BACTERIAL PATHOGENICITY

A low molecular weight outer-membrane protein of Escherichia coli O157:H7 associated with adherence to INT407 cells and chicken caeca

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Enterohaemorrhagic Escherichia coli (EHEC) O157:H7 and O26:H11 have been shown to produce a low mol. wt outer-membrane protein (OMP) that is unique to a few serotypes of E. coli. A mutant (A10) of E. coli O157:H7 strain HA1 deficient in the OMP was constructed by TnphoA mutagenesis and assayed for its adherent ability. Adherence of A10 to intestinal epithelial cells (INT407) was significantly less than that of its parent strain (HA1). Adherence of HA1 to INT407 cells was significantly decreased by treatment with a monoclonal antibody (4E8C12) that specifically binds to the OMP. When chickens were infected experimentally with E. coli O157:H7 strains, the average number of cfu of strain A10 recovered from chicken caeca was significantly less than those of strain HA1 and wild-type strain 932 at 14 and 21 days after peroral inoculation. These data suggest that the OMP of EHEC is associated with adherence of E. coli O157:H7 to epithelial cells in vitro and chicken caeca in vivo.

Introduction

Escherichia coli serotype O157:H7, a recognised cause of haemorrhagic colitis and haemolytic uraemic syndrome, is the principal serovar within the enterohaemorrhagic E. coli (EHEC) [1, 2]. The organism can produce Shiga-like cytotoxins (SLT I or SLT II or both) that cause host cell death by inhibiting protein synthesis [1–3]. Also important to the pathogenicity of EHEC is its ability to attach to host cells [1, 2]. However, the mechanism of E. coli O157:H7 adherence to host cells is poorly understood. Previous reports have suggested that outer-membrane proteins (OMP) rather than lipopolysaccharide (LPS) or flagella of E. coli O157:H7 play an important role in mediating binding of the bacteria to epithelial cells in vitro [4–6]. The mechanism of attachment of E. coli O157:H7 to intestinal mucosal cells has been studied extensively by several investigators [4–13]. Genes and their products that are involved in colonisation have been studied, but their exact roles, especially in the initial attachment of E. coli O157:H7, remain controversial and require further elucidation [4, 7, 8, 13, 14].

Padhye and Doyle [15] produced a monoclonal antibody (MAb) 4E8C12 that reacts with a low mol. wt (originally described as 5–6 kDa, but determined to be 8 kDa in the present study) OMP of EHEC O157:H7 and O26:H11. Because of the association of the OMP with EHEC [16], it was suggested that the OMP may be associated with the virulence of EHEC, perhaps associated with the organism’s adherence. TnphoA mutagenesis allows the creation of mutants deficient in bacterial factors that normally are secreted or exported to the bacterial cell surface [6, 17, 18]. As all bacterial proteins implicated as virulence factors are extracellular or surface associated, TnphoA mutants can be used to identify genes that encode adherence factors [5, 18–21]. Construction and analysis of EHEC TnphoA mutants can provide a foundation for the study of the molecular basis of the pathogenicity of EHEC. The objective of this study was to obtain a transposon TnphoA mutant deficient in the low mol. wt OMP of EHEC, and to determine its ability to adhere to human intestinal epithelial cells (INT407) and chicken caeca.

Materials and methods

Bacterial strains

E. coli O157:H7 strain 932 was used as a positive control in the chicken inoculation study. E. coli O157:H7 strain HA1, derived from strain 932, was used as the recipient cell in conjugation studies. Strain HA1, which produces both SLT-I and SLT-II, was used for the development of MAb 4E8C12 for EHEC [15].

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E. coli strain SM10, *pir* (thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km) was the donor cell in conjugation studies with *E. coli O157:H7* strain HA1, and was kindly provided by S. Craven (Russell Research Center, USDA/ARS, Athens, GA, USA). SM10, *pir* carries a suicide plasmid pRT733 (oriR6K tra-mob- Ap’ Km’) that bears transposon TnphoA.

**Media and chemical reagents**

Luria broth (LB) and Luria plates were used for routine culturing of the bacteria, and MacConkey agar was used for enumeration. All media were obtained from Difco. Ampicillin (Ap, 100 μg/ml), kanamycin (Km, 45 μg/ml), nalidixic acid (NA, 50 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (XP, 40 μg/ml) were added when appropriate. All chemical reagents were purchased from Sigma.

**Construction of TnphoA mutants**

A NA (50 μg/ml)-resistant strain of HA1 was obtained by plating on to MacConkey agar plates containing different concentrations (1-50 μg/ml) of NA. Transposon TnphoA from suicide plasmid vector pRT733 was introduced into the NA-resistant HA1 by plate matings with the donor cell SM10, *pir*. In-frame transposon insertion into signal-sequence containing genes resulted in phenotypes of TnphoA mutants that were Km', Na' and formed blue colonies on LB plates containing Km, Na and XP (an indicator dye for alkaline phosphatase activity). Screening TnphoA mutants by immunodot-blot assay

Approximately 800 Km', NA' blue colonies were selected from separate conjugation experiments for immunodot-blot screening. The immunodot-blot assay was performed by the following procedure. Briefly, an Immobilon-PVDF membrane (Millipore Corp., Bedford, MA, USA) was cut (8 x 12 cm), soaked in 10 ml of methanol 100% for 2 s, and placed in coating buffer (0.05 M carbonate buffer, pH 9.6) for 10 min before use. The TnphoA mutants were grown in 5 ml of LB containing Km and NA for 18 h at 37°C. Fifty μl (c. 10⁶ cfu) of each colony were placed on the membrane assembled in a dot-blot manifold (BRL, Gaithersburg, MD, USA). The membrane was air dried for 5 min after blotting the cultures, and then submerged in bovine serum albumin (BSA) 5% w/v in 50 mM Tris buffer saline, pH 7.5, containing 150 mM NaCl with Tween-20 0.05% v/v (TBS-T) plus sodium azide 0.05% w/v at 37°C for 1 h with agitation (100 rpm). The membrane was soaked in 20 ml of MAB 4E8C12 (ascites fluid diluted 1 in 400 in TBS-T) for 1 h at room temperature, washed three times with TBS-T for 10 min each, and then incubated with alkaline phosphatase-labelled goat anti-mouse IgG (diluted at 1 in 800 in TBS) for 30 min at 37°C. The membrane was washed four times, and placed in a substrate of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (Kirkegard and Perry Laboratory, Inc., Gaithersburg, MD, USA) for 1 min at room temperature. The reaction was stopped by rinsing the membrane with distilled water for 1 min. A dark purple spot and a light purple spot indicated positive and negative reactions with MAB 4E8C12, respectively. *E. coli O157:H7* HA1 and SM10, *pir* served as positive and negative controls, respectively.

**Tricine-SDS-PAGE and Western blot analysis**

The TnphoA mutant, designated as A10 that gave a negative result in immunodot-blot assay, was selected for Tricine-SDS-PAGE and Western blot analysis to confirm that it did not express the low mol. wt OMP. The OMP of strains A10, HA1 and SM10, *pir* were each loaded on gels consisting of a separating 20% gel, a spacer 10% gel and a stacking 4% gel in a double-slab electrophoresis unit (Protean; BioRad Laboratories, Richmond, CA, USA) and run at constant 90 mA for 7 h. Low range mol. wt markers (Sigma) were included on the gels. Protein bands were visualised by staining with Coomassie Blue 2.5% w/v for 2 h and destaining in methanol 40% v/v and acetic acid 20% v/v. Western blotting was performed according to the procedure described by Padhye and Doyle [15]. After electrophoresis, proteins were transferred to a PVDF membrane in a buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine, methanol 20% v/v and SDS 0.05% w/v in a transblot apparatus (Multiphor II; Pharmacia, Piscataway, NJ, USA) at 400 mA for 40 min. The procedures for immunostaining were the same as the dot-blot immunodotassay described above, except that MAB 4E8C12 was diluted 1 in 1600 and goat anti-mouse antibody was diluted 1 in 2000 in TBS-T.

**Southern blot hybridisation**

The genomic and plasmid DNA of strain A10 were extracted by a mini-preparation procedure [24, 25]. For each DNA preparation, 2 μg were digested at 37°C for 3 h with restriction endonuclease EcoRV (BRL) that does not cleave within TnphoA. The DNA fragments were separated by electrophoresis in agarose 0.8% w/v gel and transferred on to a Nylon membrane (Sigma). The genomic DNA of strain HA1 was also isolated and subjected to Southern hybridisation to serve as a control. The plasmid DNA of pRT733 was extracted by mini-preparation, digested with restriction endonuclease BglII, and separated in agarose 0.8% w/v gel. A 2.8-kbp BglII fragment that contains the Km resistance gene found in TnphoA was isolated from the gel, purified by GeneClean II (Bior, 101, La Jolla, CA, USA), and labelled with α-³²P dATP by the
Adherence assay

INT407 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in Basal Medium Eagle (BME) plus fetal bovine serum (FBS; BRL) 15%. The adherence assay was performed by the method of Junkins and Doyle with minor modification [27]. Briefly, INT407 cells were inoculated into each well (35 mm in diameter) of a six-well tissue culture plate with lid (Corning Glass Works, Corning, NY, USA) at a concentration of $10^6$–$10^7$ viable cells, and grown in BME plus FBS 15% at 37°C until confluent (2–3 days). Overnight bacterial cultures were sedimented by centrifugation (16,000 g, 1 min) and resuspended to an OD$_{600nm}$ of 0.5 with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) plus 10 $\mu$M ZnCl$_2$. The bacterial suspension (2 ml) was applied to an INT407 monolayer and incubated at 37°C for 2 h without agitation. After incubation, the suspension was removed from the monolayer, and non-adherent bacterial cells were removed by rinsing five times with 3 ml of TBS plus 10 $\mu$M ZnCl$_2$ plus Tween 20 0.1% v/v. The monolayer was then treated with 2 ml of 2 mM Na$_2$EDTA in TBS for 1 min. The bacteria were separated from INT407 cells by vortex mixing. The number of viable bacteria in the suspension was determined by plating serial (1 in 10) dilutions of the suspension on MacConkey agar plates, followed by overnight incubation at 37°C. The number of adherent bacteria/INT407 cells was then calculated. To minimise experimental error, positive (HA1) and negative (SM!0$_{10}$ pir) controls were always included in each tissue culture plate.

Adherence inhibition assay

Six adherence inhibition assays also were performed. The procedures of the adherence inhibition assay were the same as those of the adherence assay except that E. coli O157:H7 strain HA1 was treated with MAb 4E8C12 (ascites fluid) before being added to the INT407 monolayer. The MAb (diluted 1 in 200 and 1 in 400) was added to 2 ml of bacterial suspension and incubated at 37°C for 1.5 h. Strains A10 and HA1 that were not treated with MAb served as controls in each test. Data of the adherence and adherence-inhibition assays were analysed statistically by the t-test [28].

Chicken colonisation study

The methods used for the chicken colonisation study were described previously [29]. Briefly, E. coli O157:H7 strains 932, HA1 and mutant A10 were cultured at 37°C in Trypticase Soy Broth (TBS; BBL Microbiology System, Cockeysville, MD, USA) with appropriate chemicals for 24 h. Cells were washed three times and resuspended in 0.01 M phosphate-buffered saline (PBS) pH 7.5. Two trials of the chicken inoculation study were conducted (Table 1). In trial I, 105 1-day-old chicks (Peterson Arbor-Acre) were divided into four groups, of which 15 chicks served as controls and received 0.25 ml of PBS perorally into the crop via a 1-ml syringe, and the remaining 90 chicks received c. $10^9$ cfu of E. coli (30 chick/strain) by the same procedure. Five chicks from the control and 10 chicks from each test group were killed at 7, 14 and 21 days post-inoculation for bacterial enumeration. In trial II, two more sampling times (3 and 5 days) were added (Table 1).

Immediately after they were killed, the internal organs of chicks were dissected and caeca were removed aseptically and placed in plastic sterile tubes. The tissue was weighed, diluted (1 in 10) in cold PBS and

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>E. coli log$_{10}$ cfu/g of caecal tissue and contents (SD) at post-inoculation times</th>
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<tr>
<td></td>
<td>3 days (g)</td>
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<tr>
<td>Trial I</td>
<td></td>
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<tr>
<td>932</td>
<td>-</td>
</tr>
<tr>
<td>HA1</td>
<td>-</td>
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<td>A10</td>
<td>-</td>
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<tr>
<td>Trial II</td>
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<tr>
<td>932</td>
<td>4.65 (0.78)</td>
</tr>
<tr>
<td>HA1</td>
<td>4.05 (0.88)</td>
</tr>
<tr>
<td>A10</td>
<td>5.89 (0.90)</td>
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*Strain A10 (<10 cfu/g of caeca: minimum level of sensitivity) detected in five of 10 chicks.
†Strain A10 (<10 cfu/g) detected in 10 chicks.
‡Strain A10 (<10 cfu/g) detected in four of 10 chicks.
homogenised aseptically for 1 min with a Polytron tissue homogeniser (Brinkman Industries, Inc., Westbury, NJ, USA). Samples were serially diluted (1 in 10) in PBS, and each dilution was plated in duplicate on sorbitol-MacConkey agar containing nalidixic acid 30 µg/ml. For enumeration of the mutant A10, kanamycin 40 µg/ml also was added to the medium. The plates were incubated at 37°C for 48 h, and colonies typical of _E. coli_ were counted. At least one typical _E. coli_ colony from a plate at the highest dilution of tissue was confirmed serologically as _E. coli_ O157 by latex agglutination with O157-specific antiserum (Unipath, Basingstoke). Data of the chicken study were analysed statistically by the _t_-test [28].

Results

**Construction of low mol. wt OMP-deficient mutants of _E. coli_ O157:H7**

Approximately 800 Km', NA' blue colonies were selected from hundreds of separate conjugation experiments for immunodot-blot screening. A mutant, designated A10, did not react with MAb 4E8C12 in the dot blot assay. Strain A10 retained many of the characteristics of its parent strain, HAI, such as production of SLT I and SLT II, agglutination with O157 polyclonal antiserum, genomic DNA profile by pulsed-field gel electrophoresis and inability to hydrolyse 4-methylumbelliferyl β-D-glucuronide (MUG), except that A10 was Km', obtained from TnphoA.

**OMP analysis**

Tricine-SDS-PAGE and Western blotting were performed to analyse the OMP of mutant A10, the parent strain HAI and donor strain SM10. The tricine-SDS-PAGE profiles indicated that one band representing the c. 8-kDa OMP that was present in strain HAI was missing from strain A10. The OMP profile of SM10, _pir_ was very different from those of strain HAI and A10 (Fig. 1A). Western immunoblot analysis of the OMP revealed that the HAI OMP reacted with MAb 4E8C12, whereas those of strains A10 and SM10, _pir_ did not (Fig. 1B), further confirming that strain A10 was deficient in the low mol. wt OMP.

**Genetic analysis**

Hybridisation of genomic and plasmid DNA of strain A10 with the 2.8-kbp Km' gene probe showed that A10 contained a single TnphoA insertion located on the chromosomal DNA, and that the plasmid DNA of strain A10 had no TnphoA insertion (Fig. 2). The hybridised _EcoRV_ fragment of A10 genomic DNA, which contained TnphoA flanking the target gene, was c. 9.4 kb (Fig. 2). The genomic DNA of strain HAI did not hybridise with the probe (data not shown).
Adherence assay

Fifteen independent adherence assays were performed. The mutant strain (A10) was shown to be significantly less (p < 0.005) adherent to INT407 cells than the parent strain (HA1). The means and standard deviations (SD) of adhering bacteria per INT407 cell for strains A10, HA1 and SM10, pir were 1.62 (0.97), 133.1 (6.17) and 2.75 (2.69), respectively.

Six adherence inhibition assays also were performed. After treatment of strain HA1 with MAb 4E8C12 at 1 in 200 and 1 in 400 dilutions at 37°C for 1.5 h, HA1 was capable of adhering to INT407 cells at a mean (SD) of 3.53 (2.51) and 2.52 (2.17) bacteria/cell, respectively. As the controls, the means (SD) of adhering bacteria per INT407 cells for strains A10 and HA1 were 0.93 (0.49) and 9.49 (3.91), respectively. Results with and without treatments with MAb 4E8C12 indicate that MAb 4E8C12 significantly (p < 0.05) inhibited adherence of strain HA1 to INT407 cells. If strain HA1 was treated with the MAb at the 1 in 1600 dilution and held at 37°C for 1.5 h, the ability of HA1 to adhere to INT407 cells was not significantly different (p > 0.05) different from that of bacteria that were not treated with the MAb.

Chicken colonisation study

Results of caecal colonisation with E. coli O157:H7 strains 932, HA1 and OMP-mutant A10 in chickens after peroral inoculation are shown in Table 1. In trial I, strain A10 was not isolated at 14 and 21 days after peroral inoculation, whereas strains 932 and HA1 were still present in the caeca at levels >10⁵ cfu/g of caeca. Similar results were observed in trial II, in which strain A10 was not recovered after 14 and 21 days post-inoculation in at least four of 10 chicks. The average number of cfu of strain A10 recovered from caeca was significantly less (p < 0.05) than those of strains 932 and HA1.

Discussion

Factors involving E. coli O157:H7 adhesion and attaching and effacing (A-E) lesion formation have been studied by several investigators [4–12,30]. Donnenberg et al. [4] reported that the eae gene is necessary for intimate attachment of EHEC in vivo, but the precise role of intimin, the product of the eae gene of EHEC, in the attaching and effacing phenomena is unknown [4]. Sherman et al. [9] suggested that a 94-kDa OMP was intimin because antiserum raised to this OMP blocked adhesion and F-actin lesion formation of E. coli O157:H7 strain CL56 on tissue culture cells. In later studies, Louie et al. [8] determined by peptide sequencing, immunoassay and expression of the carboxyl-terminal 266 amino acid of intimin of E. coli O157:H7 strain CL8, that intimin was a 97-kDa OMP and was different from the 94-kDa OMP.

Dytoc et al. [5] determined that E. coli HB101 transformed with the cloned EHEC eae gene did not form attaching and effacing lesions on cultured epithelial cells in vitro or rabbit intestinal tissue in vivo. Louie et al. [8] determined that an eae insertion inactivation mutant of strain CL-8 (CL8-K01) gave negative results in the fluorescent actin staining (FAS) test, but adherence of CL8-K01 to HEp-2 cells was not significantly different from that of CL8. Furthermore, the eae gene expressed alone in E. coli HB101 did not significantly increase adherence to HEp-2 cells compared with the parent HB101, despite the fact that E. coli O157:H7 intimin was localised in the outer membrane and was surface exposed. They concluded that intimin contributes minimally if at all to the total adherence of strain CL8 to HEp-2 cells. Dytoc et al. [5] used TnphoA mutagenesis to identify factors that were necessary for O157:H7 A-E lesion formation and were not likely to be an eae gene product in two TnphoA mutants. Recently, Leroy et al. [31] reported the presence of the eae gene in non-pathogenic E. coli strains isolated from rabbits. Hence, it appears that production of A-E lesions by EHEC depends on the expression of several genes and these are affected by environmental conditions. Adherence of EHEC to epithelial cells still occurs without the eae gene. Furthermore, introduction of the eae gene into other non-adherent E. coli strains does not necessarily result in their ability to adhere to epithelial cells because other essential factors are missing.

Recent studies have revealed that a large (35 kb) region in the enteropathogenic E. coli (EPEC) chromosome, locus of enterocyte effacement (LEE), containing eaeA, eaeB, sepA, sepB, sepC and sepD (sep, for secretion of E. coli proteins), encodes the virulence determinants for A-E lesion formation of EPEC that have been identified to date [32,33]. This region is conserved among other pathogens that produce the A-E lesion, including E. coli O157:H7, and is inserted into the E. coli chromosome at the identical site where a block of virulence genes or ‘pathogenicity island’ of uropathogenic E. coli is located [32]. Therefore, more studies are needed to determine the precise role of intimin, other adherence factors, the host cell receptor and their interactions in the process of colonisation.

Although E. coli O157:H7 has not been isolated from chickens, this organism is able to colonise well in chickens, caeca being the primary site of colonisation [29]. In the present study, chickens were used to evaluate the effect of the low mol.wt OMP on the colonisation of E. coli O157:H7 in vivo. The mutant deficient in the OMP had significantly reduced ability...
to colonise chicken caeca compared to the parent strain of *E. coli* O157:H7.

Recent studies in this laboratory indicate that the OMP has a close association with the lipid A portion of LPS on the cell membrane of *E. coli* O157:H7, similar to the association of LPS and outer-membrane proteins in other *E. coli* [34, 35]. *E. coli* O157:H7 strain HA1 was obtained while attempting to isolate a variant of *E. coli* O157:H7 strain 932 that was highly adherent to Henle 407 cell monolayers [15]. Strain 932 was passaged several times on Henle 407 cell monolayers. Each time only those bacteria that remained attached to the monolayers after washing were cultured. Strain HA1 was isolated from passage 11 and was highly adherent to Henle 407 cells. This strain lacks a portion of the LPS layer of the cell outer membrane, allowing greater exposure of the unique low mol.wt OMP than its parent strain and other wild-type strains of *E. coli* O157:H7. The OMP also is expressed in greater amounts when bile salts and acriflavine are present in the growth medium [16]. Interestingly, Bilge et al. [36] reported that *E. coli* O157:H7 LPS-mutants were hyperadherent. Their studies suggested that the increased ability of the O157-mutants to adhere to HeLa cells could result from increased contact between the bacterial adhesin and the epithelial cell surface in the absence of O side chains. It appears that the environment of the intestinal tract of animals and man could influence *eae* genes and other factors, including expression of the OMP, in their involvement in the process of colonisation. In conclusion, the low mol.wt OMP is associated with the adherence of *E. coli* O157:H7 to cultured human epithelial cells and chicken caeca, and the mechanism of this involvement remains to be determined.

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**References**


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