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Invasion of tissue culture cells by diarrhoeagenic strains of *Escherichia coli* which lack the enteroinvasive *inv* gene

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Abstract

Invasive *Escherichia coli* strains of certain serotypes invade by the same mechanism as the *Shigella* sp. It has been proposed that invasion of epithelial cells by EPEC strains may also occur; this is a previously overlooked property. In the present study *E. coli* strains isolated from patients with diarrhoea or ulcerative colitis, lacking the *inv* plasmid mediating classical invasion, but hybridizing with probes for different adhesins, were analyzed for their ability to invade HeLa and Caco-2 cells. The majority of strains invaded Caco-2 cells to a higher extent than HeLa cells. Adhesion to Caco-2 cells was a prerequisite for subsequent invasion of the cells but EAF, *eae*, EAgg and other known virulence factors were not sufficient to mediate invasion. In 8/9 *E. coli* strains invasion was enhanced after growth under iron restriction. Growth during anaerobic conditions did not influence subsequent invasion by *E. coli* strains whereas 6/9 strains had their invasive ability significantly decreased after growth in the presence of 1% glucose. The invasive process was inhibited by mannose but not by lactose, fucose or galactose. Our data indicate that strains of *E. coli* may invade Caco-2 cells by novel mechanisms which require adhesion to the cells but which differ from those of *Salmonella* sp., *Yersinia* sp., *Shigella* sp. and classical enteroinvasive *E. coli*.

Keywords: Escherichia coli; Adhesion; Invasion; Tissue culture cell; Iron; Growth condition; Glucose; Mannose

1. Introduction

Invasion of enteric epithelial cells by pathogenic bacteria is a well recognized phenomenon. *Shigella* sp. and enteroinvasive *Escherichia coli* (EIEC) invade through the basolateral pole of human intestinal cells, multiply intracellularly and spread horizontally, killing the colonic enterocytes [1,2] but rarely spread beyond the lamina propria. *Salmonella* sp. and *Yersinia* sp. preferentially invade ileal tissue, multiply within the cells to a lesser extent than *Shigella* sp., and commonly migrate to the underlying tissue [3,4]. *Campylobacter jejuni* was recently reported to be invasive [5].

In *E. coli* isolated from intestinal infections several virulence determinants have been identified. These include the fimbrial adhesins and enterotoxins of enterotoxigenic *E. coli* (ETEC), different ad-

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hesins, probably outer membrane proteins of enteropathogenic *E. coli* (EPEC), and Shiga-like toxin and an outer membrane protein adhesin of enterohaemorrhagic *E. coli* (EHEC). The EPEC adhesins confer the ability to attach to intestinal cells in different ways including localized adherence (LA), diffuse adherence (DA), whilst the AAF/1 fimbriae of enteroaggregative *E. coli* (EAggEC) are responsible for the aggregative pattern of adherence of EAggEC [6–9]. EPEC strains adhering in a localized way harbour the EPEC Adherence Factor (EAF) plasmid of about 60 mDa. On this plasmid the *bfp*A gene has been identified, which encodes bundle-forming pili and is proposed to induce the localized adherence phenotype [10,11]. Certain EPEC strains attach to small intestinal epithelial cells, efface microvilli and induce F-actin polymerization in the enterocyte. The *eae*A gene (EPEC attachment and effacement) has been cloned from an EPEC strain [12]. Studies with deletion mutants of the *eae*A gene have shown that *eae*A mediates the close attachment to intestinal cells but not the actin polymerization [8,12]. Recently, a second gene, *eae*B, also mediating intimate attachment to intestinal cells, was identified [13].

The invasive ability of *Yersinia* sp. and *S. typhimurium* differs from that of *Shigella*, and appears mainly to be mediated by chromosomal genes [14]. In *Y. enterocolitica* and *Y. pseudotuberculosis*, both causative agents of terminal ileitis, the *inv* and *ail* genes have been cloned and shown to mediate invasion [15]. Also the plasmid-encoded *yad*A product of *Y. pseudotuberculosis* promotes cellular internalization [16]. Most interestingly, the *inv* gene of *Y. enterocolitica* shows significant sequence homology with the *eae*A gene of EPEC and also with a recently cloned *eae* gene in an EHEC strain [17].

The expression of invasive ability of *S. typhimurium* is greatly influenced by environmental conditions like growth medium, expression of type I fimbriae, anaerobiosis and growth phase of the cells [18] whereas adhesion to the host cell, a prerequisite

Table 1
Characterization of *Escherichia coli* and other strains

Organism	Serotype	Hybridizing with: [a]	Caco-2 cells	
			Adhesion [b]	Invasion [c]
2057-61	0111:H −	EAF, *eae*A, SS27	LA	2.30 ± 0.02
2050-61	0111:H −	*eae*A, SS27	NA	0.41 ± 0.11
E2348	0127:H6	EAF, *eae*A	LA	6.48 ± 1.90
CVD206	0127:H6	EAF, Δ*eae*A	LA	2.11 ± 0.06
JPN15	0127:H6	*eae*A	DA	0.18 ± 0.02
D16700	06:H3	*eae*A	DA	7.31 ± 0.91
E224	051:H49	*eae*A	DA	6.43 ± 0.91
CVD432	n/a [d]	EAgg	(AA) [e]	8.06 ± 1.98
E31	030:H4	EAgg	AA	5.82 ± 0.10
D6211	021:H2	EAgg, SS27	AA	6.59 ± 1.84
D6152	01:K1:H −		DA	7.33 ± 0.69
D15288	06:H −	SS27	DA	9.82 ± 1.06
D14258	01:K1:H −		DA	10.58 ± 1.01
E510	*E. cloacae*	EAgg	AA	5.74 ± 0.54
E116	*Y. pseudotub*	*eae*	DA	6.31 ± 0.36
Yp111	*Y. pseudotub*	inv +, *eae*	DA	6.47 ± 0.72
Yp100	*Y. pseudotub*	inv −, *eae*	DA	2.01 ± 0.16
S.t.m. [f]	04:Hi:2	ND	ND	3.42 ± 0.68

[a] Source of gene probes: EAF [6], *eae*A [12], EAgg [7], SS27 [30], inv [1].
[b] Adhesion to Caco-2 cells determined as localized (LA), autoaggregative (AA), diffuse (DA), or no adhesion (NA).
[c] Invasion of Caco-2 cells ± S.E.
[d] ND = not detemined, n/a = Not applicable.
[e] weak autoaggregative adherence.
[f] S.t.m. = *Salmonella typhimurium*.

for invasion, is not [19]. In *Yersinia* strains invasin synthesis is optimal at 28°C but the *ail* gene product is best expressed at 37°C [20].

Stimulated by the study by Donnenberg et al. [21] that epithelial cell invasion is an overlooked property among EPEC strains, and by the identification of a plasmid, pLV501, associated with EPEC invasivity [22,23], as well as by the cloning of two genes mediating invasivity of ETEC strains [24] we undertook this study on expression of invasivity of intestinal cells by intestinal *E. coli* strains which lack the *inv* gene of EIEC but carry other identified virulence determinants. In addition, one *Enterobacter cloacae* strain hybridizing with EAgg and one *Y. pseudotuberculosis* strain hybridizing with the *eae* probe were studied.

2. Materials and methods

2.1. Bacterial strains

The characteristics of the *E. coli* strains used are listed in Table 1. *E. coli* 2057-61 was isolated from a child with diarrhoea, and strain 2050-61 is a derivative, cured of the EAF plasmid, both obtained from Dr. K. Wachsmuth, CDC, Atlanta, GA. The type strain for EPEC adherence factor, E2348, and strains CVD 206 [25] and JPN15 mutated in the chromosomal *eae* locus or lacking the EAF plasmid, respectively, were obtained from Dr. J.B. Kaper, Baltimore, MD. Strain CVD 432 is *E. coli* DH5α, carrying the recombinant plasmid for enteroaggregative adherence, pCVD 432, also obtained from Dr. J.B. Kaper [7]. *E. coli* 6211, 6152, 15288, 16700, and 14258 were isolated from rectal biopsies of patients with ulcerative colitis [26]. E31 and E224 were isolated as dominating faecal isolates from Ethiopian children with sanguinolent diarrhoea, *Ent. cloacae* E510 was from a child with acute watery diarrhoea, and *Y. pseudotuberculosis* E116 from a child with persistent diarrhoea. *Y. pseudotuberculosis* YpIII and a non-invasive mutant, Yp100, were kindly obtained from Dr. H. Wolf-Watz, Umeå, Sweden. *S. typhimurium* was isolated from a Swedish patient with acute enteritis. *E. coli* HB101 was used as negative control. Strains were typed to the species level according to Kelly et al. [27], and were stored at −80°C in TSB with 20% glycerol. The strains were cultured on colonization factor antigen (CFA) agar at 37°C for 18 h unless otherwise stated.

2.2. Colony blot hybridization

All strains were inoculated on nylon membranes (Zeta-Probe, BioRad, Richmond, CA) after growth on blood agar (4% horse erythrocytes) for 6 h at 37°C. After lysing the colonies, nonspecific binding was blocked with 1% skimmed milk as earlier described [28]. Synthetic oligonucleotides of *E. coli* LT, ST, SLTI and SLTII [29], kindly provided by Dr. Ø. Olsvik, CDC, Atlanta, GA, were labelled with digoxenin (Boehringer-Mannheim, Mannheim, Germany) according to the manufacturer's instructions, and the oligonucleotide probe for the *inv* gene as well as EAF, *eae*A, EAgg, and SS27 [1,6,7,12,30] with ^{32}P using a random primed labelling kit (Boehringer-Mannheim). Hybridisations were performed at high stringency conditions.

2.3. Adhesion to Caco-2 cells

Adhesion to human colonic carcinoma (Caco-2) cells was performed according to the method of Cravioto et al. [31]. Briefly, the cells were cultured in RPMI 1640 with 5 mM glutamine, 10% foetal calf serum and gentamicin (100 μg/ml) on circular glass coverslips at 37°C in a 5% CO_2 atmosphere to reach next to confluent monolayers. The bacterial strains were grown overnight at 37°C in Luria-Bertani (LB) broth. From this culture 10^8 bacterial cells/ml were incubated for 5 min in 1 ml 0.07 M sodium phosphate buffered saline, pH 7.2 (PBS) with 0.5% D-mannose, and then incubated with Caco-2 cells for 1 h at 37°C in 5% CO_2. The cells were washed extensively in PBS to remove non-adherent bacteria, fixed in 70% ethanol for 15–20 min, and stained with 20% Giemsa for 15 min. The pattern of adherence was studied on at least 500 cells per slide, and defined as localized (LA), aggregative (AA), diffuse (DA), or no adhesion (NA) as described previously [32].

2.4. Invasion assay

The standard invasion assay was performed following the modification of Small et al. [14]. Briefly,

CFA-agar grown bacteria were transferred to LB broth and cultured for 2 h on a rotatory shaker at 37°C. After thorough washing in PBS, the bacterial cells were resuspended in fresh RPMI without antibiotics. The cell density was adjusted to 10^8 bacteria/ml. Caco-2 and HeLa cells were grown to next to confluent monolayers at concentrations of 3×10^6 in each well in 24-well tissue culture plates. The cells were washed three times in PBS and covered with 0.2 ml fresh prewarmed RPMI 1640.

Log phase bacterial cells were added in a 10 μl aliquot to give a density ratio of 100 bacteria/tissue culture cell. The monolayers were centrifuged at $800 \times g$ for 10 min. After 2 h incubation in 5% CO_2 the monolayers were washed three times in PBS and incubated with fresh RPMI 1640 containing gentamicin (100 μg/ml) for 1 h. The monolayers were then washed six times with PBS after which the cells were lysed with Triton X-100 (1%, 20 min). After adding 0.8 ml LB to each well, serial dilutions on MacConkey agar were done and incubated overnight at 37°C. The number of invaded bacteria was calculated and expressed as percentage of inoculated bacteria. Results are expressed as means of five experiments ± S.E.

To study the influence of growth conditions of bacteria thirteen strains were grown in LB aerobically at 37°C and 20°C, and anaerobically at 37°C, in LB broth supplemented with 1% glucose, and in minimal medium (Volcani's medium) [33]. To study the influence of iron, fourteen strains were grown in Volcani's medium with Desferal (100 mg/ml).

The influence of varying the bacteria/cell ratio was studied by inoculating bacterial cells of six *E. coli* strains at concentrations corresponding to 10:1, 100:1 and 1000:1 bacteria to tissue culture cells.

To establish the time of incubation necessary for subsequent invasion, bacteria were inoculated on Caco-2 cells for 5 min, 15 min, 30 min, 1 h, 1.5 h and 2 h after which the cells were washed and the standard invasion assay was proceeded.

The requirement of calcium ions for tissue cell invasion was analyzed by adding 1% $CaCl_2$ to the RPMI medium, and in a parallel experiment 1% EDTA. The influence of type I fimbriae on invasiveness was studied in RPMI supplemented with 1% D-mannose. Inhibitory effect of other sugars was studied in RPMI with 1% D-lactose, D- and L-fucose, and D-galactose.

2.5. Statistical analyses

Analyses between groups was determined by the two-tailed paired Student's *t*-test.

2.6. Chemicals

Gentamicin, penicillin, and EDTA were purchased from Sigma Chemical Co., St. Louis, MO. Agar bases were from Lab M, Salford, UK. RPMI

Table 2
Invasion [a] of Caco-2 and HeLa cells by *Escherichia coli* strains cultured under different growth conditions

Strain	Caco-2 cells 37°C	HeLa cells 37°C	Caco-2 cells 20°C	Caco-2 cells anaerobiosis
2057-61	2.30 ± 0.02	0.43 ± 0.0	1.22 ± 0.0 ***	2.73 ± 0.41
E2348	6.48 ± 1.90	4.92 ± 1.81	1.14 ± 0.21 *	6.03 ± 1.82
CVD206	2.11 ± 1.03	1.22 ± 0.63 ***	0.62 ± 0.21 *	2.04 ± 0.31
JPN15	0.18 ± 0.02	1.23 ± 0.58	0.49 ± 0.41	2.01 ± 0.69 *
CVD432	8.06 ± 1.98	3.96 ± 0.29 *	6.49 ± 1.02	7.61 ± 1.69
E31	5.82 ± 0.10	6.19 ± 0.91	0.62 ± 0.04 ***	2.81 ± 0.87 **
D6211	6.59 ± 1.84	6.11 ± 1.98	3.28 ± 0.39	4.28 ± 0.21
D6152	7.33 ± 0.69	4.11 ± 0.58 **	0.59 ± 0.11 ***	0.89 ± 0.51
S.t.m. [b]	3.42 ± 0.68	2.49 ± 0.30	3.41 ± 0.11	6.49 ± 0.48 ***
YpIII [b]	6.47 ± 0.59	2.39 ± 0.88	6.49 ± 0.61	8.32 ± 1.41

[a] Invasion expressed as percentage intracellular cells compared to initially incubated cells ± S.E. Statistical significance has been calculated in relation to invasion of Caco-2 cells by bacterial strains grown at 37°C in aerobic atmosphere. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.
[b] S.t.m. = *Salmonella typhimurium*, YpIII = *Yersinia pseudotuberculosis*.

1640 from IBN Biomed Inc., Costa Mesa, CA, and foetal calf serum from Flow Labs, Irvine, UK. D-Mannose, D- and L-fucose, D-glucose, D-lactose and D-galactose were from Merck AG, Darmstadt Germany. All buffers and salts were of analytical grade.

3. Results

3.1. Adhesion

Expression of ability to adhere to the Caco-2 cells appeared to be a prerequisite for invasion (Table 1). The strains which expressed localized or enteroaggregative pattern of adhesion invariably hybridized with the EAF and EAgg probes respectively. The strains expressing a diffuse type of adhesion hybridized to *eae*A or did not hybridize with any of the probes employed. *E. coli* HB101 did not adhere to Caco-2 cells.

3.2. Influence of growth conditions on invasion

Anaerobic growth enhanced invasivity for *S. typhimurium* whereas invasion of *E. coli* strains with different adhesins (EAF, EAgg) was generally not affected with the exception of JPN15 (Table 2). *E. coli* cells grown at 37°C generally invaded more efficiently than bacteria grown at 20°C (Table 2). Invasion by *S. typhimurium* and *Y. pseudotuberculosis* was similar at 37° and 20°C. Growth in LB broth with 1% glucose decreased invasion of Caco-2 cells by 7 *E. coli* strains studied and by *Y. pseudotuberculosis* III but enhanced that of *E. coli* 2057-61 (EAF, *eae*A) (Fig. 1). Growth in minimal medium decreased invasiveness of strain E31 (EAgg), E2348, D6152 and E224 but enhanced invasive ability of CVD206, JPN15 and 2057-61 (Figs. 1 and 2). *E. coli* HB101 did not invade Caco-2 cells or HeLa cells.

For several *E. coli* strains, invasion of the intesti-

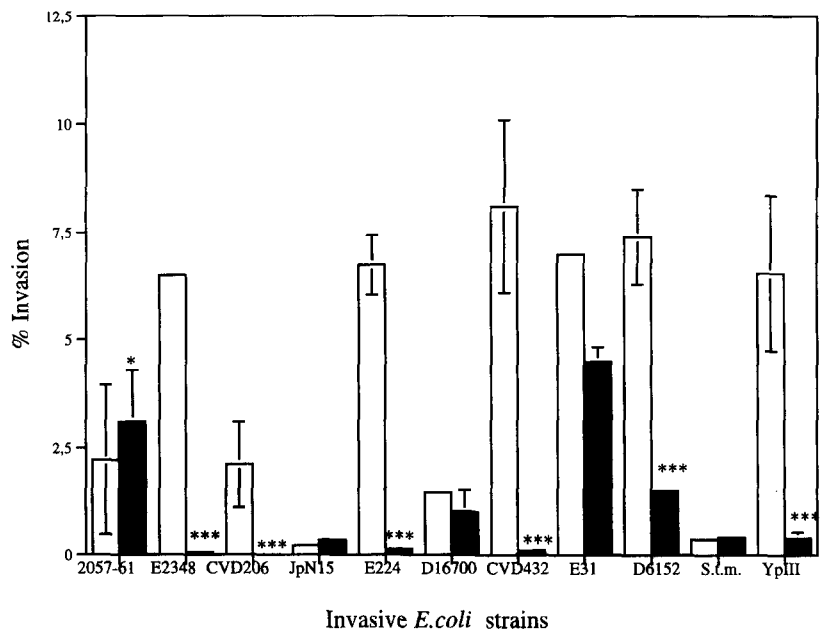

Fig. 1. Influence of glucose in the culture medium on invasion of Caco-2 cells by *E. coli* strains with different virulence characteristics (Table 1). Strains were grown in LB broth (□) or LB broth with 1% glucose (■). S.t.m. = *S. typhimurium*, YpIII = *Y. pseudotuberculosis*. The number of surviving bacteria was calculated and expressed as percentage of inoculated bacteria [14]. Each bar represents the mean of five experiments ±S.E. * $P < 0.05$, ** $P < 0.01$, *** $P <= 0.001$, as compared to strains grown in LB broth without glucose, Student's *t*-test.

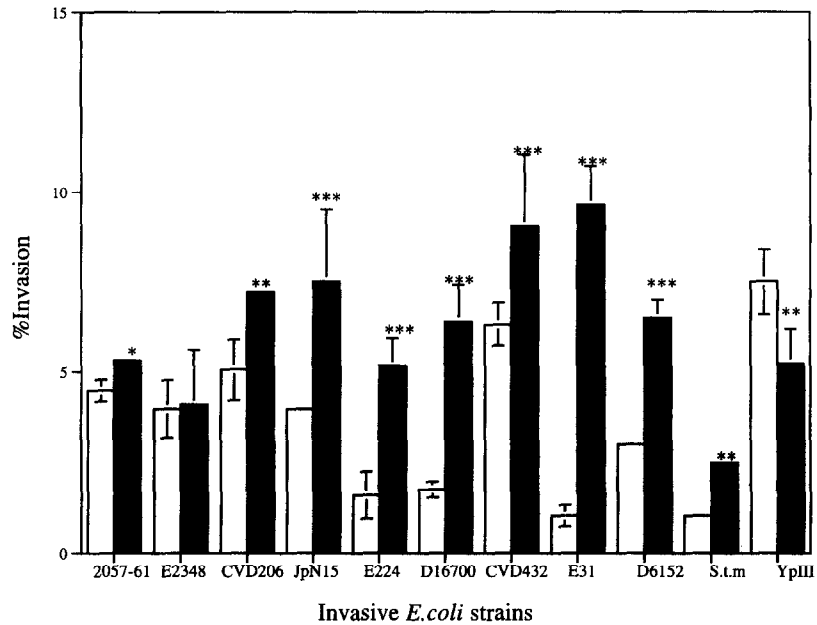

Fig. 2. Influence of iron in the culture medium on invasion of Caco-2 cells by *E. coli* strains with different virulence characteristics (Table 1). The strains were cultured in minimal medium (□) and minimal medium with Desferal (100 mg/ml, ■). S.t.m. = *S. typhimurium*, YpIII = *Y. pseudotuberculosis*. Each bar represents the mean of five experiments ±S.E. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to strains grown in minimal broth without Desferal, Student's *t*-test.

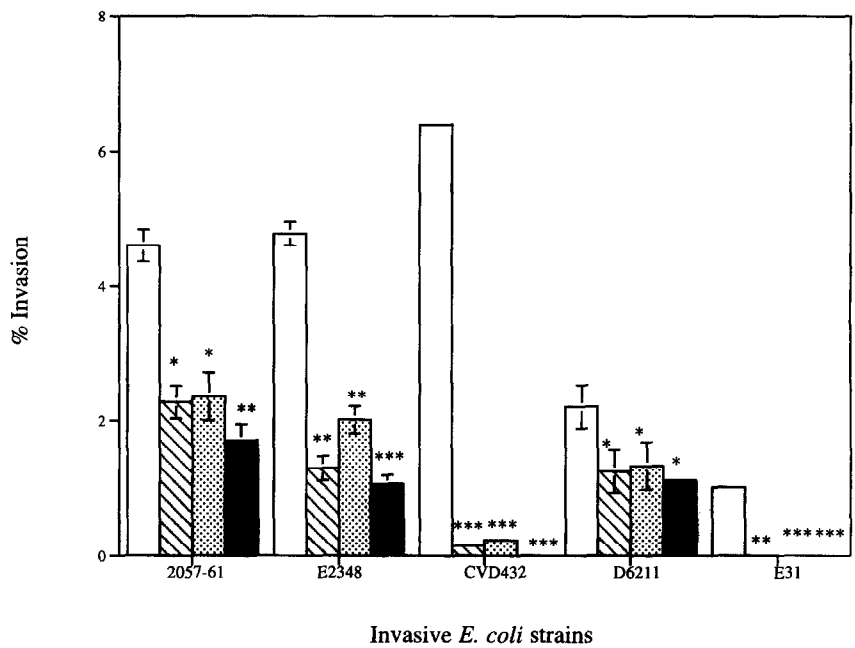

Fig. 3. Effect of calcium and mannose on invasion of Caco-2 cells by *E. coli* strains grown in minimal medium. The RPMI 1640 tissue culture medium was supplemented with 1% $CaCl_2$ (striped bars), 1% EDTA (dotted bars), 1% D-mannose (solid bars), or no supplementation (white bars). Each bar represents the mean of five experiments ±S.E. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to invasion in unsupplemented RPMI 1640, Student's *t*-test.

nal cells (Caco-2) was more efficient than of HeLa cells which are of non-intestinal origin (Table 2). A ratio of 100 bacteria per tissue culture cells was optimal. The time of incubation (5 min to 2 h) influenced subsequent invasion of the cells. Invasion increased during incubation up to 1.5 h, and did not occur when cells were incubated less than 15 min (data not shown). In the standard assay, cells were incubated with bacteria for 2 h.

3.3. Invasion in relation to adhesion

E. coli strains E2348 and 2057-61 carrying EAF as well as the *eae*A gene invaded at higher numbers than the strains carrying the *eae*A but not EAF, although strain 2057-61 invaded poorly (Tables 1 and 2). Strain CVD 206 which adheres in a localized way but has a deletion in *eae*A invaded to a significantly lower extent than its parent strain (Table 1). Two strains hybridizing with the *eae*A gene probe only, E224 and D16700, showed diffuse adhesion to Caco-2 cells but invaded the cells to the same extent as strain E2348. The enteroaggregative *E. coli* strains CVD 432 and E31, and *Ent. cloacae* E510 hybridizing with the probe for EAgg and adhering in an autoaggregating way invaded both HeLa and Caco-2 cells efficiently (Table 2). Strains D6211 (EAgg, SS27), D6152, D15288 (SS27) and D14258 adhered in an autoaggregative or diffuse way to the Caco-2 cells and invaded both Caco-2 and HeLa cells to a similar extent as *E. coli* 2348 (Tables 1 and 2).

3.4. Influence of iron on invasion

In 8/9 *E. coli* strains carrying different virulence factors and with *S. typhimurium*, significantly higher numbers of bacteria invaded after growth in iron-depleted medium (Fig. 2). For strain 2057-61 the significance was $P < 0.05$, and only strain E2348 was not significantly affected.

Mannose inhibited invasion of strains carrying EAFand Eagg (Fig. 3). No effect was found with the other sugars tested, fucose, galactose and lactose.

In the presence of calcium ions invasion was also decreased, but since the effect was not reverted in parallel experiments with 1% EDTA as a chelating agent our data do not support a specific effect for calcium ions (Fig. 3).

4. Discussion and conclusion

While the invasive process of intestinal cells has been well elucidated for *Shigella*, *Salmonella* and *Yersinia* [1–4], and to some extent for *C. jejuni* [5] we know very little about how other putatively invasive bacterial species such as *E. coli* carrying different types of intestinal virulence factors invade intestinal cells.

The different methods of cell invasivity have several draw-backs. First, the tissue specificity of small and/or large intestinal cells may not be expressed even on cells of intestinal origin. Technical procedures like centrifugation of the bacteria on the tissue culture cells may influence the results [34,35]. In this study, the cells were moderately distorted after centrifugation.

The growth conditions of the bacterial strains may be important with some bacterial species. Growth during anaerobic conditions was e.g. shown to promote invasion by *Salmonella* strains [18]. In the present study, we could confirm this with the *S. typhimurium* isolate but anaerobic conditions did not significantly enhance invasion by the different *E. coli* strains used (Table 2). Growth under iron restriction is well known to affect the synthesis of outer membrane proteins [4,14,36,37]. In this study invasion of Caco-2 cells was enhanced in 8/9 *E. coli* and one *S. typhimurium* strain cultured without available iron (Fig. 2). Whether this is correlated to synthesis of different cell envelope proteins when strains are grown under iron-restricted conditions is presently under investigation. Furthermore, strictly human pathogens like *Shigella* require prior growth at 37°C whereas food-borne pathogens like *Yersinia* and *Salmonella* express invasive properties also after growth at room temperature. In the present study all strains were grown at 37°C which may be suboptimal for expression of virulence genes of *Yersinia* sp. but may for example enhance fimbrial expression of *E. coli* [38,39]. The decreased invasion of strains after growth in the presence of 1% glucose is interesting since expression of CFA/II earlier was shown to be subject to catabolite repression, and hence not expressed during growth in glucose-rich medium [40]. The decreased invasion in our study (Fig. 1) could similarly be due to the inhibition of another surface structure required for the invasive process.

Although strains 2057-61 and E2348 invaded to a higher extent than their mutants which do not carry EAF our data do not support that EAF is involved in the invasive process since the derivative strain CVD206 which carries EAF but has a deletion in *eae*A invaded to a much lower extent than the parent strain (E2348, Table 1), and furthermore *E. coli* 16700 and E224 hybridizing only with *eae*A invaded to the same extent as the EAF-positive strains (Table 1). This supports earlier data that the *eae*A gene mediates intimate attachment to epithelial cells and forms part of the invasive process [25], possibly by interference with mammalian cell (integrin) receptors [42]. Jerse et al. showed that *eae*A-mutants were not taken up by nonphagocytic cells [12]. Our data also support that adhesion to the tissue culture cells is a prerequisite for the invasive process [13]. *E. coli* strains, isolated from bovine septicaemia, which cause diarrhoea and penetrate the intestinal epithelium to the bloodstream commonly express the fimbrial adhesin CS31A. Using CS31A-deficient mutant strains it was shown that this adhesin was neither required nor sufficient to mediate invasion of tissue culture cells [41]. Our data that mannose inhibited invasion by *E. coli* strains could indicate that a mannose-sensitive adhesin like type I fimbriae, bundle-forming pilus or the reported fimbrial adhesin of EAggEC could be involved in the invasive process. Interestingly, *Yersinia* strains cured of the *inv* gene still express invasive ability mediated by the yadA product [16,43].

Certain serogroups of *E. coli* like O124 are correlated with invasiveness [44]. None of the strains in this study belonged to classical invasive serotypes (Table 1). Several of the invading *E. coli* strains were immotile, and hence, potentially, more *Shigella*-like. These strains may carry an additional invasive determinant, or alternatively are more easily adapted to, and survive better in the large intestinal environment, like *Shigella* strains. On the other hand, with *S. typhimurium* and *C. jejuni* strains motility was shown to enhance subsequent invasion of epithelial cells [5,45].

Most of the tested strains invaded Caco-2 cells to significantly higher extent than HeLa cells which may reflect the tissue specificity for intestinal cells (Table 2). None of the tested *E. coli* strains hybridized with the *inv* probe for EIEC, emphasizing the presence of other invasive determinants in different strains of enteric *E. coli,* and that tests for invasivity utilizing rabbit keratoconjunctivitis or hybridization with the *inv* probe are not sufficient.

The *Y. pseudotuberculosis* strain isolated from an Ethiopian child with persistent diarrhoea (E116), hybridized with the *eae*A probe. This confirms earlier reports on sequence homology between the invasive determinant of *Y. pseudotuberculosis* and *E. coli eae*A. The *eae*A gene or *eae*-like genes have been detected in strains of other species, like *Citrobacter freundii* and *Hafnia alvei* [46,47]. It is worth noting that one strain of *Ent. cloacae* hybridized with the probe for EAgg, and expressed invasive ability. Plasmids carrying *E. coli* enterotoxins were earlier shown to spread among other members of Enterobacteriaceae but were found to be less stable in non-*E. coli* strains than in *E. coli* strains. Spread of EAgg to non-*E. coli* has not been described earlier, and we are currently studying the stability of expression of EAgg in this strain. However, spread of virulence determinants among strains of different Enterobacterial species within the intestine may be an overlooked property.

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References

[1] Sansonetti, P.J. (1991) Genetic and molecular basis of epithelial cell invasion by *Shigella* species. Rev. Infect. Dis.13, S285–S292.

[2] Mounier, J., Vasselon, T., Hellio, R., Lesourd, M. and Sansonetti, P.J. (1992) *Shigella flexneri* enters human colonic Caco-2 epithelial cells through the basolateral pole. Infect. Immun. 60, 237–248.

[3] Finlay, B.B. and Falkow, S. (1990) *Salmonella* interactions with polarized human intestinal Caco-2 epithelial cells. J. Infect. Dis. 162, 1096–1106.
[4] Grützkau, A., Hanski, C., Hahn, H. and Riecken, E.O. (1990) Involvement of M cells in the bacterial invasion of Peyer's patches: a common mechanism shared by *Yersinia enterocolitica* and other enterinvasive bacteria. Gut 31, 1011–1015.
[5] Russell, R.G. and Blake Jr., D.C. (1994) Cell association and invasion of Caco–2 cells by *Campylobacter jejuni*. Infect. Immun. 62, 3773–3779.
[6] Nataro, J.P., Baldini, M.M., Kaper, J.B., Black, R.E., Bravo, N. and Levine, M.M. (1985) Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. J. Infect. Dis. 152, 560–565.
[7] Baudry, B., Savarino, S.J., Vial, P., Kaper, J.B. and Levine, M.M. (1990) A sensitive and specific DNA probe to identify enteroaggregative *Escherichia coli*, a recently discovered diarrheal pathogen. J. Infect. Dis. 161, 1249–1251.
[8] Finlay, B.B., Rosenshine, I., Donnenberg, M.S. and Kaper, J.B. (1992) Cytoskeletal composition of attaching and effacing lesions associated with enteropathogenic *Escherichia coli* adherence to HeLa cells. Infect. Immun. 60, 2541–2543.
[9] Nataro, J.P., Deng, Y., Maneval, D.R., German, A.L., Martin, W.C. and Levine, M.M. (1992) Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect. Immun. 60, 2297–2304.
[10] Girón, J.A., Ho, A.S.Y. and Schoolnik, G.K. (1991) An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science 254, 710–713.
[11] Girón, J.A., Donnenberg, M.S., Martin, W.C., Jarvis, K.G. and Kaper, J.B. (1993) Distribution of the bundle-forming pilus structural gene (*bfp*A) among enteropathogenic *Escherichia coli*. J. Infect. Dis. 168, 1037–1041.
[12] Jerse, E., Yu, J., Tall, B.D. and Kaper, J.B. (1990) A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci USA 87, 7839–7843.
[13] Donnenberg, M.S., Yu, J. and Kaper, J.B. (1993) A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. J. Bact. 175, 4670–4680.
[14] Small, P.L.C., Isberg, R.R. and Falkow, S. (1987) Comparison of the ability of enteroinvasive *Escherichia coli*, *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* to enter and replicate within HEp-2 cells. Infect. Immun. 55, 1674–1679.
[15] Simonet, M. and Falkow, S. (1992) Invasin expression in *Yersinia pseudotuberculosis*. Infect. Immun. 60, 4414–4417.
[16] Yang, Y. and Isberg, R.R. (1993) Cellular internalization in the absence of invasin expression is promoted by the *Yersinia enterocolitica yad*A product. Infect. Immun. 61, 3907–3913.
[17] Yu, J. and Kaper, J.B. (1992) Cloning and characterization of the *eae* gene of enterohaemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol. 6, 411–417.
[18] Ernst, R.K., Dombroski, D.M. and Merrick, J.M. (1990) Anaerobiosis, Type 1 fimbriae and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. Infect. Immun. 58, 2014–2016.
[19] Kusters, J.G., Mulders-Kremers, G.A.W.M., van Doornik, C.E.M. and van der Zeist, B.A.M. (1993) Effects of multiplicity on infection, bacterial protein synthesis, and growth phase on adhesion to and invasion of human cell lines by *Salmonella typhimurium*. Infect. Immun. 61, 5013–5020.
[20] Miller, V.L., Bliska, J.B. and Falkow, S. (1990) Nucleotide sequences of the *Yersinia enterocolitica ail* gene and characterization of the Ail protein product. Infect. Immun. 171, 1062–1069.
[21] Donnenberg, M.S., Donohue-Rolfe, A. and Keusch, G.T. (1989) Epithelial cell invasion: an overlooked property of enteropathogenic *Escherichia coli* associated with the EPEC adherence factor. J. Infect. Dis. 160, 452–459.
[22] Fletcher, J.N., Saunders, J.R., Batt, R.M., Embaye, H., Getty, B. and Hart, C.A. (1990) Attaching effacement of the rabbit enterocyte brush border is encoded on a single 96.5-kilobase-pair plasmid in an enteropathogenic *Escherichia coli* O111 strain. Infect. Immun. 58, 1316–1322.
[23] Fletcher, J.N., Embaye, H.E., Getty, B., Batt, R.M., Hart, C.A. and Saunders, J.R. (1992) Novel invasion determinant of enteropathogenic *Escherichia coli* plasmid pLV501 encodes the ability to invade intestinal epithelial cells and HEp-2 cells. Infect. Immun. 60, 2229–2236.
[24] Elsinghorst, E.A. and Kopecko, D.J. (1992) Molecular cloning of epithelial cell invasion determinants from enterotoxigenic *Escherichia coli*. Infect. Immun. 60, 2409–2417.
[25] Donnenberg, M.S. and Kaper, J.B. (1991) Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. 59, 4310–4317.
[26] Schumacher, G., Kollberg, B., Sandstedt, B., Jorup, C., Grillner, L., Ljungh, Å. and Möllby, R. (1993) A prospective study of non-relapsing colitis and first attacks of inflammatory bowel disease. Scand. J. Gastroenterol. 28, 1077–1085.
[27] Kelly, M., Brenner, D.J. and Farmer III, J.J. (1985) Enterobacteriaceae. In: Manual of Clinical Microbiology (Lennette, E.H., Balows, A., Hausler, W.J.J. and Shadomy, H.J., Eds.), pp. 263–277. American Society for Microbiology, Washington DC.
[28] Sullivan, P.B., Coles, M.A.T., Aberra, G. and Ljungh, Å. (1994) Enteropathogenic and enteroadherent-aggregative *Escherichia coli* in children with persistent diarrhoea and malnutrition. Ann. Trop. Paed. 14, 105–110.
[29] Olsvik, Ø., Hornes, E., Wasteson, Y. and Lund, A. (1991) Detection of virulence determinants in enteric *Escherichia coli* using nucleic acid probes and polymerase chain reaction. In: Molecular Pathogenesis of Gastrointestinal Infections (Wadström, T., Svennerholm, A.M., Wolf-Watz, H., Mäkelä, H., Eds.), pp. 267–273. Plenum Press, New York.
[30] Fletcher, J.N., Batt, R.M., Ljungh, Å., Schumacher, G., Embaye, H.E., Odedra, R., Saunders, J.R. and Hart, C.A. (1994) Conservation of a plasmid-born invasive determinant in three distinct strains of *Escherichia coli* successively isolated from a patient with ulcerative colitis. Gastroenterol. 106, A681.

[31] Cravioto, A., Reyes, R.E., Ortega, R., Fernandez, G., Hernandez, R. and Lopez, G. (1988) Prospective study of diarrhoeal disease in a cohort of rural Mexican children: incidence and isolated pathogens during the first two years of life. Epidemiol. Infect. 101, 123–134.

[32] Nataro, J.P., Kaper, J.B., Robins-Browne, R., Prado, V., Vial, P. and Levine, M.M. (1987) Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. Ped. Infect. Dis. 6, 829–831.

[33] Mevarech, M. and Werczberg, R. (1985) Genetic transfer in *Halobacterium volcani*. J. Bact. 162, 461–462.

[34] Douce, G.R., Amin, I.I. and Stephen, J. (1991) Invasion of HEp-2 cells by strains of *Salmonella typhimurium* of different virulence in relation to gastroenteritis. J. Med. Microbiol. 35, 349–357.

[35] Vesikari, T., Bromirska, J. and Mäki, M. (1982) Enhancement of invasiveness by *Yersinia enterocolitica* and *Escherichia coli* in HEp-2 cells by centrifugation. Infect. Immun. 36, 834–836.

[36] Isberg, R.R., Voorhis, D.L. and Falkow, S. (1987) Identification of Invasin: a protein that allows enteric bacteria to penerate cultured mammalian cells. Cell 50, 769–778.

[37] Wilcox, M.H., Williams, P., Smith, D.G., Modun, B., Finch, R.G. and Denyer, S.P. (1991) Variation in the expression of cell envelope proteins of coagulase-negative staphylococci cultured under iron-restricted conditions in human peritoneal dialysate. J. Gen. Microbiol. 137, 2561–2570.

[38] Williams, P.H. and Hinson, G. (1987) Temperature-dependent transcriptional regulation of expression of fimbriae in an *Escherichia coli* strain isolated from a child with severe enteritis. Infect. Immun. 55, 1734–1736.

[39] Mekalanos, J.J. (1992) Environmental signals controlling expression of virulence determinants in bacteria. J. Bact. 174, 1–7.

[40] Evans, D.G., Evans Jr., D.J., Karjalainen, T.K. and Lee, C.-H. (1991) Production of colonization factor antigen II of enterotoxigenic *Escherichia coli* is subject to catabolite repression. Curr. Microbiol. 23, 71–74.

[41] Korth, M.J., Lara, J.C. and Moseley, S.L. (1994) Epithelial cell invasion by bovine septicemic *Escherichia coli*. Infect. Immun. 62, 41–47.

[42] Isberg, R.R. and Leong, J.M. (1990) Multiple b_1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell 60, 861–871.

[43] Bliska, J.B., Copass., M.C. and Falkow, S. (1993) The *Yersinia pseudotuberculosis* adhesin YadA mediates intimate bacterial attachment to and entry into Hep-2 cells. Infect. Immun. 61, 3914–3921.

[44] Ørskov, F. and Ørskov, I. (1978) Serotyping of *Escherichia coli*. In: Methods of Microbiology (Bergan, T. and Norris, J.R., Eds.), pp. 1–77. Academic Press, London.

[45] Jones, G.W., Richardson, L.A. and Uhlman, D. (1981) The invasion of HeLa cells by *Salmonella typhimurium*: Reversible and irreversible bacterial attachment and the role of bacterial motility. J. Gen. Microbiol. 127, 351–360.

[46] Schauer, D.B. and Falkow, S. (1993) The *eae* gene of *Citrobacter freundii* biotype 4280 is necessary for colonization in transmissible murine colonic hyperplasia. Infect. Immun. 61, 4654–4661.

[47] Ridell, J., Siitonen, A., Paulin, L., Mattila, L., Korkeala, H. and Albert, M.J. (1994) *Hafnia alvei* in stool specimens from patients with diarrhea and healthy controls. J. Clin. Microbiol. 32,2335–2337.