Analysis of type 1 fimbriae expression in verotoxigenic *Escherichia coli*: a comparison between serotypes O157 and O26

Andrew J. Roe, Carol Currie, David G. E. Smith and David L. Gally

Author for correspondence: David Gally. Tel: +44 131 651 1342. Fax: +44 131 650 6531.
e-mail: d.gally@ed.ac.uk

Zoonotic and Animal
Pathogens Research
Laboratory, Medical
Microbiology, Teviot Place,
University of Edinburgh,
Edinburgh EH8 9AG, UK

Previous research has shown that verotoxin-producing *Escherichia coli* (VTEC) O157 strains appear unable to express type 1 fimbriae although other serotypes such as O26 and O118 can. This study has investigated the molecular basis of this difference. The study confirmed the presence of a 16 bp deletion within the regulatory region of *fimA* (*fim* switch) in 63 VTEC O157 strains but not in other VTEC serotypes tested. The *fim* switch was shown to be detectable only in the phase off orientation in VTEC O157, but detection of the switch in the phase on orientation correlated with the degree of mannose-sensitive yeast agglutination in VTEC O26. Repair of the 16 bp deletion in the VTEC O157 *fim* switch region restored phase-variable expression of *fimA* in a permissive background. Non-O157 VTEC, especially O26 and O118, can be pathogenic in cattle; the role of type 1 fimbriae in this and colonization is discussed.

Keywords: *Escherichia coli* O157, VTEC, type 1 fimbriae, phase variation, adherence

INTRODUCTION

Ruminants, particularly cattle, act as the main reservoir for verotoxigenic *Escherichia coli* (VTEC) (Beutin, 1999; Borczyk *et al.*, 1987; Chart, 1998; Mead & Griffin, 1998; Ørskov *et al.*, 1987). *E. coli* O157 is the most common serotype associated with VTEC infections in the UK and the USA, other serotypes such as O26 and O118 being prevalent in other countries (Beutin, 1999; Mead & Griffin, 1998). Except in the case of neonatal cattle (DeanNystrom *et al.*, 1997), VTEC O157 does not cause disease in the bovine host, but diarrhoea and haemorrhagic colitis have been associated with other VTEC serotypes, especially O26 (Pearson *et al.*, 1999; Stordeur *et al.*, 2000). The very fact that VTEC O157 does not cause disease in ruminants may have helped its successful spread to 20–30% of herds in England and Scotland as well as 40–70% of herds in the USA in recent years (Elder *et al.*, 2000; Gansheroff & O'Brien, 2000; Synge, 1999, 2000).

In humans, the serious sequelae of haemorrhagic colitis and haemolytic uraemic syndrome stem from the

activity of the shiga-like toxins, and include gastrointestinal tract colonization and damage involving intimate attachment and attaching and effacing (A/E) lesion formation (Frankel *et al.*, 1998; Kaper, 1998). Intimin is the key candidate initiating bacterial binding (DeVinney *et al.*, 1999; Kenny, 1999) in the human host, possibly at the Peyer's patches of the small intestine (Phillips & Frankel, 2000).

There is a great deal of interest in understanding the successful dissemination of VTEC O157 among beef and dairy cattle and the reasons why it does not cause colitis and diarrhoea in this host. A logical starting point for such investigations is the carriage and expression of established bacterial adhesins, although to date there is no clear evidence that *E. coli* O157 colonizes the bovine gastrointestinal tract by binding to the epithelium. *E. coli* O157 does not carry genes for many of the established adhesins that could play a role in colonizing the bovine host, such as K99 and F41 (our unpublished data), but it does carry the genes for type 1 fimbriae. Type 1 fimbriae are the most common adhesin produced by *E. coli* and mediate adherence to mannose-containing glycoproteins found on the surfaces of many eukaryotic cells. Type 1 fimbriae have been shown to play an important role in the colonization of *E. coli* in the human urinary tract (Connell *et al.*, 1996; Donnenberg & Welch, 1996; Mulvey *et al.*, 1998) and, from their

Abbreviations: A/E, attaching and effacing; IHF, integration host factor; VTEC, verotoxigenic *E. coli*.

prevalence, it is assumed that they are important in the faecal–oral cycle, for binding within the gastrointestinal tract and/or for transfer between hosts (Orndorff & Bloch, 1990). For cattle, it has been established that type 1 fimbriae bind well to both immature and mature (keratinized) rumen epithelium *in vitro* (Galfi *et al.*, 1998).

While the majority of VTEC O157 strains do not appear to be able to express type 1 fimbriae, other VTEC serotypes, notably O26 and O118, can express this adhesin (Enami *et al.*, 1999). Initial adherence by factors such as type 1 fimbriae may make subsequent intimate adherence and A/E lesion formation more likely to occur (Mack *et al.*, 1999). Expression of type 1 fimbriae is phase variable, being dependent on an invertible DNA element (*fim* switch) that contains the promoter for *fimA*, the gene for the main fimbrial subunit (Abraham *et al.*, 1985; Klemm, 1984; Olsen & Klemm, 1994). In the off orientation, type 1 fimbriae cannot be produced. Inversion of the *fim* switch requires the activity of two recombinases, FimB and FimE, as well as numerous co-factors (Gally *et al.*, 1996; Klemm, 1986; McClain *et al.*, 1991).

The research presented follows on from the findings of Li *et al.* (1997), who identified a 16 bp deletion in *E. coli* O157 within the regulatory switch region that controls expression of type 1 fimbriae. They designed a primer that bridged this deletion as an effective probe for *E. coli* O157 strains. We have confirmed the presence of this deletion in 63 human and cattle *E. coli* O157 strains and have demonstrated that the phase switch controlling type 1 fimbrial expression is detectable only in the off orientation in VTEC O157. Repair of the 16 bp deletion restores phase-variable expression of *fimA*.

METHODS

Media and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The growth media used in this work were L-broth (per litre, 5 g NaCl, 5 g yeast extract and 10 g tryptone; Oxoid), L-agar (1.5% agar) and 6% sucrose agar (Blomfield *et al.*, 1991), which was used to select for recombinant bacteria. Sucrose agar consisted of L-agar with the addition of 6% sucrose in the place of NaCl. Indicator plates of minimal MOPS supplemented with X-Gal were used as described previously (Gally *et al.*, 1994).

Strains. VTEC strains were obtained from various sources. ZAP1 (O157:H7, VT1[−] VT2⁺) was provided by Dr Fiona Thompson-Carter of the National Reference Laboratories, Aberdeen, and was isolated from a patient infected during the Redhouse Dairy (Lothian, Scotland) outbreak of 1994. The strains used in the *fim*-region screen (Fig. 1) were also provided by Dr Fiona Thompson-Carter and represent a cross-section of human-disease serotypes (O26, O111, O86, O128 and O157) and bovine isolates of different phage types (2, 14, 21/28) and PFGE patterns. ZAP26 (strain 413/89-1, O26:H[−] VTEC) was a bovine isolate provided by Dr Trinad Chakraborty (Djafari *et al.*, 1997). MPRL4269 (ZAP77) and MPRL4270 (ZAP78) are both O26 human-disease-associated strains, shown to be VT1[−]/VT2[−] and VT1⁺/VT2[−], respectively.

Agglutination assay. A single colony of the strain to be tested was used to inoculate 5 ml LB broth, cultured at 37 °C without shaking, then subcultured twice for overnight static growth. Agglutination was assayed on glass slides by mixing 15 µl bacterial culture with an equal volume of bakers' yeast suspension (10 mg ml^{−1}). Mannose inhibition of agglutination was confirmed using 3% α-D-mannose in the yeast suspension.

Switch-orientation assay. Primers *fim*5' and *fim*3' (Table 1) were used to amplify a 604 bp region that incorporates the invertible *fimA* promoter element. The resulting PCR product was digested with *Hinf*I, and the products separated using a 6% polyacrylamide Tris/borate EDTA gel run at 100 V for 2 h. The products were visualized after staining with ethidium bromide using Flowgen gel-analysis software and hardware.

Cloning of *fim* switch regions into pDG19 and allelic exchange. The *fim* switch region from the strains was amplified using primers C1 and C2 (Table 1). Primer C1 incorporates a *Sph*I site and C2 incorporates a *Bam*HI site at its 5'-terminus, allowing the amplified fragment to be ligated into pDG19 (Table 1). Allelic exchange of the cloned switch region into BGEC144 (Table 1) was performed as described previously (Blomfield *et al.*, 1991; Leathart & Gally, 1998).

'Repair' of the O157 *fim* switch. The 16 bp of K-12 sequence absent in *E. coli* O157 ZAP1 was introduced into the plasmid carrying the ZAP1 *fim* switch region (pAJR1; Table 1) by using specific primers. Two primers were designed (rep1 and rep2; Table 1), each of which had an 8 bp 5'-terminus that complemented the K-12 region absent from the sequence of ZAP1. In conjunction with primers C1 and C2 (Table 1), amplification using Pwo polymerase (Roche) produced two blunt-ended products of 317 bp and 424 bp. These products were cleaned using a Qiagen PCR clean-up kit and ligated using standard conditions; 1 µl of this ligation reaction was then used as a template for PCR using primers C1 and C2 (Table 1) to produce a *fim* switch region that was identical to that of ZAP1 (apart from the 'repair' of the 16 bp deletion).

Switching-frequency assay. Strains were plated on minimal glucose X-Gal agar at 37 °C. Using a sterile cork-borer, six 'off' (white) colonies were excised from the plate, diluted in PBS and plated on minimal glucose X-Gal agar at 42 °C (300–1000 c.f.u.). Plates were counted for white and blue colonies, and the frequency of off-to-on inversion was calculated as described previously (Gally *et al.*, 1993).

RESULTS

Detection of *fim* switch deletion in VTEC isolates

To confirm the presence of the 16 bp deletion in *E. coli* O157, a number of human-outbreak and cattle isolates were tested. The isolates covered a range of phage types and a variety of PFGE patterns (Fiona Thomson-Carter, Aberdeen *E. coli* Reference Laboratory). All of the human (27) and bovine strains (36) tested possessed the deletion, as demonstrated by a smaller PCR product generated across the *fim* switch (Fig. 1a). A selection of these were confirmed by use of the deletion-detection primer designed by Li *et al.* (1997) (Fig. 1b, Table 1). A small number of shiga-like-toxin-positive *E. coli* O26 (3), O111 (4), O86 (1) and O128 (1) isolates were tested and, as anticipated, were shown not to contain the deletion (Fig. 1a, b). These data confirm the specificity of this deletion to *E. coli* O157 and the potential use of the

Table 1. Strains, plasmids and primers used in this study

Strain, plasmid or primer	Details	Reference
Strains		
MG1655	λ^- F ⁻ Fim ⁺	Our stocks (Guyer <i>et al.</i> , 1981)
AAEC185	F ⁻ λ^- <i>supE44 hsdR17 mcrA mcrB endA1 thi-1</i> Δ (<i>fimBEACDFGH</i>) Δ <i>recA</i>	Our stocks (Blomfield <i>et al.</i> , 1991)
ZAP1	O157 Redhouse Dairy human-outbreak strain, phage type 2	Our stocks
MPRL4269 (ZAP77)	O26 human isolate	Our stocks
MPRL4270 (ZAP78)	O26 human isolate	Our stocks
ZAP26	O26:H ⁻ STEC 413/89-1, bovine isolate	Djafari <i>et al.</i> (1997)
BGEC144	MG1655 Δ <i>lacZYA fimA-lacZYA</i> Δ <i>fimE-fimA'</i> Ω (<i>sacB-kan'</i>) <i>fimE'am18 lacZYA-fimA</i>	Gally <i>et al.</i> (1994)
SR (no.)	<i>fimB'E-fimA-lacZYA</i> : <i>fimA</i> reporter strains created by allelic exchange of switch regions using plasmids listed below	This study
SR1	pAJR1 (ZAP1 switch region) exchanged into BGEC144	This study
SR2	pAJR2 (MPRL4269 switch region) exchanged into BGEC144	This study
SR3	pAJR3 (MPRL4270 switch region) exchanged into BGEC144	This study
SR4	pAJR15 (ZAP26 switch region) exchanged into BGEC144	This study
SR5	pAJR13 (13 bp repair) exchanged into BGEC144	This study
SR6	pAJR16 (16 bp repair) exchanged into BGEC144	This study
Plasmids		
pDG019	<i>fimE-fimS-fimA'-lacZ'</i>	Gally <i>et al.</i> (1994)
pAJR1	Replacement of pDG19 <i>fim</i> region with that of ZAP1	This study
pAJR2	Replacement of pDG19 <i>fim</i> region with that of MPRL4269	This study
pAJR3	Replacement of pDG19 <i>fim</i> region with that of MPRL4270	This study
pAJR15	Replacement of pDG19 <i>fim</i> region with that of ZAP26	This study
pAJR13	pAJR1 with insertion of 13 bases from K-12 sequence (see text)	This study
pAJR16	pAJR1 with insertion of 16 bases from K-12 sequence (see text)	This study
Primers		
<i>fim</i> 5'	GCCGGATTATGGGAAAGA	Leathart & Gally (1998)
<i>fim</i> 3'	AGTGAACGGTCCCACCAT	
STS-1	CTCAAGCATAAAAAATTTAACTAA-CTG	Li <i>et al.</i> (1997)
CON-2	ACGTCCCCGTAACCTGGGTAGGTTA	Li <i>et al.</i> (1997)
C1	CCTCATATGTTAAGGCATGC	Leathart & Gally (1998)
C2	CCGGATCCGGCAGCGCCAGAGCC	Leathart & Gally (1998)
rep1	TGTCGTTTTTTAAAATTTTATGCTT-GAGAAAA	This study
rep2	AAAAGCATCTAACTGTTTGATATGT-AAATTATTTC	This study

Fig. 1. (a) PCR amplification of the *fim* region of VTEC and non-VTEC strains. *E. coli* O157 strains can be seen to have a smaller product when analysed using primers C1 and C2 (described in Table 1). The lanes contain the following: 100 bp markers (bright band of 600 bp) (lane 1); *E. coli* K-12 MG1655 (lane 2); ZAP28, ZAP32, ZAP36, ZAP40 (lanes 3–6), all O157 strains of differing phage types; ZAP21 (lane 7, O26), ZAP78 (lane 8, O26), ZAP72 (lane 9, O111), ZAP73 (lane 10, O111), ZAP74 (lane 11, O128), ZAP76 (lane 12, O86); ZAP1, ZAP48, ZAP52, ZAP56 (lanes 13–16, further phage types of O157); a blank control (lane 17); a repeat of the 100 bp ladder (lane 18). Further details of the strains are given in Table 1. (b) PCR analysis of the *fim* deletion region using O157-specific primers (see text and Table 1). The same strains were analysed as described for (a).

deletion PCR primer as a molecular probe (Li *et al.*, 1997).

Genotypic and phenotypic analyses of type 1 fimbrial expression

To confirm that *E. coli* O157 does not produce type 1 fimbriae whereas *E. coli* O26 can, a simple slide agglutination test was performed on one human *E. coli* O157 outbreak strain and three *E. coli* O26 strains (two human and one bovine). The O157 strain was negative for agglutination, whereas the O26 strains varied in their ability to agglutinate, but all three were positive (Fig. 2, bottom panel).

To determine whether agglutination ability correlated with the phase state of the *fim* switch in each culture, a PCR and restriction digestion of the *fim* switch was carried out as described in Methods. As shown in Fig. 2, the higher the proportion of the bacterial population with the *fim* switch in the on orientation, the better the ability to agglutinate. Moreover, the O157 strain appeared to be locked in the off orientation (>99% off). Without phase switching from the off to the on phase

orientation, *E. coli* O157 will be unable to express type 1 fimbriae.

Cloning and analysis of *fim* switch regions

After differences in the phase state of the *fim* switch between VTEC isolates had been established, the contribution of the *fim* switch region alone to this behaviour was determined. To do this, isogenic *fimA*–*lacZYA* strains containing the three O26 switch regions and one O157 switch region were created, allowing quantification of switching frequencies. This approach uses allelic exchange to replace the *fim* switch of *E. coli* K-12 MG1655 with the relevant VTEC switch region adjacent to a *fimA*–*lacZYA* transcriptional fusion (Leathart & Gally, 1998; see Methods). This allows switching frequencies to be measured with the same recombinase and host factors present. SR1 resulted from the exchange of the *E. coli* O157 switch region into BGEC144 (*fimB*⁺*E*[–]). The frequency of FimB-promoted off-to-on switching in *E. coli* K-12 containing its wild-type switch was measured as 3×10^{-3} per cell per generation (Fig. 3). However, SR1 gave no detectable switching ($< 1 \times 10^{-5}$ per cell per generation; Fig. 3). SR2–4 resulted from exchange of the three *E. coli* O26 regions into BGEC144 and all three strains had frequencies similar to that of *E. coli* K-12 (2×10^{-3} to 3.5×10^{-3} ; Fig. 3). These data show that the *E. coli* O157 switch region is unable to invert in a permissive background, proving that sequence changes in this switch region prevent off-to-on inversion of the switch to allow expression of *fimA*. Interestingly, while there are clear differences in the degree of expression of type 1 fimbriae (and switch orientation) between the three O26 strains (Fig. 2), these differences were not transferred with the respective switch regions and cannot be attributed to sequence variation in these regions.

All four VTEC switch regions were sequenced (Fig. 4). While numerous single base-pair variations were evident, the previously characterized 16 bp deletion in *E. coli* O157 appeared to be the most likely change preventing *fim* switching.

Repair of the 16 bp deletion from *E. coli* O157

To ascertain whether this deletion is responsible for the failure of the *E. coli* O157 switch region to undergo inversion from off to on, a repair was engineered by site-specific PCR and cloning methods, followed by allelic exchange of the repaired switch into BGEC144 (SR6). This construct was confirmed by sequencing (Fig. 4). During this construction, another clone was isolated that was found to contain only 13 of the 16 bp absent in the *E. coli* O157 sequence (Fig. 4). This partial repair was also exchanged into BGEC144 to create SR5. FimB off-to-on switching was measured in both SR5 and SR6 (Fig. 3). It was clear that repair of the full 16 bp restored a wild-type switching frequency (2.5×10^{-3}). The incomplete repair was able to invert from off to on but at a 10-fold-reduced frequency (2.8×10^{-4}). In summary, these data confirm that the 16 bp deletion in the *E. coli*

Fig. 2. PCR and restriction digestion of the *fim* switch from VTEC isolates indicates the on-off status within the bacterial population. The product was run either undigested or after digestion with *Hin*fl. Lanes: 1, 100 bp marker; 2, ZAP26 uncut; 3, ZAP26 cut; 4, ZAP77, uncut; 5, ZAP77, cut; 6, ZAP78 uncut; 7, ZAP78 cut; 8, ZAP1 uncut; 9, ZAP1 cut. The results of the yeast agglutination assay are summarized below the gel image.

Fig. 3. Switching frequencies of strains containing the *fim* switch region from VTEC isolates (Table 1). SR1–4 contain the switches from ZAP1, MPRL4269, MPRL4270 and ZAP26, respectively exchanged into BGEC144. SR5 is the ZAP1 *fim* switch region containing a 13 bp repair exchanged into BGEC144, whereas SR6 contains the ZAP1 *fim* switch region with the full 16 bp repair exchanged into BGEC144 (Table 1). Switching frequencies were measured as described in Methods.

O157 switch region prevents phase-variable expression of *fimA*, the main fimbrial subunit gene.

DISCUSSION

The data presented in this work confirm that the majority of VTEC O157 strains contain a 16 bp deletion in the *fim* switch that has so far not been detected in any other *E. coli* serotype. The *fim* switch from one *E. coli* O157 human-outbreak strain (ZAP1) was shown to remain in the off orientation despite culture in conditions that favour expression of type 1 fimbriae. In contrast, the three *E. coli* O26 strains examined showed variation in their ability to produce type 1 fimbriae, and this variation correlated well with the proportion of bacteria containing the *fim* switch in the on orientation. The switch regions from all four strains were cloned and exchanged into the *E. coli* K-12 chromosome to replace the wild-type switch and create *fimA-lacZYA* transcriptional fusions. The switch region from ZAP1 failed to promote expression of the fusion and the switch was still locked in the off orientation. This proves that sequence variation within the ZAP1 *fim* switch region

prevents inversion and expression of *fimA*. All three O26 regions were able to produce phase-variable expression of *fimA* at rates equivalent (within twofold) to that of *E. coli* K-12.

The four switch regions were sequenced and the 16 bp deletion was identified as the major difference between the O26 and O157 strains. This deletion was repaired in the *E. coli* K-12 fusion background and this repaired strain was then able to phase-vary *fimA* expression at frequencies equivalent to both *E. coli* K-12 and the O26 switch replacement strains. While other changes within the *fim* region of *E. coli* O157 may prevent type 1 fimbrial expression, this single deletion within the regulatory region is sufficient to nullify expression and is the likely reason why *E. coli* O157 cannot produce type 1 fimbriae. The reason why the 16 bp deletion prevents phase variation is unclear. The regulation of type 1 fimbrial phase variation has been studied in detail, with most binding sites for required co-factors being identified within the *fim* switch (Blomfield *et al.*, 1997; Gally *et al.*, 1994, 1996; Olsen *et al.*, 1998). The 16 bp deletion lies at the edge of a known integration host factor (IHF)-binding site (Fig. 4) but does not involve the majority of bases considered important for IHF binding (Blomfield *et al.*, 1997). However, it is possible that the lack of *fimA* expression is caused by a failure of IHF to bind and bend the DNA at this site. Alternatively, the required DNA conformation for inversion may be strict and may be affected by the 16 bp deletion. A consistent distance of 314 ± 1 bp between the 9 bp inverted repeats was observed in urinary tract *E. coli* isolates (Leathart & Gally, 1998). For recombination the inverted repeats need to: (1) be brought together by DNA bending proteins (IHF and Lrp) and (2) be in phase to allow appropriate recombinase binding and activity. The 16 bp deletion could interfere with either or both of these requirements. In the course of repairing the 16 bp deletion, a 3 bp deletion was created at the same site (Fig. 4); the strain containing this deletion was able to phase vary but at a 10-fold reduced rate compared to the full repair and *E. coli* K-12. This 3 bp deletion is not within the IHF-binding site and supports the premise that the 16 bp deletion prevents recombination by interfering with strict spatial requirements.

Fig. 4. Sequences of *fim* switch regions for the VTEC isolates used in this study and for *E. coli* K-12 MG1655. The diagram shows the IHF-binding site located within the invertible element (a) (Blomfield *et al.*, 1997) and the 16 bp deletion region (b) identified by Li *et al.* (1997).

Clear differences were observed in the degree of expression of type 1 fimbriae (and switch orientation) between the three O26 strains tested. However, all three imported switch regions had similar off-to-on switching frequencies. Potentially, other genetic factors in the relevant host strain are responsible for the differences in expression levels. This could include differences in recombinase expression or activity. However, it is possible that, as shown for urinary tract isolates, regulators of other adhesins or virulence determinants may cross-talk with type 1 fimbriae to alter expression levels (Xia *et al.*, 2000). Expression of type III secretion organelles and intimate attachment may not be compatible with the presence of fimbrial adhesions on the bacterial surface. The potential for coordinate expression between type 1 fimbriae and type III secretion in enterohaemorrhagic *E. coli* (EHEC) serotypes such as O26 remains to be investigated. Certainly, O157 EHEC infections do not seem to need type 1 fimbriae but, as with rabbit diarrhoeagenic *E. coli* (RDEC), O26 strains may be more likely to form A/E lesions once the bacterium is brought into close proximity to the host-cell epithelium by a fimbrial adhesin. Our preliminary data have demonstrated that the level of type 1 expression

does correlate with ability of *E. coli* to bind to bovine colonic epithelium. Therefore, expression of type 1 fimbriae by *E. coli* O26/O118 could enhance colonization in the bovine colon as well as that in the rumen (Galfi *et al.*, 1998). Obviously, host factors such as competitive flora and mucus secretion may also modulate this activity. Other factors that could explain VTEC O26/O118 virulence in the bovine host (in comparison to VTEC O157), including the level and activity of secreted proteins, are currently being investigated.

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