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Human intestinal tissue tropism of intimin epsilon O103 *Escherichia coli*

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Abstract

Human intestinal in vitro organ culture was used to assess the tissue tropism of human isolates of *Escherichia coli* O103:H2 and O103:H- that express intimin ϵ . Both strains showed tropism for follicle associated epithelium and limited adhesion to other regions of the small and large intestine. This is similar to the tissue tropism shown by intimin γ enterohaemorrhagic (EHEC) O157:H7, but distinct from that of intimin α enteropathogenic (EPEC) O127:H6.

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1. Introduction

The characteristic intestinal epithelial cell lesion produced by enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli* is termed the ‘attaching/effacing’ (A/E) lesion (reviewed in Frankel et al. [1]). The first gene to be associated with A/E activity, the *eae* gene, encodes the intimate bacterial adhesin intimin [2]. This gene is part of the locus of enterocyte effacement (LEE) pathogenicity island, which also encodes a type III secretion system, an intimin receptor (Tir) that is translocated into host cells, and other EPEC secreted proteins (EspA, EspB, and EspD, EspF, Map) [3]. Intimin can also bind to β -1 integrins [4], and to cell surface-located nucleolin [5], although the in vivo consequences of this are unknown. The Tir receptor binding activity of intimin is localised at the carboxy-terminal 280 amino acids of the polypeptide, and at least five different intimin types, designated α , β , γ ,

δ , and ϵ have been described [6,7], although other, as yet unpublished, intimin types have been identified and their sequences deposited in GenBank (ζ , accession number: AJ298279; η , accession number: AJ271407; ι (*sic*), accession number: AJ308551; and κ , accession number: AJ308552).

Intimin α is specifically expressed by human EPEC strains belonging to one evolutionary branch known as EPEC clone 1, while intimin β is mainly associated with human and animal EPEC and EHEC strains belonging to their respective clone 2. Intimin γ is associated with EHEC O157:H7, EPEC O55:H7 and O55:H-, while intimin δ is expressed by EPEC O86:H34 [6]. Intimin ϵ is expressed by human and bovine EHEC strains of serogroups O8, O11, O45, O103, O121 and O165 [7]. Intimin types ζ and η have been identified in O84:NM and O84:H2 respectively, although their sequences appear identical, intimin ι in O145:H4, and intimin κ in O118:H5.

Intimin is required for colonisation and pathogenesis following experimental infection of humans by EPEC [8], and by EHEC O157:H7 in calves [9] and pigs [10]. Recent results show that different intimin types play a role in determining the pattern of colonisation and tissue tropism

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in the host. Among these are the observations of intimin γ O157:H7 adhesion being restricted to follicle associated epithelium (FAE) [11], whereas intimin α EPEC colonise both small intestine and FAE [11]. Furthermore intimin exchange studies using human intestinal explants show that EPEC O127:H6 engineered to express intimin γ instead of intimin α , change tropism to a FAE restricted phenotype [12] and that intimin γ EHEC O157:H7 engineered to express intimin α changes to an EPEC-like tropism phenotype [13]. Intimin ϵ shows overall sequence similarity to intimin β , but the last 280 C-terminal amino acids which contain the Tir and cell binding region show a greater similarity with intimins α and γ [7].

In this paper we have investigated the human intestinal tissue tropism shown by two O103 intimin ϵ strains.

2. Methods

2.1. Bacterial strains

Intimin ϵ positive O103:H2 strain PMK5, was isolated from a patient with haemolytic uremic syndrome (HUS) in France [14]. In order to perform IVOC in our category two laboratory a derivative of PMK5 that does not express Shiga toxin 1 was generated from the parent wild-type strain in category three facilities at the Institute for Animal Health (see below). The other intimin ϵ positive strain E77804 (O103:H-) was isolated from an adult male with diarrhoea in the UK. This strain is *stx* negative and EPEC adherence factor probe (EAF) negative.

2.2. Bionumeric analysis

Amino acid sequences of the different intimin types were retrieved from GenBank. These included human EPEC O127:H6 strain E2348/69 (M58154), calf EHEC O26:H-strain 413/89 (AJ223063), human EHEC O157:H7 strain EDL933 (Z11541), dog EPEC strain 4221 (U66102), human EHEC O103:H2 strain PMK5 (AF116899) and bovine EHEC O84:H4 strain 4795/97 (AJ308551). Multiple alignment and cluster analysis were carried out on these sequences using Bionumerics software (Applied Maths, Belgium). A dendrogram was constructed by the unweighted pair group method using arithmetic averages (UPGMA). Bootstrap analysis was performed with 1000 resampled data sets.

2.3. Construction of an EHEC O103:H2 *stx1* mutant [15]

Sequences flanking the *stx1A* gene were amplified and the products were gel-purified and combined in an overlapping polymerase chain reaction (PCR) [16]. The secondary PCR product was cloned into the suicide vector pCVD442 [17] and the resulting plasmid was introduced into a nalidixic acid resistant derivative of PMK5 by con-

jugation from *E. coli* S17-1 λ pir [18] and a merodiploid isolated on LB agar containing ampicillin and nalidixic acid. Double recombinants were selected on medium containing 5% sucrose at 30°C, screened for the deletion by colony PCR and verified by Southern hybridisation. The resulting PMK5 Δ *stx1* mutant contained a large internal deletion of the *stx1A* gene that also removed the start codon for the B subunit. Culture supernatants of the PMK5 Δ *stx1* mutant showed no cytotoxicity for VERO cells but PMK5 Δ *stx1* still demonstrated attaching and effacing activity on cultured epithelial cells (data not shown).

2.4. Tissue samples

Samples were obtained, with fully informed parental consent and ethical approval, during routine investigation of patients for intestinal disorders. Mucosal biopsies of small intestine, ileal Peyer's patches and colon were taken during endoscopy (Fujinon EG/EC-41 paediatric endoscope). Only macroscopically normal appearing areas were sampled, and results were only used when subsequent intestinal histology was reported to be normal. Patients' ages are listed in Table 1.

2.5. Organ culture adhesion assay

8 h IVOC was performed [19]. Each bacterial strain was examined on at least three occasions using tissue from different children. An uninoculated specimen was included each time to exclude in vivo bacterial colonisation. After incubation, specimens were washed thoroughly to remove non-adherent bacteria and prepared for scanning electron microscopy (SEM). Samples were fixed with 2.5% glutaraldehyde, post-fixed in osmium tetroxide, critically point dried using liquid carbon dioxide, and viewed in a JEOL 5300 scanning EM. Some SEM samples of proximal intestinal incubations were reprocessed for light microscopy to visualise internal detail. This was to resolve the question whether bacterial adhesion was to a follicular area or to a mounded villus. Samples were placed in 2,2-dimethoxypropane, infiltrated with resin, polymerised and then ultramicrotome sectioned. 0.5 μ m sections were stained with 1% aqueous toluidine blue in 1% sodium tetraborate.

3. Results

Fig. 1 shows the similarity matrix and dendrogram constructed from the multiple alignment and cluster analysis using the UPGMA method. Analysis of the similarity matrix values of the complete amino acid sequence of the various intimins showed that intimins ϵ and γ were more similar to each other (86%) than to the other intimins (81.1–84.8%). In addition, the similarity between intimins α and η is higher (92.9%) than with the other intimins

Table 1
Attaching/effacing lesion formation in intestinal regions expressed as a proportion of biopsies inoculated

Strain	Intestinal region				
	Small intestine		Large intestine	FAE	
	D4	Ileum		D4 Isolated	Ileal PP
O103:H- (E77804)	3/9	0/8	0/17	2/2	2/3
Age (months)	140	151	142	103, 111	34, 110, 181
Median (range)	(19–185)	(83–181)	(46–191)		
O103:H2 (PMK5 $\Delta stx1$)	2/11	0/7	1/16	NA	5/6
Age (months)	122	166	141	–	141
Median (range)	(19–185)	(83–181)	(46–191)		(46–191)

Age is expressed individually, unless more than three samples were used when median and range are given.

Abbreviations: FAE, follicle associated epithelium; D4, fourth part of the duodenum; PP, Peyer's patch; NA, not available.

(81.1–87.9%). The dendrogram illustrates these relationships. The similarity values between the different intimin sequences are all generally high because the sequences are highly conserved in the N-terminal region.

The IVOC results are shown in Table 1. Both O103 strains showed some degree of attaching/effacing lesion formation on duodenal mucosa. However, in four of the five instances when this occurred, the bacteria adhered to mounded areas of the sample which may have represented follicular regions, rather than the villous surface, although this was not clear on SEM (Fig. 2a). Sections cut through the region of adhesion showed no evidence of follicular morphology (data not shown), so it was deduced that the regions were villous. No adhesion was evident on distal small intestine. By chance, two duodenal samples that were incubated with strain E77804 (O103:H-), were clearly observed to contain isolated lymphoid follicles (Fig. 2b). Both samples showed bacterial adhesion with attaching/effacing lesion formation on FAE (Fig. 2c), but not on neighbouring villous epithelium. Both intimin ϵ strains demonstrated A/E lesions on FAE of ileal Peyer's patches (Fig. 2d). Biopsy samples were taken from multiple levels of the colon (caecum, ascending, transverse, descending, and sigmoid) and only one sample (descending colon) incubated with PMK5 showed A/E lesion formation. Thus both strains showed a distinct tropism towards FAE overlying lymphoid follicles with limited adhesion to other intestinal regions.

4. Discussion

The application of IVOC to the study of EPEC and

EHEC human intestinal tract adhesion has demonstrated two distinct phenotypes of tissue tropism. Firstly, a more widespread pattern of adhesion to the small intestine and FAE of Peyer's patches, with limited adhesion to the large bowel epithelium; this is typically shown by intimin α expressing EPEC [11]. Secondly, a restricted range of adhesion to only FAE of Peyer's patches, typified by intimin γ positive O157:H7 [11]. This suggested that intimin type influenced tissue tropism. This had also been indicated by animal based data where the expression of intimin α instead of intimin γ by O157:H7 was associated with the spread of infection from the colonic region into the small intestine in neonatal piglets [20]. Similar experiments, performed using the human IVOC model, demonstrated that O157:H7 expressing intimin α colonised the small intestine as well as FAE of Peyer's patches [13], and that O127:H6 EPEC showed a FAE restricted phenotype when expressing intimin γ [12]. The observation of some O103 strain adhesion to duodenal mucosa, in addition to the more pronounced follicular adhesion, is similar to that shown by O127:H6 when mutated to express intimin γ [12]. The current study has shown that intimin ϵ strains O103:H- and O103:H2 have a similar human intestinal tropism to intimin γ expressing strains, and also show adherence to FAE of isolated lymphoid follicles of the duodenum. Lymphoid follicles are spread along the length of human intestine [21,22], indicating the potential colonisation of intimin ϵ O103 *E. coli* along the length of the gut, not just in the terminal ileal region.

It has not been established whether this tropism changes during the infection, as IVOC has not been extended beyond an 8 h period. There may be parallels with animal pathogens which show an initial restricted adhesion to

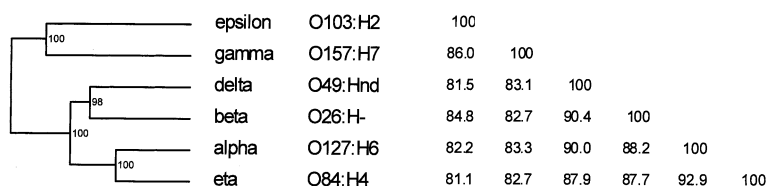


Fig. 1. A dendrogram and similarity matrix of the amino acid sequences of α , β , γ , δ , ϵ and η intimins, constructed using the UPMGA method. Numbers at branch points indicate bootstrap values (as a percentage) performed with 1000 resampled data sets.

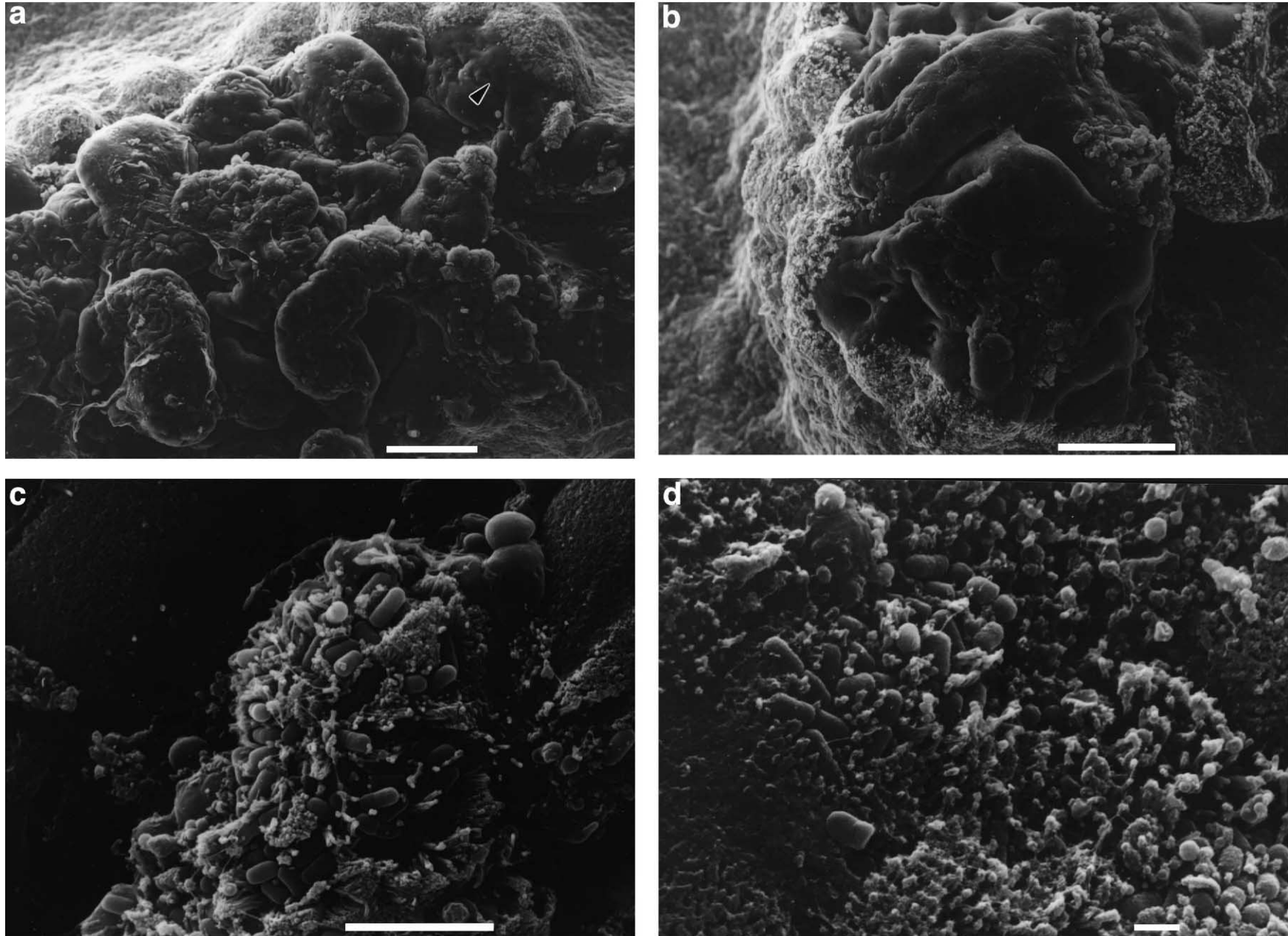


Fig. 2. SEM of IVOC. a: Follicular-like region (arrow) within duodenal sample. b: Isolated lymphoid follicle within duodenum. c: Strain E77804 showing A/E lesion on follicular-like epithelium in duodenum. d: Strain PMK5 showing A/E lesion on FAE of Peyer's patch. Bar = 100 μm (a,b), 5 μm (c), and 1 μm (d).

FAE overlying Peyer's patches, but which, with time, spread to other intestinal surfaces, e.g. the intimin β expressing rabbit strains RDEC-1 *E. coli* O15:H- [23] and *E. coli* O103:K:H2 [24]. RDEC-1 adheres to Peyer's patch lymphoid follicles within 24 h, but does not attach to ileal or colonic mucosa until 3 days after inoculation. The authors deduced that the intestinal mucosa was probably colonised by bacteria shed from the Peyer's patches. Similar observations in rabbit EPEC O103:H2 led the authors to suggest that this might be a phenomenon common to all EPEC infections [24]. The difference in tropism we have observed between O127:H6 and O157:H7 [11] indicates that some strains may be able to colonise the small intestinal mucosa directly without an intermediate stage of FAE colonisation. Although we have evidence that intimin type influences initial tissue tropism, the factors that are responsible for the change in tropism that occurs during an infection have not been determined. Furthermore, it is unclear how intimin influences tissue tropism and it will be of interest to study the distribution of host cell receptors for intimin in the intestine.

The similarity matrix values of the complete amino acid sequences of the various intimins showed that intimins ϵ and γ are more 'related' to each other than to the other intimins and we have found that strains expressing these intimins show a similar human intestinal tissue tropism. The reasons for this are unclear, however, it will be of interest to determine if the apparent parallel between relatedness and tissue tropism holds true for intimin α and η strains.

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