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Phenotypic and genetic features of *Escherichia coli* strains showing simultaneous expression of localized and diffuse adherence

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Abstract

We have previously shown that some *Escherichia coli* strains isolated from children with diarrhea present the so-called 'localized and diffused adherence (LA/DA) pattern' in which both localized adherence (LA) and diffused adherence (DA) are expressed simultaneously. In the present study, we show that the LA adherence of these strains is genetically and phenotypically similar to that so far described for enteropathogenic *E. coli* (EPEC) as determined by DNA hybridization and electron microscopy. On the other hand, the DA is encoded by genes not homologous to the DAEC or AIDA-I DNA probes. In addition, the LA/DA strains are able to invade eukaryotic cells both in vitro and in vivo. In the rabbit ileal loop assay their invasion capacity goes beyond the enterocyte and reaches the muscularis mucosae as determined by transmission electron microscopy. These findings suggest that the LA/DA adherence pattern may be linked to a new *E. coli* virulence category which in the case of the strains studied may be associated to other virulence traits that enable them to more deeply invade the intestinal mucosa. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Diarrhea; *Escherichia coli*; Adhesion; Localized adherence; Diffuse adherence; Invasion

1. Introduction

Bacterial adherence to the intestinal mucosa has been identified as being characteristic of *Escherichia coli* strains that cause diarrhea in infants [1]. In a previous paper [2], we have shown that *E. coli* strains can adhere to HeLa cells in two different patterns: localized adherence (LA), in which microcolonies are formed at one or more regions on the cell, and diffuse adherence (DA), where bacteria uniformly cover the cells. Subsequently, Nataro et al. [3] described a third *E. coli* adherence pattern in HEp-2 cells, called aggregative adhesion (AA), in which the bacteria have a characteristic stacked brick appearance on both the surface of the cells and the glass slide. In a study on the correlation between adherence patterns and *E. coli* serotypes, we reported that some serotypes simultaneously exhibited the LA and DA phenotypes [4], further designated as the LA/DA pattern [5]. This pattern is characterized by clusters of bacteria attached to localized areas of the cells and by single bacteria covering the whole surface of the same cells. The lack of information on the

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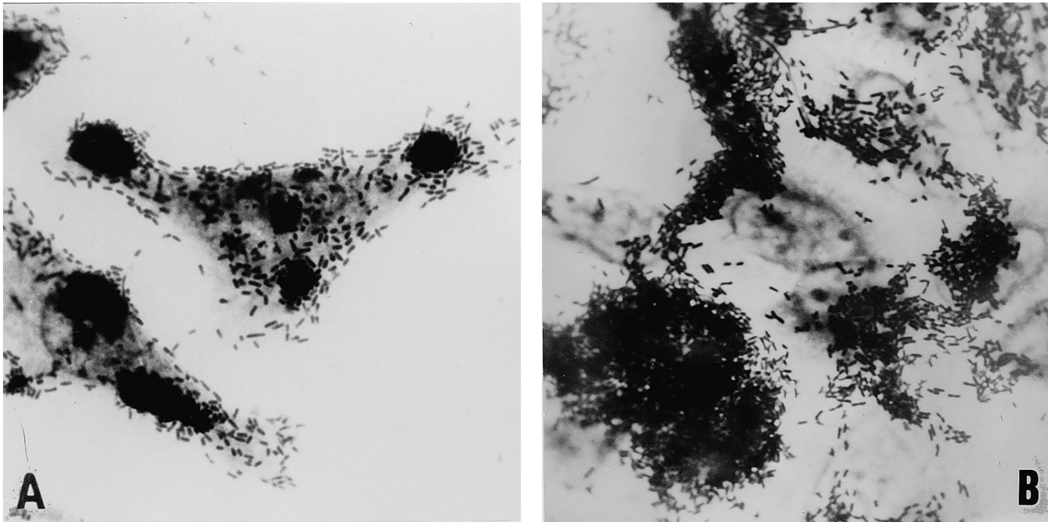

Fig. 1. Adherence of *E. coli* strains to HeLa cells. (A) Localized and diffuse pattern displayed by strain 163/10 HC. (B) Aggregative pattern displayed by strain 17-2. A, ×770; B, ×847.

virulence characteristics displayed by the LA/DA strains both in vitro and in vivo led us to determine their virulence traits as well as their pathogenic potential in the rabbit ileal loop assay. Since in vitro adhesion tests may raise doubts on whether the strain has an LA/DA or AA pattern depending on the incubation period, we compared these two patterns by using both adhesion and DNA hybridization assays.

2. Materials and methods

2.1. Bacterial strains

We have studied three *E. coli* strains from serotype O55:H$^-$ isolated from cases of children with diarrhea and characterized in previous work as presenting the LA/DA adherence pattern [4]. *E. coli* strains 71-82 HSJ, 11LCH and 17-2 were used as controls of

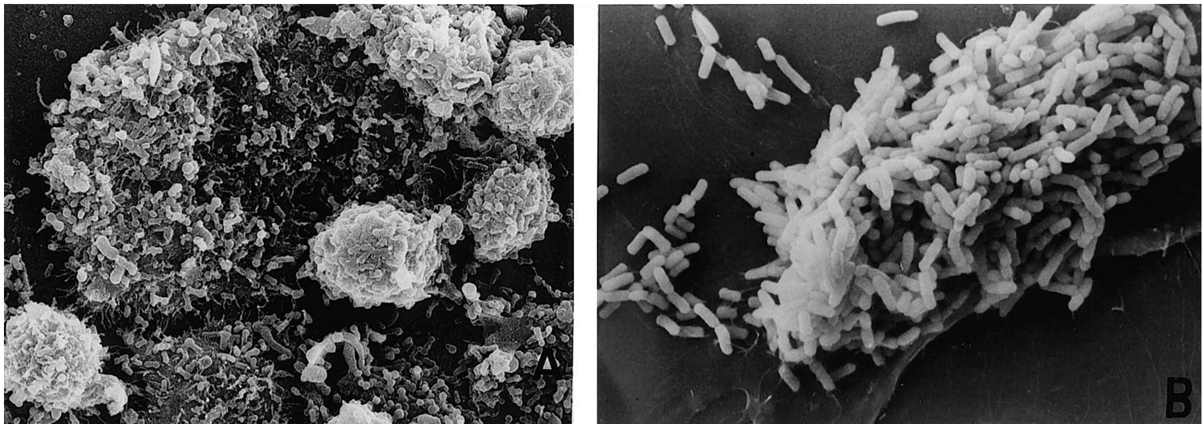

Fig. 2. Scanning electron microscopy of HeLa cells incubated with LA/DA strain 163/10 HC (left). Bacteria can be seen in clusters and diffusely attached to the epithelial cell surface. Right: AA strain 17-2. Bacteria are seen covering the surface of the cell. Left, ×1360; right, ×3400.

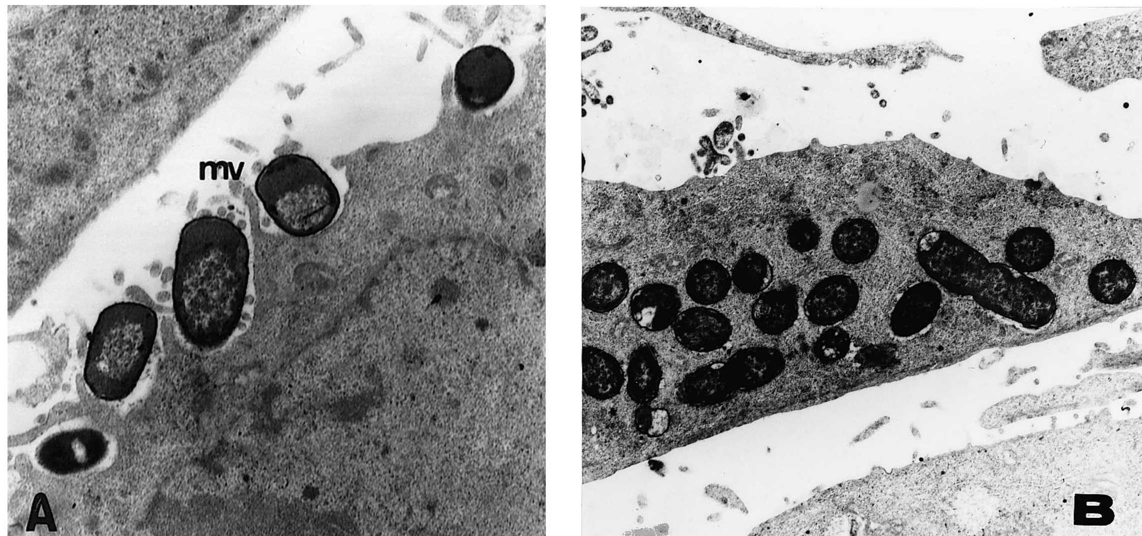

Fig. 3. Transmission electron micrographs of HeLa cells incubated with LA/DA strain 163/10 HC. (A) Bacteria can be seen adhered to cell membrane with microvilli effacement (mv). (B) Bacteria are seen enclosed in membrane-bound vacuoles. A, ×10 080; B, ×5040.

typical LA, DA and AA phenotypes, respectively. All the strains used were kept at −70°C in 15% glycerol since their isolation.

2.2. Adhesion and invasion assays

Bacterial cell adhesion and invasion assays were performed as described previously [2]. Briefly, monolayers of 10^5 HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) in 35-mm tissue culture dishes (Corning, Charlotte, NC). Bacterial strains were grown statically in 3 ml of tryptic soy broth (Difco, Detroit, MI) for 16–18 h at 37°C. The monolayers were infected with, approximately 3×10^7 bacteria (40 μl of bacterial cultures added to 1 ml of DMEM) and incubated at 37°C for 3 h. The infected monolayers were washed with sterile phosphate-buffered saline (PBS), fixed with methanol, stained with May–Grunwald–Giemsa stain, and examined under the light microscope. For invasion assay, the DMEM was removed after 3 h of infection, the monolayers were washed three times with PBS and further incubated for 1 h in DMEM containing 100 μg ml^{-1} gentamicin. Preparations were fixed, stained and examined as described above. To evaluate the number of intra-

Table 1
Adherence pattern, invasion ability, hybridization pattern and FAS test of the *E. coli* strains studied

Strain	Adherence pattern	% Invasion[a,b]	Hybridization pattern	FAS test
135/12HC	LA/DA	1.57	EAF, *bfp*A, *eae*A	+
163/10HC	LA/DA	1.88	EAF, *bfp*A, *eae*A	+
172/1HC	LA/DA	1.67	EAF, *bfp*A, *eae*A	+
71-82HSJ	LA	1.62	EAF, *bfp*A, *eae*A	+
11LCH	DA	ND[c]	*eae* A	+
17-2	AA	ND	AA	−

[a]Percentage of initial inoculum surviving treatment with gentamicin.
[b]The percentage of invasion of *E. coli* HB101 (negative control) and an enteroinvasive *E. coli* O28ac (positive control) were 0.002% and 2.95%, respectively.
[c]ND, not detected.

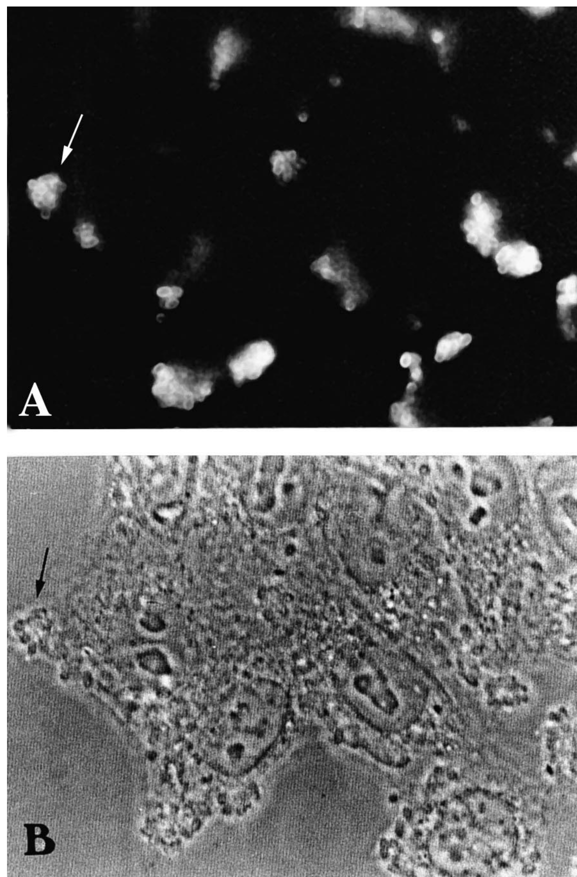

Fig. 4. Accumulation of actin at sites of adherent LA/DA strain 163/10 HC. HeLa cells were infected for 3 h with bacterial cells and stained for filamentous actin with FITC-phalloidin. Fluorescence (A) and corresponding phase-contrast (B) micrographs are shown. Bacteria showing fluorescence at the site of clustered bacteria (arrow). ×830.

cellular bacteria after exposure to gentamicin, the monolayers were treated with 1% Triton X-100 to release the internalized bacterial cells, and viable counts were determined. The amount of intracellular bacteria was expressed as the percentage of the initial inoculum surviving treatment with gentamicin. For transmission electron microscopy (TEM), the monolayers were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 1 h, gently scraped and washed with 0.1 M cacodylate buffer. The cell pellets mounted in agar blocks were cut and fixed in 1% osmium tetroxide, dehydrated in graded series of alcohol and embedded in POLY/BED 812 (Polysciences). Ultrathin sections were stained with uranyl acetate and lead citrate [6], and visualized with a JEOL JEM 1200 electron microscope. For scanning electron microscopy, the fixed cells were dehydrated with acetone, critical-point dried, and coated with gold.

2.3. Fluorescent actin staining (FAS)

The FAS test was performed after 3 h incubation with HeLa cells as described [18].

2.4. Probes

The following specific gene probes were used: EAF (EPEC adherence factor), a 1.0-kb *Bam*HI–*Sal*I fragment from plasmid pMAR2 [7]; *eae*A (*E. coli* 'attaching and effacing' gene A encoding intimin), a 1-kb *Sal*I–*Kpn*I fragment from plasmid pCVD434 [8]; *bfp*A (bundle-forming pilus gene), a 852-bp *Eco*RI fragment from pMSD207 [9]; DAEC, a 350-bp *Pst*I fragment of pSLM852 (this fragment is located within the *daa*C gene whose product is associated with the assembly of the F1845 fimbria of diffusely adherent *E. coli* strains) [10]; AIDA-I factor, a 6.2-kb *Sph*I–*Cla*I fragment of pIB264 (this factor is a protein associated with the DA phenotype) [11]; AA factor (enteroaggregative *E. coli* adherent plasmid), a 1-kb *Eco*RI–*Pst*I fragment of pCVD432 [12]; pIS2 (a 6.6-kb plasmid isolated from *E. coli* O111:H^{-} which codifies for invasion of epithelial cells) [13]; pLV527, a 4.5-kb *Eco*RI–*Sal*I fragment of pLV501 (an invasion plasmid of *E. coli* O111:H^{-}) [14]; and INV, a 2.5-kb *Hin*dIII fragment from pSF55 (a cloned DNA fragment of invasion plasmid of *Shigella flexneri*) [15]. The DNA probe fragments were labeled with [α-^{32}P]dATP by nick translation [16], and the hybridization conditions were described elsewhere [17]. The recombinant plasmids containing the respective DNA probe-fragments were used as positive controls, and pBR322 was the negative control.

2.5. Rabbit ileal loop assay

The rabbit ileal loop assay was carried out by the procedure of Moon et al. [19]. New Zealand white rabbits, 9–10 weeks old, were fasted for 48 h before ileal loop surgery. Six ligated loops, approximately

5-cm long each, separated by 2-cm interloops, were made in each rabbit. Each loop was inoculated with 0.5 ml of a suspension of 10^9 bacteria per ml. The animals were killed after 24 h and segments from each intestinal loop were fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) and prepared for light microscopy and TEM.

3. Results

3.1. Examination of the LA/DA pattern by microscopy

Microscopy examination of adhesion tests performed using a 3-h incubation period confirmed the LA/DA pattern exhibited by the O55:H^- strains (Fig. 1A), which is very much different from what is seen in a typical AA pattern (Fig. 1B).

Scanning electron microscopy of HeLa cells infected with all the LA/DA strains studied showed the same association pattern, meaning microcolonies adhered in a typical LA pattern as well as single bacteria diffusely attached to the microvilli similar to a DA pattern, confirming their LA/DA phenotype

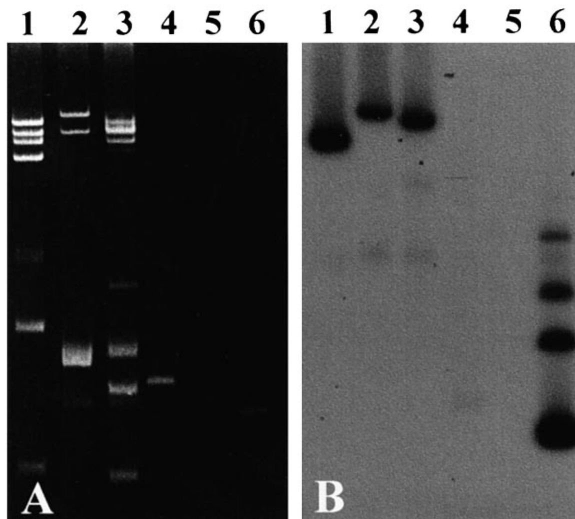

Fig. 5. (A) Agarose gel electrophoresis and Southern blot hybridization [20]. Plasmid DNA was isolated from strains 135/12 HC (lane 1), 163/10 HC (lane 2), 172/1 HC (lane 3), pBR322 (lane 4), empty slot (lane 5), pMSD207 (lane 6). (B) Southern blot hybridization with *bfp*A probe.

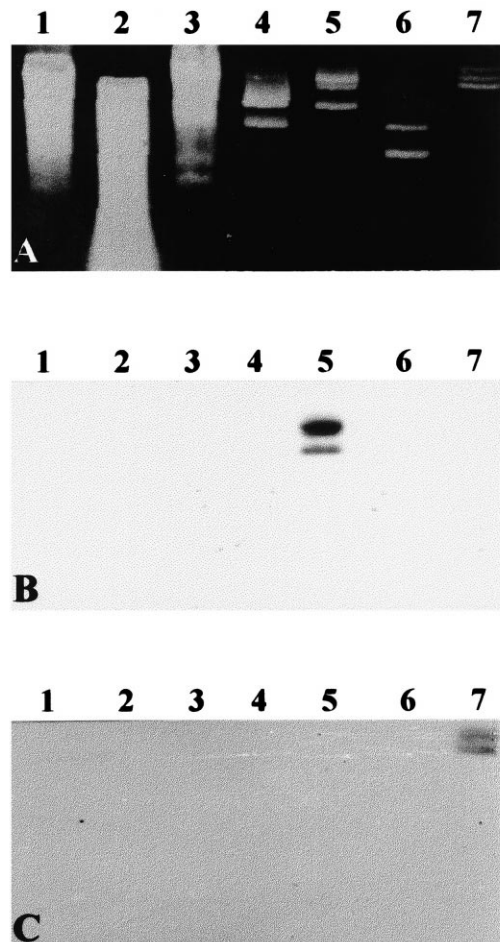

Fig. 6. (A) Agarose gel electrophoresis of total DNA digested with *Hin*dIII. Lanes: 1, 135/12 HC; 2, 163/10 HC; 3, 172/1 HC; 4, pBR322; 5, pSLM 852; 6, pUC8; 7, pIB264. (B,C) Southern blot hybridization with (B) DAEC probe and (C) AIDA-I probe.

(Fig. 2, left) which again can be very easily distinguished from a typical AA pattern (Fig. 2, right). Visualization of the LA/DA pattern by TEM showed bacteria in intimate contact with the eukaryotic cell membrane, and effacement of the microvilli (Fig. 3A) as well as invasion of the HeLa cells where bacteria were found inside endocytic vesicles (Fig. 3B). The LA/DA strains were as invasive as the LA control strain, but about 700× more invasive than *E. coli* HB101 (negative control), and about 2× less invasive than an enteroinvasive *E. coli* O28ac (positive control) (Table 1).

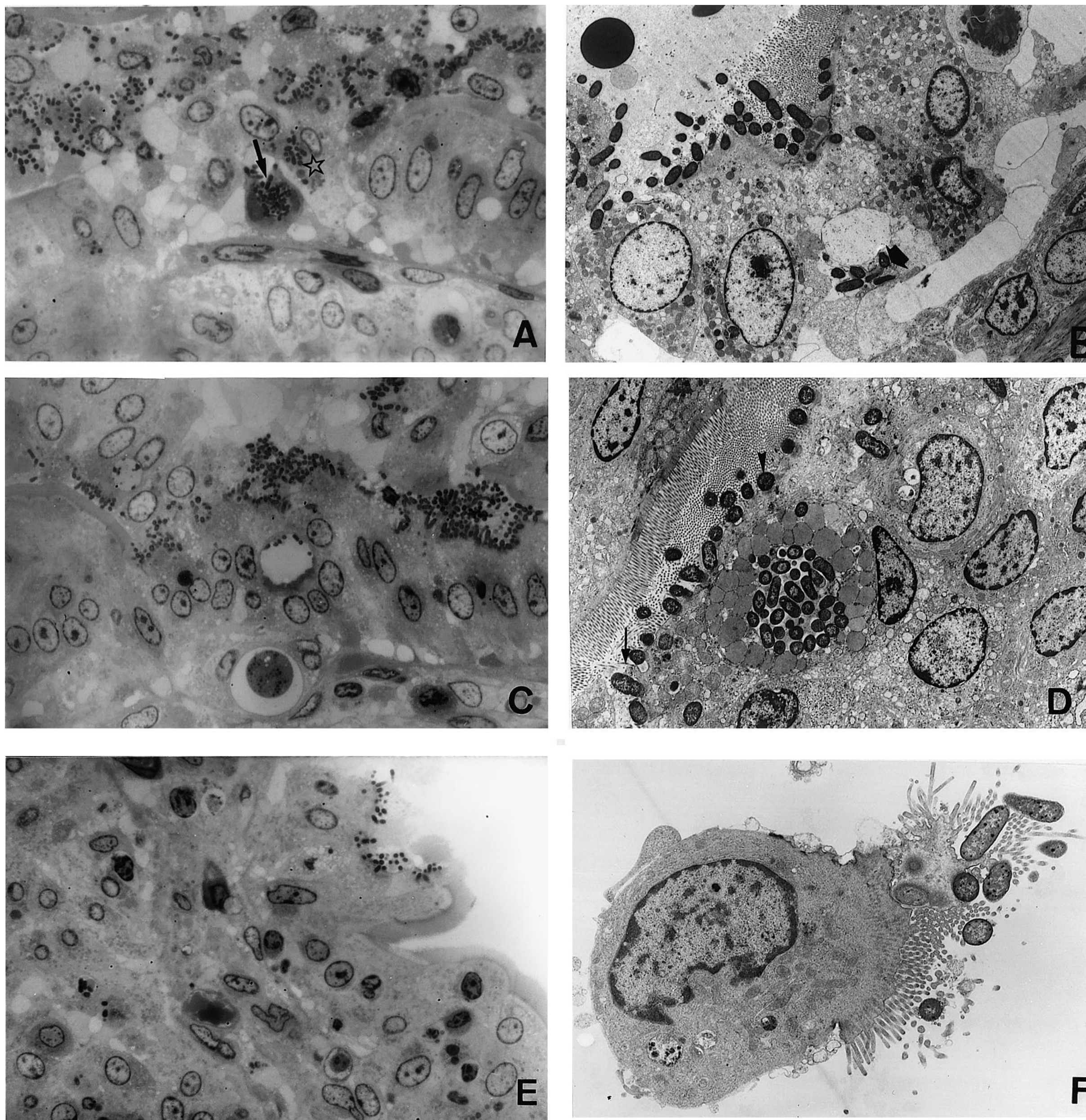

Fig. 7. Light and electronmicroscopy of sections of rabbit intestinal loop infected with: (A,B) LA/DA strain 163/10HC. Bacteria adhering to and invading enterocytes (star), a cell membrane invagination containing many bacterial cells (arrow), and bacterial cells enclosed in membrane-bound phagosome-like vacuole localized in the muscularis mucosae (arrowhead) are evident; (C,D) LA strain 71-82HSJ. Bacterial cells are seen adhered to and invading the enterocytes; characteristic effacement of microvilli (arrow), membrane cupping, and pedestal formation at the sites of attachment (arrowhead) can also be seen; (E,F) DA strain 11LCH. Single bacteria are seen simply adhered to the enterocyte membrane with no cellular alterations at the site of attachment. A, ×1890; B, ×1890; C, ×1890; D, ×2646; E, ×1890; F, ×3906.

3.2. Accumulation of actin filaments

The LA/DA strains exhibited a positive reaction, showing intense fluorescence at the sites of clustered bacteria (Fig. 4).

3.3. Hybridization analysis

The LA/DA strains were tested for the presence of genes associated with adhesion and invasion phenotypes. As shown in Table 1, the LA/DA O55:H$^-$ strains reacted with the same DNA probes as the LA control strain. Fig. 5 shows the results obtained with the *bfp*A probe. The strains did not show homology either with the AA, DAEC and AIDA-I probes or with the invasive probes. Fig. 6 shows the results obtained with the DAEC and AIDA-I probes.

3.4. Analysis of the LA/DA strains in the rabbit ileal loop assay

The pathogenicity of the LA/DA strains was evaluated in vivo by the rabbit ileal loop assay [19]. Examination of sections of the loops inoculated with these strains revealed bacteria in intimate contact with the apical epithelial cell membrane, causing distortion of the microvilli with pedestal formation, as well as bacteria within the enterocytes (Fig. 7A,B) similar to what was seen with the LA strain (Fig. 7C,D). The difference was that only LA/DA strains were seen deeper invading the muscularis mucosae (Fig. 7B). Inoculation of the intestinal loops with the DA control strain resulted in no visible alterations of the enterocyte at the site of attachment (Fig. 7E,F).

4. Discussion

The results presented herein show that O55:H$^-$ *E. coli* strains displaying the LA/DA adherence pattern harbor all the phenotypic and genetic characteristics found in typical EPEC strains. Besides, they present a diffuse adherence which is independent of the LA phenotype. We have also shown that this DA phenotype is encoded by genes not related to the AIDA-I and DAEC probes that are being used by other researchers to characterized DAEC strains. In fact those two DNA probes have been shown not to react with many *E. coli* isolates displaying the diffuse adherence phenotype [21,22] suggesting that there might be a family of adhesins genetically different, but expressing the same adherence pattern in cultured cells.

As we have shown, the LA/DA adherence pattern can be well distinguished from the AA pattern by both DNA hybridization and in vitro adhesion tests when they are carried out using a 3 h incubation period.

The report from Donnenberg et al. [23] on the capability of EPEC to invade eukaryotic cells in vitro or in vivo indicates that this is a complex process involving multiple genetic loci. However, these strains have never been shown internalized beyond the epithelial cells of the intestinal mucosa, even when they possess extra plasmids that enable them to invade eukaryotic cells as shown by Fletcher et al. [14] and by Scaletsky et al. [13]. Considering that the O55:H$^-$ strains studied have all the characteristics of typical EPEC and do not possess extra genetic invasion determinants as those referred above, the ability of these strains to invade the rabbit intestinal mucosa as deep as the muscularis mucosae is a phenomenon of which the mechanisms involved remain to be elucidated.

On the other hand, epidemiological studies are necessary to evaluate the frequency of *E. coli* strains associated with diarrheal disease and presenting the multiplex virulence phenotype described herein. It is also important to carry out investigations to address if the peculiar invasion capacity of these strains renders them able to cause a more severe and/or long-lasting diarrheal disease.

Acknowledgments

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