-secreted protein genes, *espA* and *espB*, and demonstrate that triggering of host epithelial signal transduction pathways and invasion are mediated by these proteins. We also compared them functionally to EPEC-secreted proteins and studied their temperature regulation.

MATERIALS AND METHODS

Bacterial strains, media, plasmids, enzymes, and oligonucleotides. The phenotypes and genotypes of bacterial strains used in this study are listed in Table 1. Multicopy plasmid pBluescript SK+ and low-copy-number plasmid pMW118 were obtained from Stratagene and Nippongene, respectively. Plasmid pACYC184 is a low-copy-number vector described elsewhere (28). Plasmid pCVD442 is a positive selection vector containing *sacB*, *bla*, and *pir*-dependent R6K replicon described elsewhere (7). Other plasmids used in this study are listed in Table 1. Oligonucleotides used in this study are listed in Table 2. Vent DNA polymerase (New England Biolabs) was used for PCR to amplify chromosomal DNA from RDEC-1 and EPEC strains. The PCR reaction was carried out for 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 2 min. Inverse PCR is described elsewhere (36). Briefly,

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Comments	Reference
Strains			
E2348/69	EPEC	Prototypic O127:H6 EPEC strain	24
UMD864	E2348/69 Δ*eaeB*		9
UMD872	E2348/69 Δ*eaeA*	Nonpolar mutation in *espA*	
RDEC-1	Nalidixic acid resistant	Prototypic O15:K$^-$:H$^-$ rabbit EPEC strain	4
AAF001ΔA	RDEC-1 EspA$^-$	Nonpolar mutation in *espA*	This study
AAF001ΔB	RDEC-1 EspB$^-$	Nonpolar mutation in *espB*	This study
AAF001ΔAB	RDEC-1 EspA$^-$ EspA$^-$	Nonpolar mutation in *espA* and *espB*	This study
AAF002	RDEC-1 *espA* back mutation	Gene replacement of nonpolar mutation in *espA* to intact *espA*	This study
AAF003	RDEC-1 *espB* back mutation	Gene replacement of nonpolar mutation in *espB* to intact *espB*	This study
SM10λ*pir*	*thi thr leu tonA lacY supE resA*::RP4-2-Tc::Mu Km	Strain for conjugal transformation	33
Plasmids			
pORF123B	RDEC-1 *espA espD espB* Amp^r	4.3-kbp PCR fragment of RDEC-1 *esp* genes was cloned into pBluescript	This study
pORF23B	RDEC-1 *espA espD espB* Amp^r	2.7-kbp PCR fragment of RDEC-1 *esp* genes was cloned into pBluescript	This study
pORF23	RDEC-1 *espA* Amp^r	1.1-kbp *Eco*RI-*Bgl*II fragment of pOR23B was cloned into pBluescript	This study
pORF23Δ	RDEC-1 Δ*espA* Amp^r	Non-polar mutation in *espA* of pORF23	This study
pAA23Δ	RDEC-1 Δ*espA sacB* Amp^r	1.1-kbp *Sal*II-*Sac*I fragment of pORF23Δ was cloned into pCDV442	This study
pAA23	RDEC-1 *espA sacB* Amp^r	1.1-kbp *Sal*II-*Sac*I fragment of pORF23 was cloned into pCDV442	This study
pBxb	RDEC-1 *espB* Amp^r	1.4-kbp *Xba*I fragment of pOR23B was cloned into pBluescript vector	This study
pBxbΔ	RDEC-1 Δ*espB* Amp^r	Nonpolar mutation in *espB* of pBxb	This study
pAABxbΔ	RDEC-1 Δ*espB sacB* Amp^r	1.1-kbp of *Sal-Sac*I fragment of pBxbΔ was cloned into pCVD442	This study
pAABxb	RDEC-1 *espB sacB* Amp^r	1.4-kbp of *Sal-Sac*I fragment of pBxb was cloned into pCVD442	This study
pMWespAB	EPEC *espA espD espB* Amp^r	2.8-kbp PCR fragment of EPEC *esp* genes was cloned into pMW118 in orientation of *lacZ* promoter	This study
pMWΔespD	EPEC *espA* Δ*espD espB* Amp^r	Disruption of *Bgl*II site in *espD* of pMWespAB	This study
pMWespA	EPEC *espA* Amp^r	1.7-kbp *Bgl*II-*Sal*I deletion in pMWespAB	This study
pMWespB	EPEC *espB* Amp^r	1.1-kbp *Bam*HI-*Bgl*II deletion in pMWespAB	This study

PCR was carried out with primers facing opposite directions with circular plasmid DNA as a template. By self-ligation of the resulting PCR fragment, a mutation was introduced into a preferred position within the *esp* genes.

HeLa cells. HeLa cells were maintained and assayed in minimal essential medium (MEM) containing 10% (vol/vol) fetal calf serum at 37°C in a 5% CO_2 incubator. For the infection studies using RDEC-1 mutant strains harboring plasmids, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to the MEM.

DNA sequencing. The 4.3-kbp DNA fragment containing *eaeA* 3′ flanking region, *espA*, and *espB* was amplified by PCR using primers AA01 (+) and MS11 (−) (Table 2) and RDEC-1 (4) chromosomal DNA as the DNA template. The resulting blunt-end fragment was digested with *Sal*I and cloned into the *Sal*I-*Sma*I site of the pBluescript II SK+ vector to obtain pORF123B. The DNA sequences of RDEC-1 *espA* and *espB* were determined for both strands, using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems).

Database search. Amino acid sequences were analyzed by BLAST (server of the National Center for Biotechnology Information) and BEAUTY (server of the Human Genome Center, Baylor College of Medicine) searches.

Construction of the nonpolar stop codon mutations in RDEC-1 *espA* and *espB* genes. The 2.7-kbp DNA fragment encoding the *espA* and *espB* genes was amplified by PCR using primers BK25 (+) and MS11 (−) (Table 2) and pORF123B as the DNA template. The resulting blunt-end fragment was digested

TABLE 2. Oligonucleotides used in this study

Designation[a]	Sequence[b]	Description (location[c])
AA01 (+)	GCG TCG ACC GAT TTT TGG CAG AGA ACC TTT *Sal*I	PCR primer for downstream region of RDEC-1 *eaeA* (3911 to 3932)
BK25 (+)	GGA ATT CTC CAT GGA TAC ATC AAC TGC AAC *Eco*RI	PCR primer for RDEC-1 *espA* (−1 to 14)
MS11 (−)	TTA GAT CTC ACT TCA TCA TTA AAC GTA *Bgl*II	PCR primer for EPEC *espB* (980 to 998)
EespA (+)	CGG GAT CCT TAT AGT TTT TGT CAT GCT AAG *Bam*HI	PCR primer for EPEC *espA* (−45 to −23)
EespB (−)	GCG TCG ACA CAC TTC ATC ATT AAA CGT ATC *Sal*I	PCR primer for EPEC *espB* (978 to 999)
ΔespA (+)	AAG ATC TAT GGC TAA TCT TGT GGA TGC TA *Bgl*II	Inverse PCR primer for RDEC-1 *espA* (238 to 259)
ΔespA (−)	TT**A** TTG GGC TGT GGT TGA CTC CTT 235	Inverse PCR primer for RDEC-1 *espA* (224 to 237)
ΔespB (+)	AAG ATC TTC TGA TAC TGC GAC TAA AAC *Bgl*II	Inverse PCR primer for RDEC-1 *espB* (422 to 443)
ΔespB (−)	CAT CTT GCC CAG GAG TT**A** TTG AAT 154	Inverse PCR primer for RDEC-1 *espB* (148 to 171)

[a] Oligonucleotides corresponding to plus and minus DNA strands are represented by (+) and (−).
[b] Boldface letters represent positions of stop codon insertions relative to start codons. Restriction sites are underlined.
[c] Relative to the start codon.

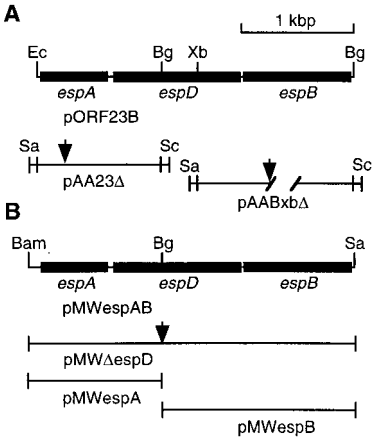

FIG. 1. Genetic maps of the plasmids containing RDEC-1 (A) and EPEC (B) *espA*, *espD*, and *espB* genes. Arrows indicate positions of stop codon insertions in *espA* and *espB* (A) and the frameshift mutation engineered into the *Bgl*II site in *espD* (B). The 250-bp deletion in *espB* is marked by //, and solid boxes represent ORFs. Restriction enzymes are indicated as follows: *Bam*, *Bam*HI; Ec, *Eco*RI; Bg, *Bgl*II; Xb, *Xba*I; Sa, *Sal*I; and Sc, SacI.

with *Eco*RI and cloned into the *Eco*RI-*Sma*I site of the pBluescript II SK+ vector to obtain pORF23B (Fig. 1A). A 1.1-kbp *Eco*RI-*Bgl*II DNA fragment from pORF23B containing *espA* was cloned into the *Eco*RI-*Bam*HI site of pBluescript II SK+ to obtain pORF23. To construct a nonpolar mutation in *espA*, inverse PCR was carried out with primers ΔespA (+) and ΔespA (−), which contain a *Bgl*II restriction site and a stop codon, and circular pORF23 as a DNA template. The PCR product was then blunt-end ligated to obtain pORF23Δ. The resulting plasmid contained a stop codon and a *Bgl*II site 235 bp downstream from the *espA* start codon, which was confirmed by DNA sequencing. The 1.1-kbp *Sal*I-*Sac*I DNA fragment containing the *espA* mutation from pORF23Δ was inserted into the same sites of the suicide vector pCVD442 (7), which contains the *sacB* gene for positive selection and an ampicillin resistance gene, to obtain pAA23Δ (Fig. 1A). The resulting plasmid was introduced into *E. coli* SM10λ*pir* (33) and transconjugated into RDEC-1 harboring pACYC184 (28) as described elsewhere (7). For the nonpolar mutation in *espB*, inverse PCR was carried out with primers ΔespB (+) and ΔespB (−) and pBxb as a DNA template. pBxb contains the 1.4-kbp *Xba*I fragment from pORF23B encoding *espB* cloned into the pBluescript vector. The resulting PCR product was self-ligated to obtain pBxbΔ, which contained a stop codon and a *Bgl*II site introduced by primers ΔespB (−) and ΔespB (+). The resulting *esp* gene in pBXbΔ contained a stop codon in addition to 250-bp deletion, starting 154 bp downstream of the *espB* start codon. The 1.1-kbp *Sal*I-*Sac*I site DNA fragment containing the *espB* mutation and the deletion of 250 bp from pBxbΔ was inserted into the same site of pCVD442 to obtain pAABxbΔ (Fig. 1A). The resulting plasmid was transformed into *E. coli* SM10λ*pir* and transconjugated into RDEC-1 harboring pACYC184. To establish double mutations in *espA* and *espB*, pAABxbΔ was introduced into strain AAF001ΔA (EspA$^-$). RDEC-1 mutant strains were selected by resistance to sucrose and chloramphenicol and sensitivity to ampicillin. To confirm the deletion mutation and stop codon insertions in *espA* and *espB*, chromosomal DNA was prepared from each mutant strain, and PCR was performed with two sets of primers encompassing the *espA* and *espB* genes, respectively. The resulting PCR products were digested with *Bgl*II to confirm the presence of this engineered restriction site. The mutant strains containing the stop codon in *espA* or/and *espB* were designated AAF001ΔA (EspA$^-$), AAF001ΔB (EspB$^-$), and AAF001ΔAB (EspA$^-$ EspB$^-$).

Construction of back mutations in EspA$^-$ and EspB$^-$ strains. The 1.1-kbp *Sal*I-*Sac*I DNA fragment from pORF23 containing *espA* was inserted into the *Sal*I-*Sac*I sites of pCVD442 to obtain pAA23. The 1.4-kbp *Sal*I-*Sac*I fragment of pBxb was inserted into the *Sal*I-*Sac*I site of pCVD442 to obtain pAABxb. pAA23 and pAABxb were introduced into SM10λ*pir* and transconjugated into AAF001ΔA and AAF001ΔB. The resulting back mutant strains were confirmed as described above and designated AAF002 (EspA$^+$) and AAF003 (EspB$^+$).

Cloning of the EPEC *esp* genes. The 2.8-kbp DNA fragment encoding *espA* and *espB* was amplified by PCR using primers EespA (+) and EespB (−), with EPEC 2348/69 (24) chromosomal DNA as the template. This fragment was digested with *Bam*HI and *Sal*I and introduced into the *Bam*HI-*Sal*I site of the low-copy-number vector pMW118 under control of the *lacZ* promoter, to obtain pMWespAB (Fig. 1B). pMWespAB was digested with *Bgl*II, which has a restriction site in the *espD* open reading frame (ORF), blunt ended with the Klenow fragment of *E. coli* DNA polymerase I, and then self-ligated to obtain pMWΔespD. pMWespAB was also digested with *Bgl*II and *Bam*HI and then self-ligated to obtain pMWespB. pMWespAB was digested with *Bgl*II-*Sal*I, filled with Klenow fragment, and then self-ligated to obtain pMWespA.

Preparation of RDEC-1-secreted proteins. Bacterial overnight cultures were diluted 1:100 into Dulbecco modified Eagle medium (DMEM) and incubated to an optical density of 1.0 at 600 nm. For RDEC-1 mutant strains containing EPEC *esp* recombinant plasmids, IPTG was added in DMEM to induce transcription. Bacteria were removed by centrifugation (18,000 × *g*, 10 min), and the supernatant was precipitated by addition of 10% ice-cold trichloroacetic acid and incubated on ice for 1 h. After centrifugation, the pellets were resuspended in Laemmli sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (23) on a 12% polyacrylamide gel.

Immunoblotting analysis. Western blot analyses were performed as described elsewhere (30). Rabbit polyclonal antibodies against EPEC (E2348/69) EspA and EspB were used in this study. EPEC supernatant proteins were resolved by SDS-PAGE (10% gel) and transferred to a nitrocellulose membrane; EspA and EspB visualized with Ponceau S were excised, fragmented, and injected into rabbits to obtain polyclonal antisera. Antisera for EspA and EspB specifically recognize the respective secreted proteins.

Adherence and invasion assay. HeLa cells (10^5/well) were seeded, grown overnight, and then infected with RDEC-1 or a mutant strain (multiplicity of infection of 1:100) for 3 h. For coinfection experiments, each strain (multiplicity of infection of 1:100) was mixed and inoculated. HeLa cells were washed seven times with phosphate-buffered saline (PBS) and lysed with 1% (vol/vol) Triton X-100 for 5 min, and then serially diluted samples were plated on LB plates to determine the number of adherent bacteria. For invasion assays, HeLa cells were infected as described above, then washed three times with PBS, and incubated in MEM containing 10% fetal calf serum and 100 μg of gentamicin per ml for 1 h to kill external bacteria. The resulting cells were washed three times with PBS, lysed with Triton X-100, and then plated as described above.

Immunofluorescence microscopy. HeLa cells (10^5) were seeded and grown overnight on 12-mm-diameter round glass coverslips and then infected with RDEC-1 or a mutant strain for 3 h. Cells were washed three times with PBS, fixed with 3.0% paraformaldehyde in PBS (pH 7.2), and then washed twice with PBS. The resulting fixed cells were permeabilized with 20 μl of 0.1% Triton X-100 in PBS in the presence of phalloidin-rhodamine (to stain filamentous actin) or antiphosphotyrosine antibody 4G10 (UBI Inc.). Fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G and immunoglobulin M antibody (ICN Inc.) was used as the secondary antibody for antiphosphotyrosine. Stained samples were visualized and photographed as described elsewhere (13).

Nucleotide sequence accession numbers. Nucleotide and amino acid sequences have been deposited in the EMBL/GenBank database, and their accession numbers are as follows: RDEC-1 *espA*, U80908; RDEC-1 *espB*, U80796; EPEC strain E2348/69 *espA*, Z54352; EPEC strain E2348/69 *espB*, Z21555; EPEC strain E2348/69 *espD*, Y09228; EHEC strain EDL933 serotype O157 *espB*, X96953; and EHEC strain 413/89-1 serotype O26 *espB*, X99670.

RESULTS AND DISCUSSION

Cloning and sequence analysis of *espA* and *espB* genes. The DNA fragment encoding RDEC-1 *espA* and *espB* was obtained by PCR from RDEC-1 chromosomal DNA by using primers derived from the published sequences of EPEC and RDEC-1. The resulting 4.3-kbp product was ligated into pBluescript, and both strands were sequenced (Fig. 2A and B). ORFs were found in the cloned region, and these DNA sequences were similar to those of EPEC *espA* and *espB*. Accordingly, we designated the two ORFs *espA* and *espB*. The predicted molecular masses of EspA (192 amino acids) and EspB (314 amino acids) were 20,533 and 33,219 Da. EspA of RDEC-1 was very similar to that of EPEC serotype O127 (88.5% identity) (Fig. 2C). To our surprise, RDEC-1 EspB protein was identical to the recently reported EspB from EHEC strain 413/89-1 serotype O26 (11), which was originally isolated from a calf and also isolated from patients with hemolytic uremic syndrome (31), although there were two nucleotide differences, at positions 12 (T to C) and 729 bp (G to T). Furthermore, RDEC-1 EspB showed 70.3 and 69.8% identity, respectively, to EHEC serotype O157 EspB and EPEC serotype O127 EspB. Small sequence deletions were found in RDEC-1 and EHEC (O26 and O157) EspB at the same positions as in the EPEC sequences. These results suggest that RDEC-1 encodes *espA* and *espB* genes and that the predicted EspA polypeptide is highly conserved in RDEC-1 and EPEC O127. However, RDEC-1 EspB is less conserved and is most closely related to EHEC O26 EspB. The predicted product of an ORF

downstream from *espA* (Fig. 2A) showed similarity to EPEC EspD (Fig. 2C), a secreted protein that modulates EspB secretion and is needed for triggering of host signal transduction pathways (EMBL/GenBank accession no. Y09228, submitted by M. S. Donnenberg). These results indicate that the *espD* homolog is probably also located between *espA* and *espB* in RDEC-1.

Construction of nonpolar mutations in *espA* and *espB* and back mutations. To confirm the role of RDEC-1 *espA* and *espB* in host epithelial signal transduction pathways, a stop codon mutation was engineered into *espA* and *espB* as described in Materials and Methods. The positions of these stop codons and the introduced *Bgl*II sites are illustrated in Fig. 1A. Two suicide vectors were constructed and introduced into the RDEC-1 wild-type strain by transconjugation as described in Materials and Methods. The resulting mutant strains, AAF001ΔA (EspA$^-$), AAF001ΔB (EspB$^-$), and AAF001ΔAB (EspA$^-$ EspB$^-$), were confirmed by *Bgl*II digestion (data not shown). We also reverted these mutations to wild type (back mutation) to confirm that suicide vectors do not affect flanking regions or other loci. Two back mutant strains were obtained by transconjugation of the suicide vectors pAA23 and pAABxb into strains AAF001ΔA (EspA$^-$) and AAF001ΔB (EspB$^-$). The resulting back mutant strains, AAF002 and AAF003, were confirmed by PCR and *Bgl*II digestion.

Secretion profile of RDEC-1 and mutant strains in tissue culture media. We have already shown that EPEC secretes five proteins, of 110 kDa (EspC), 40, 39, and 37 kDa (EspB), and 25 kDa (EspA), in culture media (20). RDEC-1 showed a similar secretion profile except for the absence of a protein with an apparent molecular weight similar to that of EspC (Fig. 3A). We have already shown that EspC is not required for EPEC induction of host signal transduction pathways (36). Although two secreted proteins (40 and 39 kDa) were difficult to resolve, these proteins could be resolved by using different conditions of SDS-PAGE, and we have confirmed that EPEC EspB antisera do not react to these proteins (data not shown). RDEC-1 secreted two proteins similar in mobility to EPEC EspA and EspB. Both proteins cross-react with EPEC EspA and EspB antisera in Western immunoblots (Fig. 3B), indicating that RDEC-1 also secretes EspA and EspB. Mutant strains AAF001ΔA, AAF001ΔB, and AAF001ΔAB do not secrete EspA, EspB, and EspA plus EspB, respectively, as judged by their secretion profiles and Western blot analysis. The 39- and 40-kDa proteins are still apparent in AAF001ΔAB, indicating that these proteins are not related to EspA or EspB.

EspB, whose gene is located downstream from *espA*, was still secreted in the mutant strain AAF001A (EspA$^-$), indicating that the stop codon insertion mutation does not affect downstream gene expression. These results also confirm that RDEC-1 EspA and EspB proteins are encoded by the sequences designated *espA* and *espB*. Furthermore, the two back mutant strains AAF002 and AAF003, originally derived from AAF001ΔA (EspA$^-$) and AAF001ΔB (EspB$^-$), now expressed the parental secreted proteins, indicating that each nonpolar mutation in AAF001ΔA and AAF001ΔB is as predicted and does not affect downstream genes and other loci. However, the amounts of the other secreted proteins were decreased in the EspA$^-$, EspB$^-$, and EspA$^-$ EspB$^-$ strains

A

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ttaatgattggtaaagtaattgattataaggaggatgttatttgatattggttttttaat 60

cgtttttggtcttgctaagaaagattattaagaggtatatacATGGATACATCAACTGCA 120
                                        M  D  T  S  T  A

ACATCAGTTGCTAGTGCGAACGCGAGTACTTCGACATCGACAGTCTATGACTTAGGCAGT 180
T  S  V  A  S  A  N  A  S  T  S  T  S  T  V  Y  D  L  G  S

ATGTCGAAAGACGAAGTAGTTCAGCTATTTAATAAAGTCGGTGTTTTTCAGGCTGCGCTT 240
M  S  K  D  E  V  V  Q  L  F  N  K  V  G  V  F  Q  A  A  L

CTCATGTTTGCCTATATGTATCAGGCACAAAGCGATCTGTCGATTGCAAAGTTTGCTGAT 300
L  M  F  A  Y  M  Y  Q  A  Q  S  D  L  S  I  A  K  F  A  D

ATGAATGAGGCATCTAAGGAGTCAACCACAGCCCAAAAAATGGCTAATCTTGTGGATGCT 360
M  N  E  A  S  K  E  S  T  T  A  Q  K  M  A  N  L  V  D  A

AAAATTGCTGATGTTCAGAGTAGTTCTGACAAGAATAAGAAAGCCAAACTTCCTCAAGAA 420
K  I  A  D  V  Q  S  S  S  D  K  N  K  K  A  K  L  P  Q  E

GTGATTGACTATATAAATGATCCTCGCAATGACATTACAGTAAGTGGTATTAGCGATCTA 480
V  I  D  Y  I  N  D  P  R  N  D  I  T  V  S  G  I  S  D  L

AATGCTGAATTAGGCGCTGGTGATTTGCAAACGGTGAAGGCCGCTATTTCGGCCAAATCG 540
N  A  E  L  G  A  G  D  L  Q  T  V  K  A  A  I  S  A  K  S

AATAACTTGACCACGGTAGTGAATAATAGCCAGCTTGAAATACAGCAAATGTCAAATACG 600
N  N  L  T  T  V  V  N  N  S  Q  L  E  I  Q  Q  M  S  N  T

TTAAACCTATTAACGAGTGCACGTTCTGATATTCAGTCACTGCAATACAGAACTATTTCA 660
L  N  L  L  T  S  A  R  S  D  I  Q  S  L  Q  Y  R  T  I  S

GCAATATCCCTTGGTAAATAActggagataactATGCTTAATGTAAATAGCGATATCCAG 720
A  I  S  L  G  K  *              M  L  N  V  N  S  D  I  Q

TCTATG 726
S  M
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B

```
tcgagtttaattattaaagaggaatttaattATGAATACTATTGATTATACTAATCAAGTA 60
                              M  N  T  I  D  Y  T  N  Q  V

ATGACGGTTAATTCTGTTTCGGAGAATACTACCGGCTCTAATGCAATTACCGCATCTGCT 120
M  T  V  N  S  V  S  E  N  T  T  G  S  N  A  I  T  A  S  A

ATTAATTCATCTTTGCTTACCGATGGTAAGGTCGATGTTTCTAAACTGATGCTGGAAATT 180
I  N  S  S  L  L  T  D  G  K  V  D  V  S  K  L  M  L  E  I

CAAAAACTCCTGGGCAAGATGGTGCGTATATTGCAGGATTACCAACAGCAACAGTTGTCG 240
Q  K  L  L  G  K  M  V  R  I  L  Q  D  Y  Q  Q  Q  Q  L  S

CAGAGCTATCAGATCCAACTGGCCGTTTTTGAGAGCCAGAATAAAGCCATTGATGAAAAA 300
Q  S  Y  Q  I  Q  L  A  V  F  E  S  Q  N  K  A  I  D  E  K

AAGGCCGCTGCAACAGCCGCTCTGGTTGGTGGGGCTATTTCATCAGTATTGGGGATCTTA 360
K  A  A  A  T  A  A  L  V  G  G  A  I  S  S  V  L  G  I  L

GGCTCTTTTGCAGCAATTAACAGTGCTACGAAAGGCGCGAGTGATATTGCTCAAAAAACC 420
G  S  F  A  A  I  N  S  A  T  K  G  A  S  D  I  A  Q  K  T

GCCTCTACATCTTCTAAGGCTATTGATGCGGCTTCTGATACTGCGACTAAAACGTTGACT 480
A  S  T  S  S  K  A  I  D  A  A  S  D  T  A  T  K  T  L  T

AAGGCAACGGAAAGCGTTGCTGATGCTGTTGAAGATGCATCCAGCGTGATGCAGCAAGCG 540
K  A  T  E  S  V  A  D  A  V  E  D  A  S  S  V  M  Q  Q  A

ATGACTACAGCAACGAGAGCGGCCAGCCGTACATCCGACGTTGCTGATGACATTGCCGAT 600
M  T  T  A  T  R  A  A  S  R  T  S  D  V  A  D  D  I  A  D

TCTGCTCAGAGAGCTTCTCAGCTGGCTGAAAACGCTGCAGATGCCGCTCAGAAGGCAAGT 660
S  A  Q  R  A  S  Q  L  A  E  N  A  A  D  A  A  Q  K  A  S

CGGGCAAGCCGCTTTATGGCTGCAGTAGATAAGATTACTGGCTCTACACCATTTATTGCC 720
R  A  S  R  F  M  A  A  V  D  K  I  T  G  S  T  P  F  I  A

GTTACCAGTCTTGCCGAAGGCACGAAGACATTGCCAACAACGGTATCTGAATCAGTCAAA 780
V  T  S  L  A  E  G  T  K  T  L  P  T  T  V  S  E  S  V  K

TCTAACCATGAGATTAGCGAACAGCGTTATAAGTCTGTGGAGAACTTCCAGCAGGGTAAT 840
S  N  H  E  I  S  E  Q  R  Y  K  S  V  E  N  F  Q  Q  G  N

TTGGATCTGTATAAGCAAGAAGTTCGCAGAGCGCAGGATGATATCGCTAGCCGTCTGCGT 900
L  D  L  Y  K  Q  E  V  R  R  A  Q  D  D  I  A  S  R  L  R

GATATGACAACAGCCGCTCGCGATCTCACTGATCTTCAGAATCGTATGGGTCAATCGGTT 960
D  M  T  T  A  A  R  D  L  T  D  L  Q  N  R  M  G  Q  S  V

CGCTTAGCTGGGTAAttgatcatggtcga 989
R  L  A  G  *
```

FIG. 2. Nucleotide sequences of RDEC-1 *espA* (A) and *espB* (B). Asterisks indicate stop codons. Potential ribosome binding sites are underlined. Predicted amino acid sequences of EspA and EspB are aligned in panel C. Shaded areas represent identity.

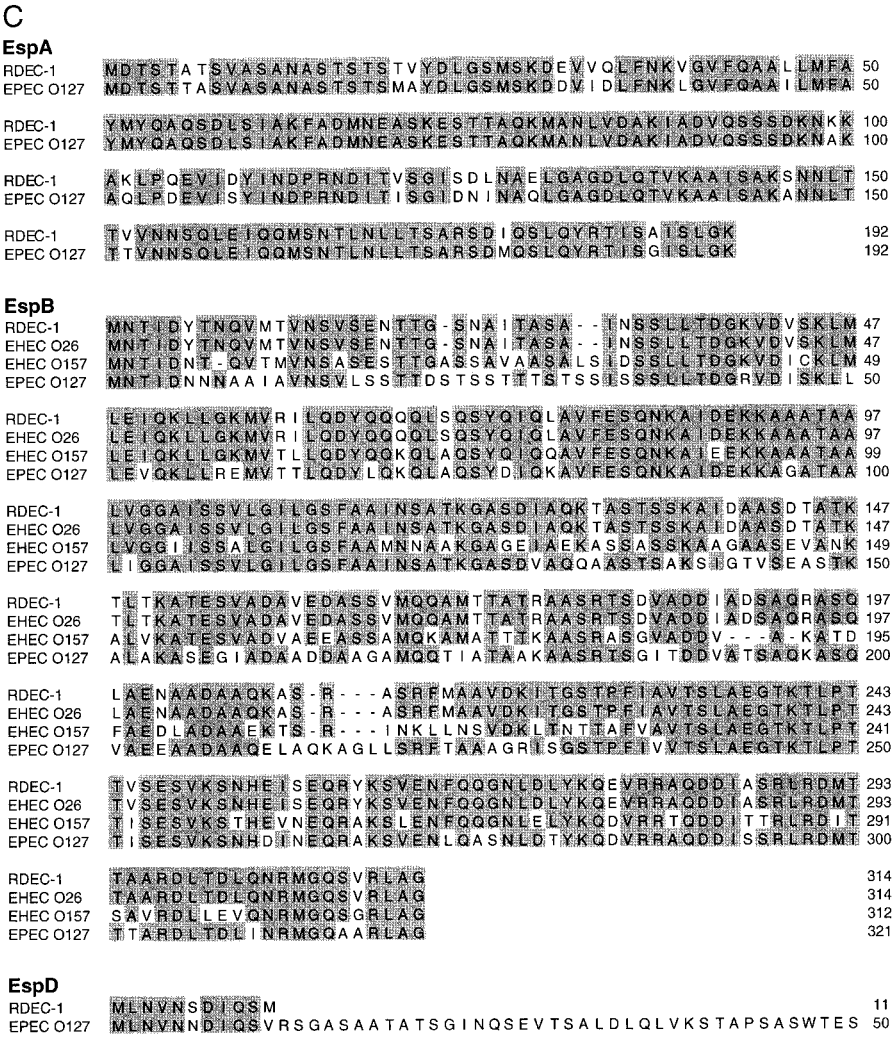

FIG. 2—*Continued.*

compared to the wild-type RDEC-1 strain. Furthermore, the decrease of detectable secretion of the 40- and 39-kDa proteins in the EspA$^-$ EspB$^-$ strain is greater than that found in the EspA$^-$ and EspB$^-$ strains. Secretion of EPEC proteins, except EspC, is mediated by a type III secretion system encoded by the *sep* cluster. It is possible that truncation of EspA or EspB by inserting a stop codon interferes with this secretion pathway or feedback regulation of this system, thereby affecting secretion of the other type III-dependent secreted proteins. Quantification of *esp* transcript levels would be required to test this possibility. Such effects on protein secretion might contribute to changes in the virulence phenotypes attributed to the loss of EspA and/or EspB.

Although the mobilities of RDEC-1 EspA and EspB in SDS-PAGE were slightly higher than that of EPEC, the calculated molecular masses of RDEC-1 EspA and EspB were greater than those of the EPEC proteins. The secretion of EspA and EspB is mediated by a type III secretion system (18). Homologous systems are found in *Yersinia*, *Shigella*, and *Salmonella* species, and proteins are exported without a cleavage of N-terminal signal sequence. RDEC-1 *espA* and *espB* have no discernible signal sequences, and EPEC EspA and EspB pro-

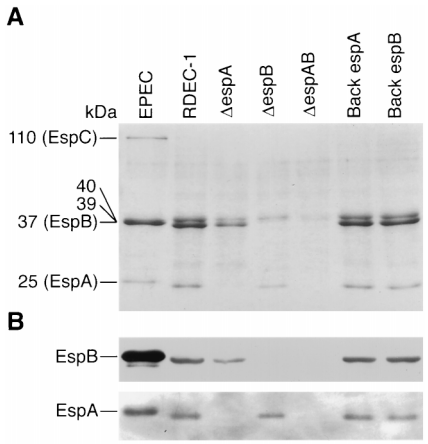

FIG. 3. (A) Secretion profiles of EPEC, RDEC-1, and RDEC-1 mutant strains. Bacteria were grown in DMEM, and secreted proteins were precipitated by addition of 10% trichloroacetic acid, then analyzed by SDS-PAGE (12% gel), and stained with Coomassie blue. (B) Immunoblot of secreted proteins. Anti-EPEC EspA and EspB antisera were used for detection of RDEC-1 EspA and EspB. Strains: EPEC, wild-type EPEC; RDEC-1, wild-type RDEC-1; ΔespA, AAF001ΔA; ΔespB, AAF001ΔB; ΔespAB, AAF001ΔAB; Back espA, AAF002; Back espB, AAF003.

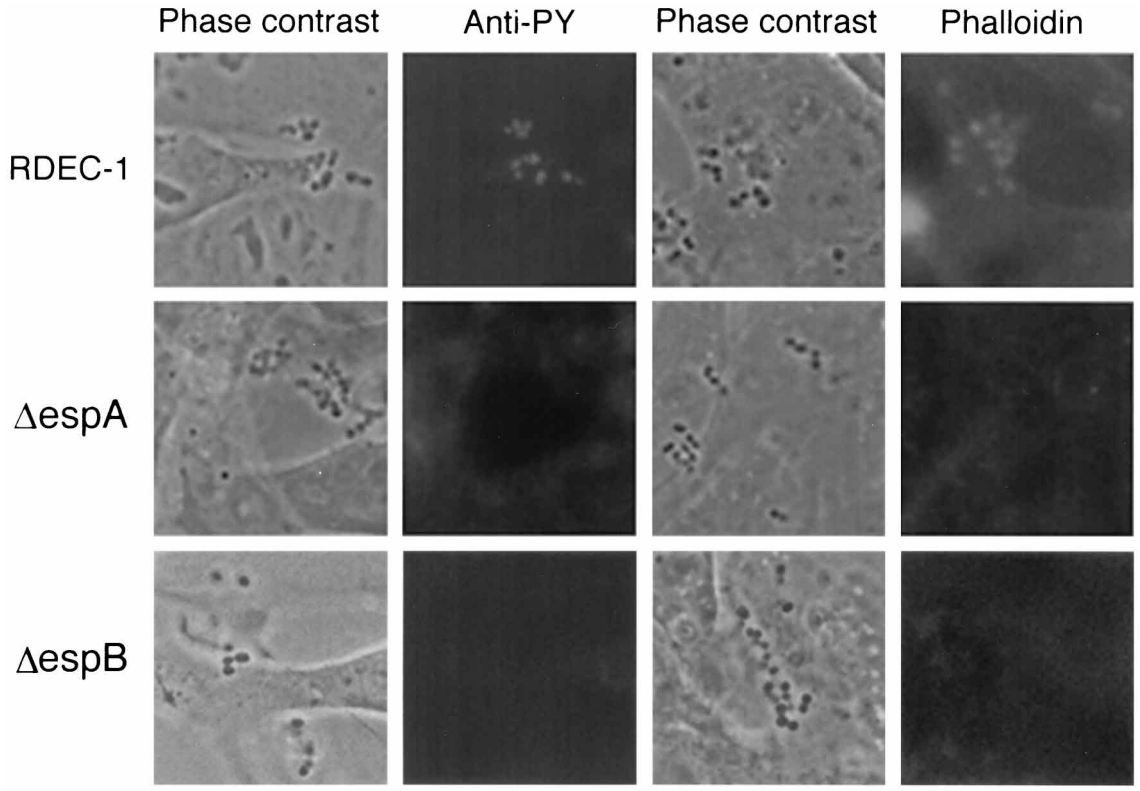

FIG. 4. Accumulation of host cytoskeletal components. HeLa cells (10^5) were infected for 3 h with wild-type RDEC-1, AAF001ΔA (ΔespA), and AAF001ΔB (ΔespB). Infected HeLa cells were fixed by the addition of 3.0% paraformaldehyde; then antiphosphotyrosine (Anti-PY) and phalloidin-rhodamine were used for detection of colocalization of tyrosine-phosphorylated protein and actin by immunofluorescence microscopy.

teins are secreted without N-terminal cleavage (20). The faster migration of RDEC-1 Esp proteins in SDS-PAGE is unclear, although it may reflect amino acid compositions and/or structural features.

Esp proteins are needed for triggering of the host signal transduction pathway. EPEC EspA and EspB proteins induce host signal transduction pathways resulting in accumulation of tyrosine-phosphorylated proteins, cytoskeletal actin, and other components beneath the adherent bacteria. To determine whether RDEC-1 EspA and EspB trigger these events in HeLa cells, cytoskeletal actin and tyrosine-phosphorylated proteins were stained with phalloidin-rhodamine and fluorescently labelled antiphosphotyrosine antibody (Fig. 4 and Table 3). Although the level of accumulation of cytoskeletal actin and tyrosine-phosphorylated protein beneath the attached RDEC-1 is lower than that of EPEC, the staining pattern was indistinguishable

TABLE 3. Phenotypes of various mutant strains

Strain	Relevant characteristics	Phosphotyrosine accumulation	Actin accumulation	Invasion efficiency (%)[a]
Single infection				
EPEC	Wild type	+	+	9,313
RDEC-1	Wild type	+	+	100
AAF001ΔA	$EspA^-$	−	−	6
AAF001ΔB	$EspB^-$	−	−	36
AAF001ΔAB	$EspA^-$ $EspB^-$	−	−	35
AAF002	Back mutation of *espA*	+	+	174
AAF003	Back mutation of *espB*	+	+	142
AAF001ΔA[pMWespA]	$EspA^-$ [EPEC EspA]	+	+	175
AAF001ΔB[pMWespB]	$EspB^-$ [EPEC EspB]	+	+	54
AAF001ΔAB[pMWespAB]	$EspAB^-$ [EPEC EspA EspB EspD]	+	+	414
AAF001ΔAB[pMWΔespD]	$EspAB^-$ [EPEC EspA EspB]	+	+	93
Coinfection				
UMD872/AAF001ΔB	$EspB^-$/EPEC EspB	−	−	ND
UMD864/AAF001ΔA	$EspA^-$/EPEC EspA	−	−	ND

[a] Calculated by dividing the number of invading bacteria by the number of adherent bacteria; values are presented as a percentage of the value for wild-type RDEC-1. ND, not determined.

from that of EPEC. In contrast, RDEC-1 EspA$^-$, EspB$^-$, and EspA$^-$ EspB$^-$ strains did not accumulate cytoskeletal actin or tyrosine-phosphorylated proteins beneath the attached bacteria. However, the back mutant strains AAF002 and AAF003 accumulated these proteins similarly to the parental strains.

When plasmids containing EPEC *espA* and/or *espB* were introduced into the RDEC-1 EspA$^-$, EspB$^-$, and EspA$^-$ EspB$^-$ strains, the accumulation of cytoskeletal actin and tyrosine-phosphorylated proteins was also restored (Table 3). However, when the EPEC EspA$^-$ strain, which still secretes EspB, was coinfected with RDEC-1 EspB$^-$, induction of host signal transduction events was not restored. Furthermore, EPEC EspB$^-$ did not complement RDEC-1 EspA$^-$ in a coinfection experiment. These results suggest that functionally EspA and EspB are similar in RDEC-1 and EPEC with respect to activating host signal transduction pathways, although both proteins need to be secreted by the same strain.

Although tyrosine-phosphorylated Hp90 could be detected by immunoblotting when HeLa cells were infected with EPEC, we did not detect this phosphorylated protein with RDEC-1-infected cells. Furthermore, we have compared protein phosphorylation patterns of HeLa cells infected with RDEC-1 and *espB* mutant strains by immunoblotting analysis (data not shown). However, we could not find any differences, even though we detected tyrosine-phosphorylated proteins beneath adherent RDEC-1 by immunofluorescence. It has been reported that EHEC O157 does not induce tyrosine phosphorylation in HEp-2 and T84 cells, as judged by immunofluorescence microscopy (17). Although EspB of RDEC-1 has no extensive similarity to that of EHEC O157 (Fig. 2C), small deletions at the C terminus were conserved in RDEC-1 and EHEC (O26 and O157) but not in EPEC O127. These results suggest that the lower accumulation of tyrosine-phosphorylated proteins during RDEC-1 infection might be due to (i) lower adherence efficiency of RDEC-1 because of differences in adhesion levels, (ii) heterogeneity of Esp proteins, and/or (iii) a reduced role for tyrosine phosphorylation of Hp90.

Adherence and invasion ability. EPEC EspA and EspB are not only involved in triggering of host signal transduction pathways but also necessary for invasion of HeLa cells in vitro (21). RDEC-1 rabbit infection studies showed that this strain rarely penetrates epithelial cells (38). To investigate the role of RDEC-1 EspA and EspB in adherence and invasion, RDEC-1 *esp* mutant strains were compared to RDEC-1 (Fig. 5 and Table 3). The adherence abilities of EspA$^-$, EspB$^-$, and EspA$^-$ EspB$^-$ strains were similar to that of the wild-type RDEC-1 strain (Fig. 5A), indicating that adherence is independent of EspA and EspB expression. Although the invasive ability of wild-type RDEC-1 was about 90 times lower than that of EPEC, this ability was further decreased in the mutant strains EspA$^-$, EspB$^-$, and EspA$^-$ EspB$^-$ (Fig. 5B). The differences in invasive ability between wild-type and mutant strains were not statistically significant ($P < 0.05$, Student's t test). However, RDEC-1 wild-type invasion levels were obtained with back mutation strains AAF002 and AAF003. These results suggest that RDEC-1-secreted proteins may induce host signal transduction pathways triggering invasion into epithelial cells in vitro. We have shown the in vitro invasive ability of RDEC-1, whereas the role of this phenotype in pathogenesis is unclear.

To determine the ability of EPEC EspA and EspB to complement the RDEC-1 mutants, various plasmids containing the EPEC *esp* genes were introduced into the RDEC-1 mutant strains, and invasion efficiencies were compared to that of the wild-type RDEC-1 strain. Interestingly, the invasion level of AAF001ΔAB (RDEC-1 EspA$^-$ EspB$^-$) harboring pMWespAB (EPEC EspA$^+$ EspB$^+$) was four times greater than that of wild-type RDEC-1 (Table 3), even though the amount of secreted EPEC EspA and EspB in AAF001ΔAB strain was lower than that normally found in RDEC-1 (Fig. 6). Introduction of EPEC *esp* genes into RDEC-1 mutant strains clearly suggest that the different invasion levels observed between RDEC-1 and EPEC strains in HeLa cells can be linked to the Esp proteins, and EPEC EspA and EspB are more efficient at mediating invasion in this tissue culture model. Homology comparisons showed that EspA was highly conserved in RDEC-1 and EPEC but that EspB was more heterogeneous, suggesting that the difference of invasive abilities between RDEC-1 and EPEC may be due to the EspB protein. Interestingly, EHEC O157 adheres to but does not invade human ileocecal (HCT-8) epithelial cells (26). Although EHEC O157 EspB has no extensive similarity to EspB of RDEC-1, the C-terminal deletions were conserved in RDEC-1 and EHEC (O26 and O157) but not in EPEC O157. These

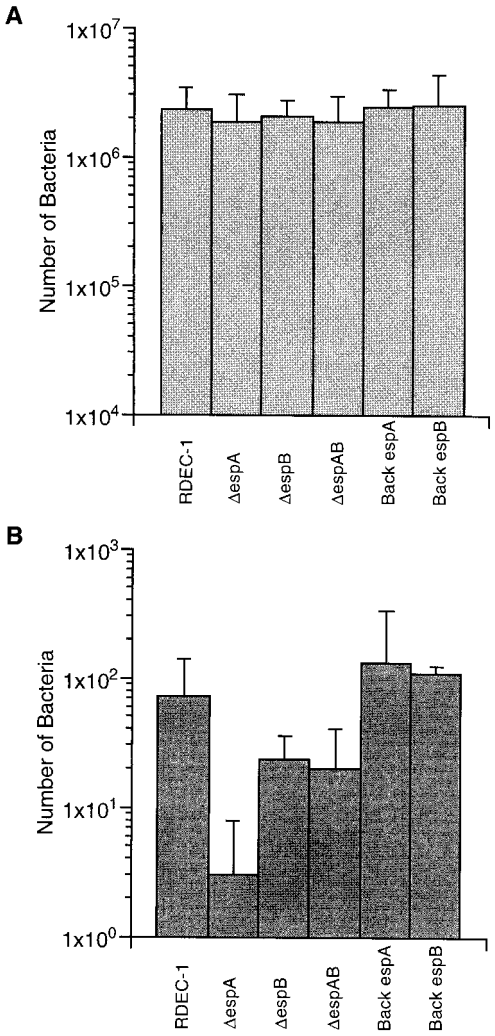

FIG. 5. Adherence (A) and invasive (B) abilities in infected HeLa cells. HeLa cells (10^5) were infected for 3 h with RDEC-1 and mutant strains. The numbers of adherent and invasive bacteria were determined as described in Materials and Methods. Strains: RDEC-1, wild-type RDEC-1; ΔespA, AAF001ΔA; ΔespB, AAF001ΔB; ΔespAB, AAF001ΔAB; Back espA, AAF002; Back espB, AAF003. The experiments were carried out three times, and error bars indicate standard deviations.

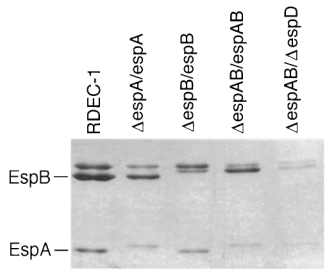

FIG. 6. Secretion profiles of RDEC-1 and mutant strains harboring cloned EPEC *esp* genes. Bacteria were grown in DMEM at 37°C; secreted proteins were analyzed by SDS-PAGE (12% gel) and stained with Coomassie blue. Strains: RDEC-1, wild-type RDEC-1; ΔespA/espA, AAF001ΔA harboring pMWespA; ΔespB/espB, AAF001ΔB harboring pMWespB; ΔespAB/espAB, AAF001ΔAB harboring pMWespAB; ΔespAB/ΔespD, AAF001ΔAB harboring pMWΔespD.

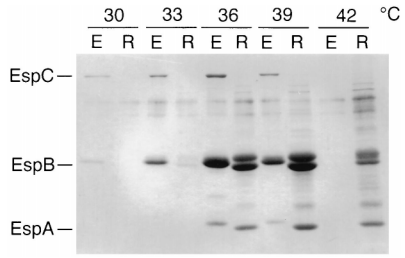

FIG. 7. Comparison of secretion profiles of RDEC-1 and EPEC strains at different temperatures. Bacteria were grown in DMEM at different temperatures, and secreted proteins were prepared from cultures at an optical density of 1.0 at 600 nm. Secreted proteins were analyzed by SDS-PAGE (12% gel) and stained with Coomassie blue. Strains: E, wild-type EPEC; R, wild-type RDEC-1.

findings suggest that Esp heterogeneity affects the in vitro invasive ability of EPEC, RDEC-1, and EHEC.

EspD mutant affects EspA and EspB secretion. EPEC contains an ORF, *espD*, located between *espA* and *espB* (Fig. 1B). To confirm the role of the *espD* product in secretion, we constructed plasmid pMWΔespD, encoding EPEC *espA*, Δ*espD* (frameshift mutation at the *Bgl*II site), and *espB*, and introduced it into the RDEC-1 double-mutant strain AAF001ΔAB. The amount of EPEC-secreted EspA and EspB proteins in AAF001ΔAB[pMWΔespD] was lower than that in AAF001ΔAB [pMWespAB], which contains a fragment encoding intact EPEC *espA*, *espD*, and *espB* genes (Fig. 6). Furthermore, invasion ability was also decreased. These results suggest that disruption of *espD* affects secretion of EPEC EspA and EspB proteins. In this study, we showed that mutations in *espA* and/or *espB* also reduced the amounts of the other secreted proteins, probably due to their truncated products. Secretion levels were lower in *espA* and *espB* double mutants than in *espA* or *espB* mutants. Thus, truncated EPEC EspD may affect the secretion of EspA and EspB in AAF001ΔAB[pMWΔespD] due to interference in type III secretion system. Whether EspD is directly involved in this secretion system is still unclear.

EPEC- and RDEC-1-secreted proteins are tightly controlled by temperatures which correspond to their relevant host body temperatures. Temperature-regulated expression of EPEC- and EHEC 413/89-1-secreted proteins has already been reported: EspB expression was greatly increased when the strains were incubated at 37°C compared to growth at lower temperatures (11, 20). Based on the hypothesis that EspA and EspB proteins are regulated by appropriate host body temperatures, wild-type EPEC and RDEC-1 strains were inoculated into DMEM, and the secreted proteins were prepared following incubation at various temperatures and then analyzed by SDS-PAGE (Fig. 7). Expression of EPEC-secreted proteins was detected at 33°C and reached the maximal secretion level at 36°C. Expression was decreased at 39°C, and no secreted proteins were seen at 42°C. In contrast, maximal expression of RDEC-1-secreted proteins occurred at 39°C, and these proteins were still expressed at 42°C. These results suggest that the maximal expression of Esp proteins in EPEC and RDEC-1 are triggered by their relevant host body temperatures 37°C (human) and 39°C (rabbit), and would presumably contribute to host specificity and pathogenicity.

In conclusion, we have identified two RDEC-1-secreted proteins, EspA and EspB. EspA is highly conserved between EPEC O127 and RDEC-1. However, EspB is less conserved, and EspB of RDEC-1 is more closely related to that of EHEC O26 than that of EPEC O127. Both proteins were needed to trigger host signal transduction pathways and invasion. Complementation experiments using EPEC *esp* genes revealed that host signal transduction events triggered by RDEC-1 and EPEC appear to be mediated by the same secreted proteins. Finally, optimal expression of RDEC-1- and EPEC-secreted proteins correlated with their natural host body temperatures. It is known that the plasmid-encoded adhesion of EPEC (BFP) and RDEC-1 (AF/RI) is host specific. In this study, we showed that the body temperature is also an important factor for strict host specificity and may contribute to the lack of successful EPEC infections in rabbits or other animals. Animal infection studies using RDEC-1 *espA* and *espB* strains will provide information about the role of these secreted proteins in virulence and may be useful for vaccine studies.

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