

Studies on *Escherichia coli* serotype O157:H7 strains containing a 60-MDa plasmid and on 60-MDa plasmid-cured derivatives

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Summary. Seventeen strains of *Escherichia coli* serotype O157:H7 producing Shiga-like toxin were examined for the presence of plasmids and for the ability to adhere to HEP-2 and Intestine 407 cells. All of the strains possessed a common 60-MDa plasmid. To determine the role of the 60-MDa plasmid, plasmid-cured strains were compared with the parent strains for their ability to produce pili and to adhere to epithelial cells in culture. The total cell lysate protein and outer-membrane protein (OMP) profiles were also compared. Both the parent strains and their plasmid-cured derivatives produced pili. Immunofluorescence assay results indicated that the plasmid-cured and parent strains adhered equally well to HEP-2 and Intestine 407 cells; overall adherence was greater with intestinal cells than HEP-2 cells. SDS-PAGE of polypeptides synthesised in an *E. coli* system *in vitro* showed that plasmid DNA encodes *c.* 35 proteins. SDS-PAGE of OMP preparations demonstrated that the 60-MDa plasmid appears to be involved in the synthesis of a 33-kDa OMP. Two strains cured of the 60-MDa plasmid, one that possessed no plasmids and one that still contained a 2.2-MDa plasmid, produced exopolysaccharide (EPS) when cultured on solid medium at 25°C. Two other strains, which were cured of the 60-MDa plasmid but contained a 4.5-MDa plasmid, did not produce visible amounts of EPS. Gas chromatography analysis showed that the EPS consisted of fucose, glucose and galactose in an approximate molar ratio of 2.0:0.9:1.1 and also had 7% of a uronic acid sugar as part of its structure.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) strains, particularly of serotype O157:H7, have been implicated in numerous foodborne outbreaks and sporadic cases of haemorrhagic colitis¹⁻⁶ and haemolytic uraemic syndrome.^{7,8} The clinical symptoms of the disease caused by this organism differ from disease symptoms caused by other groups of *E. coli* associated with human diarrhoeal illness. The mechanisms of pathogenesis of EHEC have not been fully elucidated but appear to be distinct from those of the traditional groups of *E. coli*. However, EHEC, like the classical enteropathogenic *E. coli* strains, do appear to show a specific type of attaching and effacing adherence to intestinal epithelial cells.

E. coli O157:H7 strains produce one or both of two phage-encoded Vero cell cytotoxins, Shiga-like toxin I (SLT-I) and Shiga-like toxin II (SLT-II). The mech-

anism of action and biological properties of these toxins are similar to those of Shiga toxin.⁹⁻¹¹

Most O157:H7 strains also contain a 60-MDa plasmid whose function and role in virulence has not been conclusively determined. Tzipori *et al.*¹² demonstrated that neither possession of the 60-MDa plasmid nor the production of SLT was necessary to cause attaching-effacing lesions in piglet intestines or to cause diarrhoea. Jerse *et al.*¹³ identified a chromosomal gene (*eae*) in enteropathogenic *E. coli* which is necessary for attaching and effacing lesions on tissue culture cells. The *eae* gene probe also hybridised with EHEC chromosomal DNA but not with EHEC plasmid DNA. Studies showed that plasmid-mediated factors may facilitate initial adherence to the intestinal mucosa of piglets but are not required for gut colonisation or bacterial attachment and effacement activity.¹⁴

One study showed that O157:H7 strains that possessed the 60-MDa plasmid elaborated fimbriae and adhered to Henle 407 intestinal cells whereas their plasmid-cured derivatives failed to express fimbriae or to adhere to intestinal cells.¹⁵ None of the strains adhered to HEP-2 cells. Toth *et al.*¹⁶ reported that a plasmid-cured strain, 2-45, showed decreased adher-

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ence to intestinal cells and to HEP-2 cells, but full adherence was restored to strain 2-45 transformed with the plasmid. Both the parent and plasmid-cured strains elaborated pili. Another study reported that five O157:H7 strains adhered to both HEP-2 and Intestine (INT) 407 cells with a greater overall adherence to INT 407 cells. Only one of the five strains mediated mannose-sensitive haemagglutination and possessed pili.¹⁷ Junkins and Doyle¹⁸ found that although there was some variability in adherence, a plasmid-cured strain adhered three times better, on average, than the parent strain under certain cultural and test conditions. They suggested that the strains may use two distinct mechanisms of adherence. The presence or absence of fimbriae on these strains was not demonstrated by electronmicroscopy.

It has been suggested that attachment of *E. coli* O157:H7 to epithelial cells is mediated by structures on the bacterial surface other than pili.^{16,19} Bacterial outer membranes were found to act as colonisation factors or adhesins *in vitro*, and antiserum against a 94-kDa *E. coli* O157:H7 outer-membrane protein (OMP) inhibited bacterial adherence to HEP-2 cells.^{19,20}

It is apparent that there are conflicting reports on the function of the 60-MDa plasmid and on its importance for the expression of virulence. The objective of the current study was to clarify the role of the 60-MDa plasmid by studying attachment of the plasmid-bearing and plasmid-cured strains to HEP-2 and INT 407 cells. The production of specific plasmid-encoded products was also investigated.

Materials and methods

Bacterial strains and growth conditions

The *E. coli* strains studied are listed in the table. For plasmid analysis, preparation of lysates and OMPs and for the adherence assays, the bacteria were grown to stationary phase in Brain Heart Infusion Broth (Difco) for 18 h at 37°C with agitation at 150 rpm.

Cytotoxicity assay

The method for assay of Shiga-like toxin was a modification of that of Gentry and Dalrymple.²² Vero cells were grown in Minimal Essential Medium (ICN Biomedicals, Inc./Flow Laboratories, Costa Mesa, CA, USA) containing fetal bovine serum 5%. Approximately 5×10^5 cells (100 μ l) were inoculated into 96-well microtitration plates (ICN/Flow) and the plates were incubated for 2–3 days at 37°C in the presence of CO₂ 5%. Filter-sterilised culture supernates (100 μ l), bacterial lysates (100 μ l, 0.8 mg of protein) and supernates and lysates heated at 100°C for 20 min were added to the Vero cell monolayers. Serial 1 in 2 dilutions of supernates and lysates were made in cell culture medium directly in the microtitration plates and the plates were incubated for 72 h at 37°C in the presence of CO₂ 5%. The Vero cells

were examined daily by light microscopy for cytotoxicity. The 50% cytotoxic dose (CD50) was defined as the amount of toxin (titre) that caused a cytopathic effect in 50% of the Vero cell monolayer after 72 h.

Plasmid analysis

Plasmid isolation. Plasmids of the *E. coli* strains were extracted by the method of Flamm *et al.*²³ Plasmid DNA was precipitated with a 0.58 volume of chilled isopropanol. The precipitate, suspended in a solution containing glycerol 50%, 0.06 M EDTA, pH 8.6, and bromophenol blue 0.013%, was then examined by agarose gel electrophoresis. Plasmid DNA suspended in 0.2 \times SET buffer (150 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl, pH 7.5, at 4°C) was further purified by banding in caesium chloride-ethidium bromide equilibrium gradients.²⁴

Selection of plasmid-cured strains. *E. coli* O157:H7 strains B1409 and 45753-35 were cured of the 60-MDa plasmid by incubation in the presence of ethidium bromide.²⁵ A radiolabelled probe for the 60-MDa plasmid was prepared by the Multiprime DNA Labelling System (Amersham, Arlington Heights, IL, USA) with an *EcoRI* digest of the 60-MDa plasmid. The plasmid was isolated from strain A9124-1 and purified by caesium chloride gradients. Colony filter hybridisation was performed as described by Bhaduri and Fratamico.²⁶ Pre-hybridisation and hybridisation were performed in aqueous solution at 68°C. Colonies that did not hybridise with the probe were selected and the absence of the plasmid was confirmed by agarose gel electrophoresis. The isolates were identified as *E. coli* O157:H7 serologically. Plasmid-cured strains were designated B1409P–, 45753-35P–, 933P– and 932P–.

In-vitro coupled transcription-translation. Plasmid DNA from strain B1409 purified by caesium chloride gradients was used in an *E. coli*-derived cell-free coupled transcription-translation system (Amersham) to identify plasmid-encoded gene products. The assay was performed according to the manufacturer's instructions. The purity of the plasmid DNA was tested by agarose gel electrophoresis and restriction endonuclease analysis.²⁴ The samples were mixed with an equal volume of electrophoresis sample buffer (0.125 M Tris-HCl, pH 6.8, containing glycerol 25%, SDS 2.5%, 2-mercaptoethanol 2.5% and bromophenol blue 0.0025%), heated in a boiling water bath for 6 min and then analysed by SDS-PAGE (acrylamide 4% in stacking gel; 12.6% in separating gel).²⁷ The gels were stained with Coomassie Blue R-250 0.1%, immersed in EN³HANCE autoradiography enhancer (New England Nuclear, Boston, MA, USA) for 1 h, washed with distilled water, dried, then exposed to Kodak X-OMAT AR film.

Analysis of bacterial lysate and OMP preparations

Bacterial cultures (18-h) were washed twice with phosphate-buffered saline (PBS; Dulbecco's formu-

Table. Characteristics of *E. coli* strains used in this study

<i>E. coli</i> strain no.	Source	Cytotoxicity assay (CD50)*		Plasmid profile (MDa)	Adherence to	
		CL	CS		HEp-2	INT-407
B1409†	CDC	262 144	16 384	60	2+	4+
B1409P-	This study; plasmid-cured derivative of B1409	262 144	8 192	—	2+	4+
45753-35	FSIS (meat)	262 144	512	60, 4·5	2+	4+
45753-35P-	This study; plasmid-cured derivative of 45753-35	262 144	512	—, 4·5	2+	4+
933‡	CDC (haemorrhagic colitis)	524 288	262 144	60, 2·2	1+	3+
933P-	USUHS, plasmid-cured derivative of 933 (Karch <i>et al.</i> , 1987)	524 288	262 144	—, 2·2	1+	3+
932§	FRI (haemorrhagic colitis)	1048 576	1048 576	60, 2·2	1	3+
932P-	FRI, plasmid-cured derivative of 932 (Junkins and Doyle, 1989)	1048 576	1048 576	—, 4·5, 2·2	2+	4+
A9218-C1	CDC	2097 152	524 288	60, 2·2	ND	ND
45750	FSIS (meat)	1048 576	1048 576	60, 4·5	ND	ND
A8959-C7	CDC (haemorrhagic colitis)	524 288	32 768	60, 2·2	2+	4+
45753-32	FSIS (meat)	1048 576	8 192	60, 4·5	ND	ND
A9124-1	CDC	1048 576	524 288	60	2+	4+
3417-85	CDC	32 768	65 536	60, 40, 30, 2·2	2+	4+
931	CDC (haemorrhagic colitis)	1048 576	131 072	60, 2·2	ND	ND
C984	CDC	524 288	16 384	60, 2·2	2+	4+
A9167-1	CDC (haemorrhagic colitis)	2097 152	16 384	60, 2·2	ND	ND
86-24	USUHS	262 144	524 288	60, 40, 30, 2·2	2+	4+
B1375-GSC15	CDC	524 288	524 288	60	ND	ND
505B	FRI (meat)	524 288	65 536	60	ND	ND
32381	FRI (human isolate)	524 288	131 072	60	ND	ND
K12 C600	USUHS	< 2	< 2		+/-	1+
K12 C600-LK3	USUHS, K12 C600 with 60 MDa plasmid from 933 ¹⁵	< 2	< 2	60	+/-	1+

CL, cell lysate; CS, cell supernate; ND, not determined.

CDC, Centers for Disease Control, Atlanta, GA; FSIS, Food Safety and Inspection Service, Washington, DC; USUHS, Uniformed Services University of the Health Sciences, Bethesda, MD; FRI, Food Research Institute, Madison, WI.

* CD50, amount of toxin (titre) that caused a cytopathic effect in 50% of a Vero cell monolayer.

† Produces SLT-II (determined by CDC).

‡ Produces SLT-I and SLT-II (determined by CDC).

§ Produces SLT-I and SLT-II (determined by Marques *et al.*²¹).

lation; ICN/Flow) and bacterial lysates were prepared by sonication.²⁸ OMP were prepared by the method of Portnoy *et al.*²⁹ except that Triton-insoluble proteins were dissolved in SDS 1%. Bacterial lysates and OMP samples were mixed with an equal volume of electrophoresis sample buffer and analysed by SDS-PAGE as described above.

Adherence assays

HEp-2 (ATCC CCL 23) and INT 407 cells (ATCC CCL 6) were grown to *c.* 75% confluence on circular coverslips placed in wells of 24-well tissue culture plates. The bacteria were washed and resuspended in PBS. Modified Eagle's Medium (ICN/Flow; 300 μ l) containing fetal bovine serum 10% and 100 μ l (2×10^8 bacteria) of the bacterial suspensions were added to the monolayers and the coverslips were incubated for 2 h at 37°C in CO₂ 5%. The monolayers were washed three times with PBS and incubated for 30 min with FITC-labelled antibody against *E. coli* O157:H7 (1 in 20 dilution in tissue culture medium; Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA). The coverslips were washed twice with PBS, mounted

on glass slides and examined by fluorescence microscopy. Giemsa's stain was used to detect adherence of strains K12 C600 and K12 C600-LK3 to the monolayers. Monolayers infected with the 60-MDa plasmid-cured derivatives and with the parent strains were also stained with Giemsa. The bacteria were incubated with epithelial cells in the presence of mannose 1% to determine whether the addition of mannose inhibited adherence. The monolayers were washed three times with 40 mM dithiothreitol (in PBS) after incubation with the bacteria, to determine whether the bacteria adhered firmly to the cells rather than to soluble or loosely associated cellular components.

Examination of *E. coli* O157:H7 for the presence of pili

The parent strains (60-MDa plasmid-bearing strains) and the 60-MDa plasmid-cured derivatives were grown in brain heart infusion broth for 18 h at 37°C with and without agitation or on MacConkey Sorbitol Agar (Oxoid) at room temperature for 48 h. The bacteria were suspended in sterile saline 0·85%

and a portion of the suspension was placed on Formvar-carbon coated grids, negatively stained with uranyl acetate 2% and examined with a Zeiss (Thornwood, NJ, USA) Model 10 B transmission electronmicroscope.

Production and analysis of bacterial exopolysaccharide (EPS)

Purification of EPS. *E. coli* O157:H7 strain B1409P- was grown in MacConkey broth at room temperature or on MacConkey agar (Difco) at 37°C for 18 h and incubated at room temperature until colonies had a mucoid appearance (24–48 h). Bacteria and EPS were removed from the agar and suspended in sterile water. The suspension was centrifuged for 10 min at 5500 *g* to remove the bacteria and the supernate was then dialysed against distilled water for 24 h. The EPS was precipitated with two volumes of acetone containing CaCl₂, 0.15%, collected by centrifugation (5500 *g* for 10 min), dried and resuspended in sterile water.

Gas-liquid chromatography. Carbohydrate analysis was performed with purified EPS by the method of Varma *et al.*³⁰ on a Hewlett Packard 5880A series gas chromatograph. The column (SP 2330 FS capillary column; Supelco Inc., Bellefonte, PA, USA) was temperature programmed from 150 to 250°C at 4°C/min.

Uronic acid assay. The determination of the uronic acid content of the purified EPS was performed by the method of Blumenkrantz and Asboe-Hansen.³¹

Electronmicroscopy. Plasmid-cured and parent strains were grown on MacConkey agar or MacConkey sorbitol agar at 37°C for 18 h and then incubated at room temperature for 24–48 h. Colonies surrounded by EPS were fixed in the vapour from an OsO₄ solution and immersed in absolute ethanol. Samples were cut and critical point dried from liquid CO₂, coated with gold and examined by scanning electronmicroscopy (JOEL, Peabody, MA, USA).

Results

Plasmid analysis

Examination of the plasmid profiles of the strains studied demonstrated several patterns. They possessed only a 60-MDa plasmid, 60- and 4.5-MDa plasmids, 60- and 2.2-MDa plasmids or 60-, 40-, 30- and 2.2-MDa plasmids (table). Strains 45753-35P-, 933P- and 932P- whose parent strains contained smaller plasmids and the 60-MDa plasmid were cured of the large plasmid but retained the smaller 4.5- and 2.2-MDa plasmids. Strain 932P- contained a 4.5-MDa plasmid which was not present in the parent strain. Strain B1409P- possessed no plasmids.

Proteins encoded by the 60-MDa plasmid DNA isolated from strain B1409 were synthesised *in vitro* by

means of a plasmid DNA-dependent *E. coli* cell-free coupled transcription-translation system. SDS-PAGE analysis of the protein products revealed proteins ranging in size from > 97 kDa to < 14 kDa (fig. 1, lane 3). A total of *c.* 35 protein bands was visible.

Characteristics of E. coli strains

Both the cell lysate and cell supernate preparations of all of the *E. coli* strains studied had verotoxigenic activity (table). CD50 titres ranged from 512 to 2097152. Cytotoxigenic activity was heat labile. There was no major difference between the CD50 of the parent strains (B1409, 45753-35, 933 and 932) and their 60-MDa plasmid-cured derivatives.

SDS-PAGE of cell lysates and OMP

The total cell lysate protein profiles of the four parent strains (B1409, 45753-35, 933, 932) were similar, with only minor differences (fig. 2, lanes 2, 4, 6 and 8). The profile of the plasmid-cured strain, B1409P-, (lane 3) showed an increase in concentration of proteins of approximately 49 kDa and 34 kDa (large

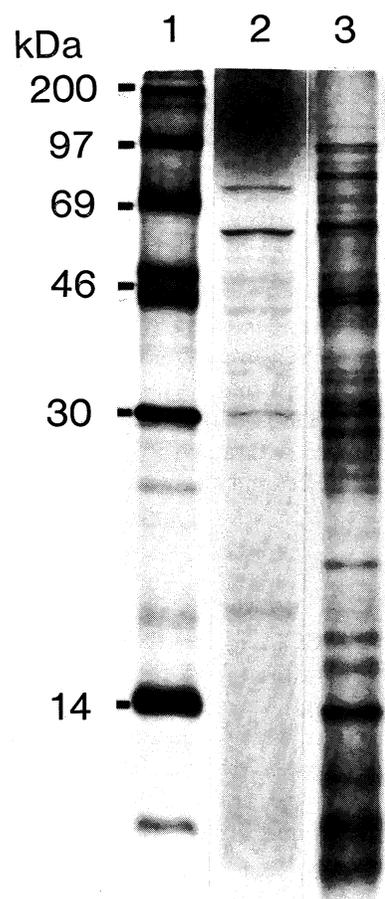


Fig. 1. Autoradiograph of SDS-polyacrylamide gel showing ³⁵S-methionine-labelled proteins encoded by plasmid DNA. Lane 1, mol. wt standards; 2, blank containing no plasmid DNA; 3, proteins encoded by 60-MDa plasmid DNA isolated from strain B1409.

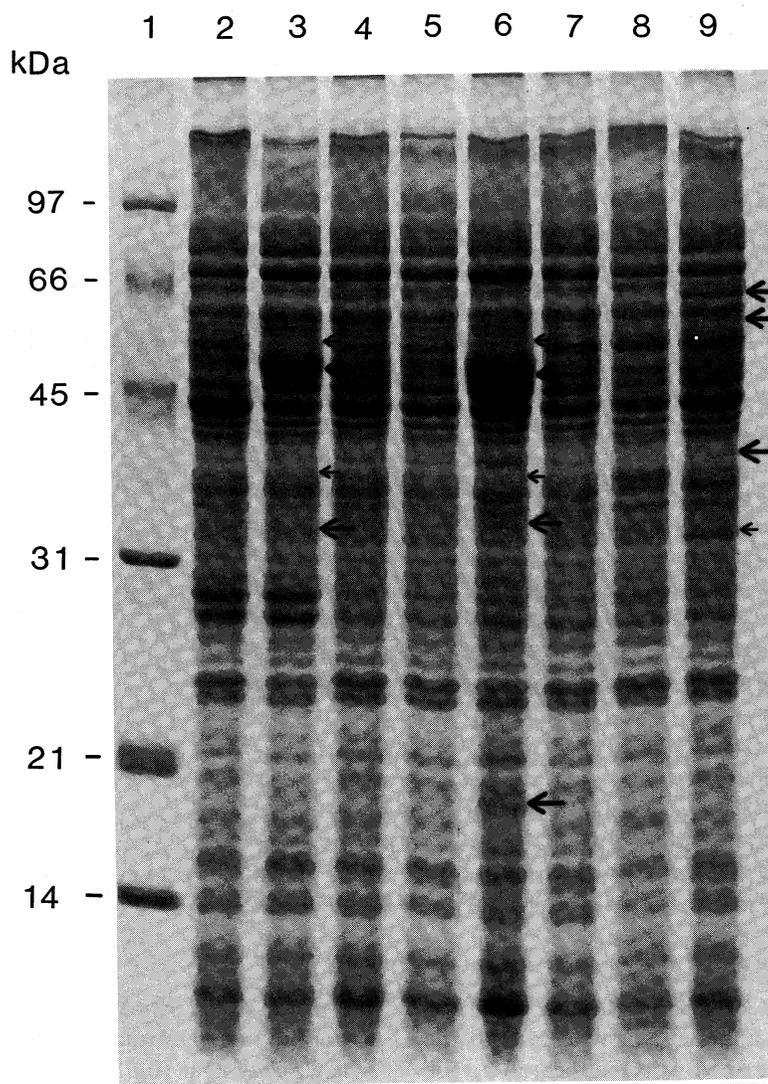


Fig. 2. SDS-polyacrylamide gel of total cell lysate protein stained with Coomassie Blue R-250; 60 μ g of protein was applied/lane. Lane 1, mol. wt standards; 2, *E. coli* B1409; 3, B1409P⁻; 4, 45753-35; 5, 45753-35P⁻; 6, 933; 7, 933P⁻; 8, 932; 9, 932P⁻ (P⁻, 60-MDa plasmid-cured derivatives); \leftarrow , protein bands which are increased in concentration; \leftarrow , bands which are decreased in concentration.

arrows) and a decrease in concentration of proteins of 53 kDa and 38 kDa (small arrows) when compared to the protein profile of the parent strain (lane 2). In contrast, parent strain 933 (lane 6) had higher levels of proteins of 49 kDa, 34 kDa and 18 kDa (large arrows) and lower levels of proteins of 53 kDa and 38 kDa (small arrows) when compared to strain 933P⁻ (lane 7). The profiles of strain 45753-35 (lane 4) and the cured derivative (lane 5) were similar. The profile of the plasmid-cured strain 932P⁻ (lane 9) showed protein bands of 64, 57 and 40-kDa (large arrows) which were not clearly visible for the parent strain, 932 (lane 8). A protein band at 32 kDa appears decreased in the profile of 932P⁻ (lane 9, small arrow).

The OMP profiles of the parent strains were similar (fig. 3, lanes 2, 4 and 6). However, the profiles of the cured derivatives (lanes 3, 5, 7) showed a significantly decreased level or disappearance of a 33-kDa protein when compared to the parent strain profiles. Lanes 9

and 10 show the profiles of *E. coli* K12 substrain C600 and K12 C600-LK3 (contains 60-MDa plasmid), respectively. There is a protein of approximately 33 kDa which is also absent or decreased in strain K12 C600 but is visible in the OMP profile of strain K12 C600-LK3.

Adherence assays

The adherence pattern of the parent and cured strains to HEP-2 and INT 407 cells was similar although overall adherence was greater with INT 407 cells than with HEP-2 cells. Adherence was scored as 4+ when approximately 80% of the intestinal cells or HEP-2 cells had adherent bacteria, 3+ when approximately 60% had adherent bacteria and 2+ and 1+ when approximately 30% and 10% of the cells had adherent bacteria, respectively. Adherence of strain 932P⁻ to both cell lines was somewhat greater than

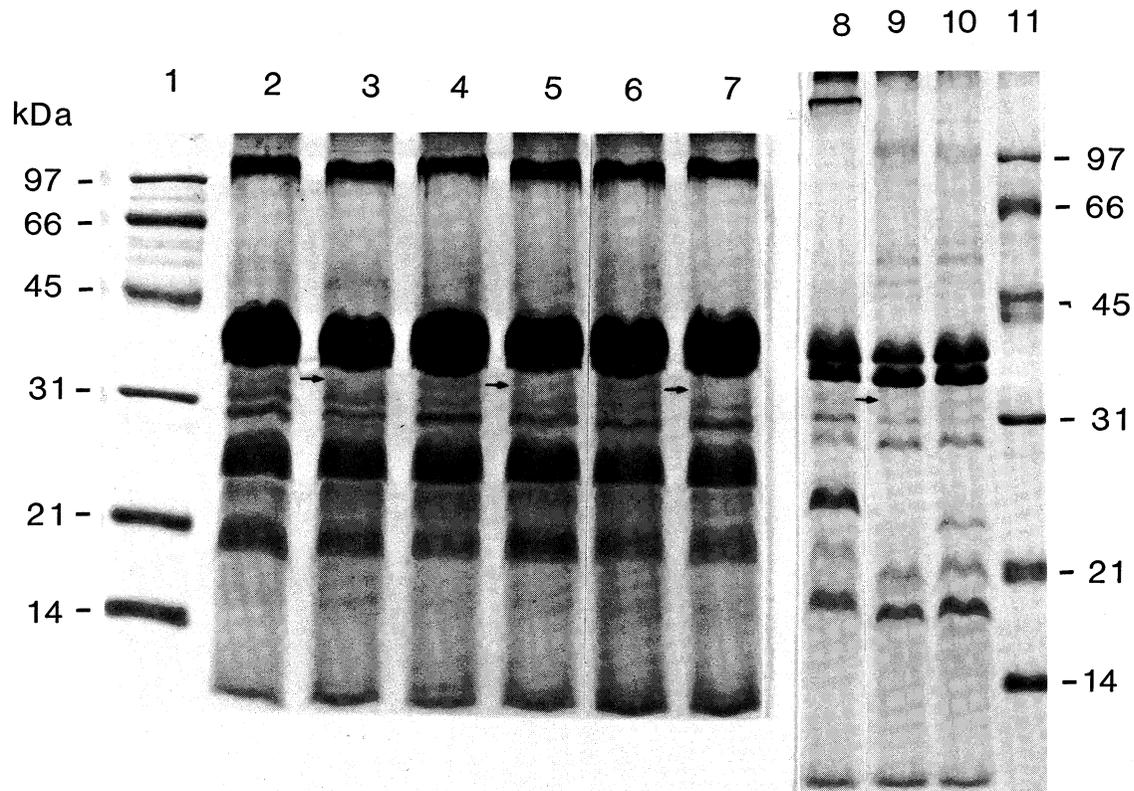


Fig. 3. SDS-polyacrylamide gel of OMP stained with Coomassie Blue R-250; 60 μ g of protein was applied/lane. Lane 1, mol. wt standards; 2, *E. coli* B1409; 3, B1409P-; 4, 45753-35; 5, 45753-35P-; 6, 932; 7, 932P-; 8, B1409; 9, K12 C600; 10, K12 C600-LK3; 11, mol. wt standards. Strain 933P- (not shown) also had a missing 33-kDa band. The arrows indicate the missing band.

adherence of the parent strain, 932 (table). All of the bacterial strains tested adhered as single cells to the monolayer and also formed what appeared to be microcolonies (fig. 4). The adherence patterns of strains K12 C600 and K12 C600-LK3 were similar. These strains adhered as individual cells and did not form microcolonies. Incubation in the presence of mannose 1% did not inhibit adherence of the plasmid-cured or parent strains and the bacteria remained firmly bound to the epithelial cells following washing with dithiothreitol (data not shown).

Visualisation of pili

Both the parent strains and their plasmid-cured derivatives expressed pili although their presence on the bacteria was sparse (fig. 5). Growth of the bacteria on MacConkey sorbitol agar or in liquid medium with or without agitation did not result in a change in the number of visible pili. Pili were not visualised by electronmicroscopy on strains K12 C600 or on K12 C600-LK3.

Production and analysis of exopolysaccharide

Strain B1409P- produced large amounts of EPS within 24-48 h when grown at 25°C on solid medium. There was no evidence of EPS when the organism was

grown at 37°C. Production of EPS was especially abundant when strain B1409P- was cultured on MacConkey agar (fig. 6). When the cultures were left at 25°C for several days, EPS covered the entire surface of the plates. Strain 933P- also produced EPS, but at somewhat lower levels than strain B1409P-. Strains 45753-35P-, 932P- and the four parent strains did not produce visible amounts of EPS even when incubation at 25°C was extended to several days. Three different B1409P- isolates were studied and all three produced visibly equivalent levels of EPS when cultured under the same conditions.

When colonies which had produced EPS were examined by scanning electronmicroscopy, a large amount of aggregated material and fibrous bundles that covered the entire surface of the B1409P- colony was visible (fig. 7a). Only bacteria and occasional fibrous bundles were visible in the B1409 colony (fig. 7b). The monosaccharide components of EPS produced by strain B1409P- were identified as fucose, glucose and galactose (approximate molar ratio of 2.0:0.9:1.1) by gas chromatography, based on their retention times when compared to the retention times of known standards. The EPS was shown to contain uronic acid 7% by a colorimetric assay.

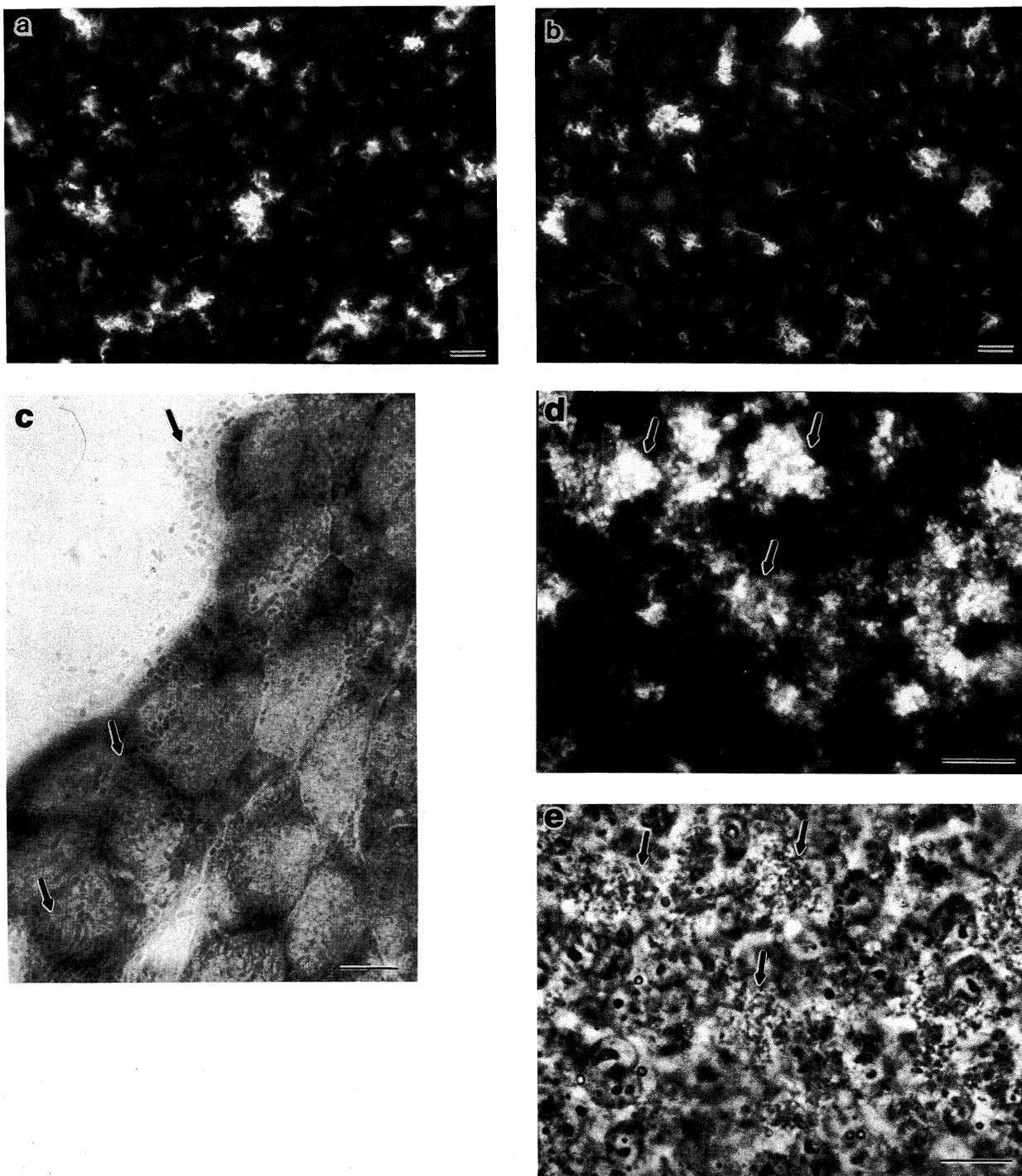


Fig. 4. Fluorescence (a, b, d) and light micrographs (c, e) showing INT 407 cells inoculated with *E. coli* B1409 (a), B1409 P–(b) and 932P–(c, d, e). A Giemsa-stained monolayer is shown in c; arrows indicate patches of adherent bacteria. Arrows in d (fluorescence micrograph) and in e (corresponding light micrograph) indicate microcolonies. a and b, bar = 10 μm ; c, d and e, bar = 5 μm .

Discussion

The function of the 60-MDa plasmid of *E. coli* O157:H7 strains was investigated. A definitive role in virulence for the plasmid was not elucidated in the present study; however, several differences were noted between the parent (60-MDa plasmid-bearing) and plasmid-cured strains. The OMP profiles suggest that plasmid DNA may encode or regulate the production of an OMP since the profiles of the plasmid-cured strains showed a disappearance or decreased pro-

duction of a 33-kDa OMP. The OMP was produced by strain K12 C600-LK3, but was not apparent in the profile of strain K12 C600 which does not possess the *E. coli* O157:H7 60-MDa plasmid. Enteropathogenic strains also possess a plasmid *c*, 60 MDa in size. The OMP profile of enteropathogenic strain E2348/69 serotype O127:H6 showed 94-kDa and 31-kDa proteins that were absent in the profile of the 60-MDa plasmid-minus derivative, MAR 20.³² Toth *et al.*³³ reported that antiserum against plasmid-specific proteins detected 82- and 92-kDa proteins in *E. coli*

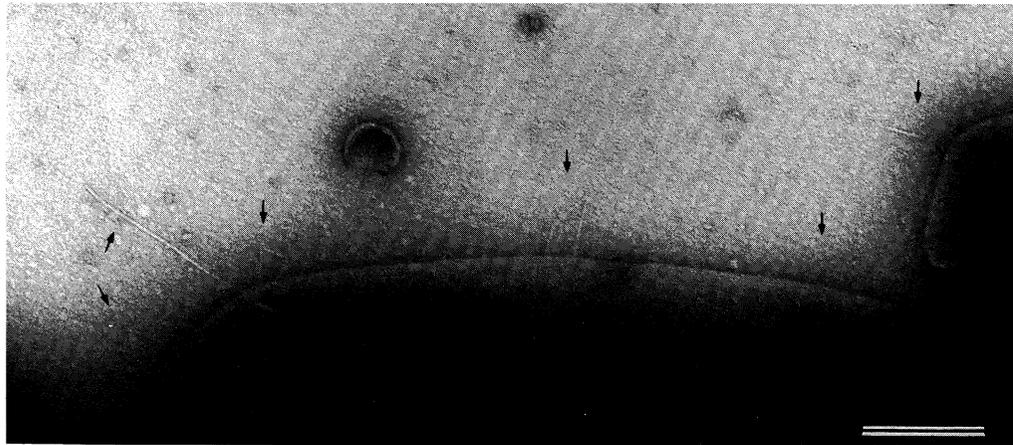


Fig. 5. Electronmicrograph of *E. coli* B1409P- demonstrating pili (→). Bar = 0.2 μm .

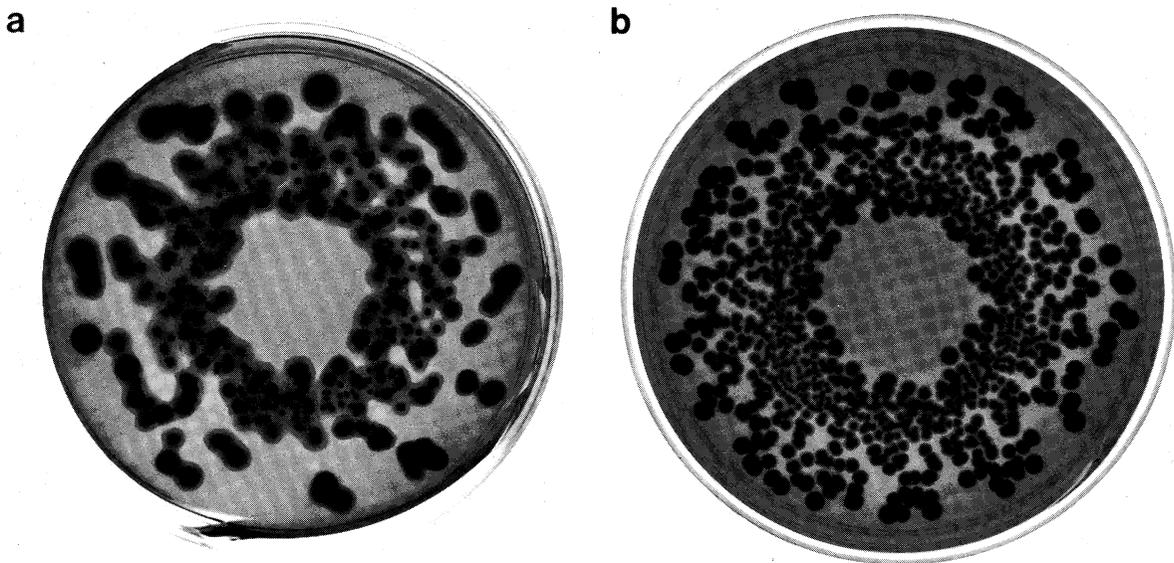


Fig. 6. Differential production of exopolysaccharide material by *E. coli* B1409P- (a) and B1409 (b) grown on MacConkey agar for 48 h at 25°C.

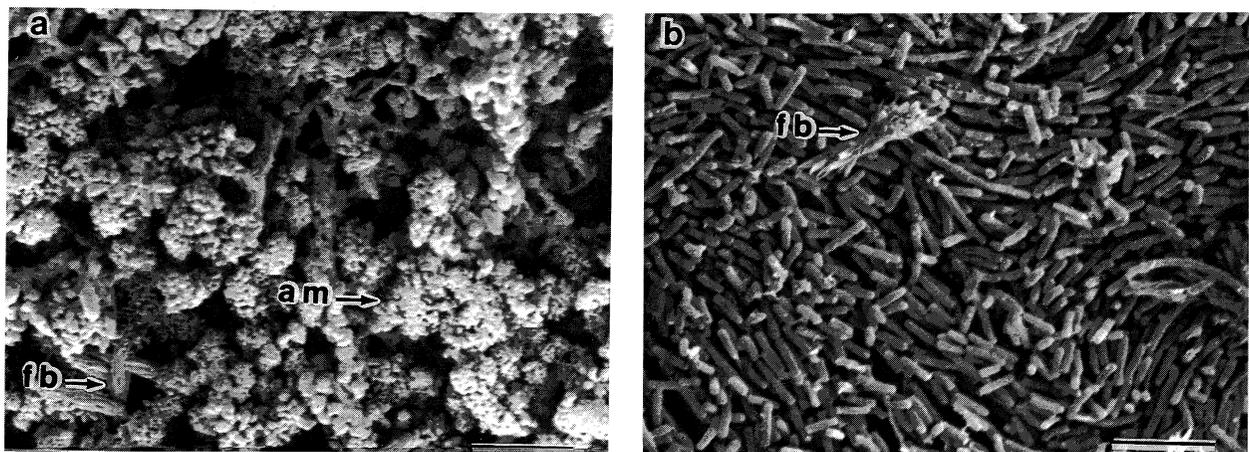


Fig. 7. Electronmicrographs of colonies of B1409P- (a) and B1409 (b) grown on MacConkey sorbitol agar for 48 h at 25°C; fb, fibrous bundles; am, aggregated material. Bar = 5 μm .

O157:H7 strains, but these proteins were not detected in plasmid-cured strains. Whether the two proteins were OMP is unclear. Results of in-vitro transcription and translation with plasmid DNA in the current

study showed that *c.* 35 proteins were encoded by plasmid DNA. SDS-PAGE analysis of the proteins revealed that there were proteins in the 33-kDa range and also in the 82–92-kDa range.

Sherman *et al.*²⁰ found that addition of antiserum against a 94-kDa OMP in adherence assays resulted in inhibition of attachment of *E. coli* to epithelial cells in tissue culture. It appears that the 33-kDa OMP is not involved in adherence to INT 407 and HEp-2 cells as plasmid-cured and parent strains adhered equally well. However, the possibility that the plasmid-cured strains produce low levels of the 33-kDa OMP that are still sufficient for adherence cannot be ruled out. Further research with *E. coli* O157:H7 OMP may include development of highly specific monoclonal or polyclonal antisera against the 33-kDa OMP that could be useful in assays for determining whether the isolates that lack the 60-MDa plasmid produce low levels of the protein.

SDS-PAGE analysis of cell lysate total protein profiles did not clearly distinguish plasmid-encoded proteins. There were differences in the total protein profiles of the parent and plasmid-cured strains, but there were no apparent differences that were consistent between each of the pairs. It is not clear why the protein profile of strain B1409P– was similar to that of strain 933, and strain B1409 had a profile similar to that of 933P–. There was an increase in production of proteins of approximately 49 (broad band) and 34 kDa in both strains B1409P– and 933.

The production of EPS by *E. coli* O157:H7 strains has been reported.³⁴ The EPS was described as a compound similar to colanic acid and it was produced most vigorously aerobically at room temperature mainly by plasmid-cured strains, including strain 932P (lacks 60-mDa plasmid). In the present study strain B1409P–, and to a lesser extent strain 933P–, produced EPS when they were cultured on solid medium at 25°C, whereas strains 45753-35P–, 932P– and the parent strains did not produce visible amounts of EPS under the conditions tested. Strains B1409P– and 933P– also produced EPS on solid medium in the absence of added sugars. Production of EPS does not appear to be the result of a random gene mutation since all three B1409P– isolates tested produced similar levels of EPS on all of the solid media used. The composition of the EPS is similar to that of an acidic polysaccharide produced by *E. coli* O8:K27(A):H– which consisted of D-glucuronic acid, D-glucose, D-galactose and L-fucose in equimolar ratio³⁵ and is also similar to the EPS described by Junkins and Doyle.³⁴

The role of bacterial EPS is not well understood, but some possible functions have been suggested.³⁶ These include protection against desiccation, against phagocytosis or against bacteriophages. EPS may also have a role in allowing increased absorption of nutrients, ions and iron, and it may have a protective role in the 60-MDa plasmid-cured strains. It was produced only when the strains were grown on solid medium and not when they were cultured in liquid medium. This would suggest that the EPS may function as a protection against desiccation on solid medium. Grant *et al.*³⁷ found that strains from the

genera *Salmonella*, *Escherichia* and *Aerobacter* produced an EPS which was identical in composition. It contained fucose, galactose, glucose and glucuronic acid in a molar ratio of 2:2:1:1. The EPS was identified as colanic acid. Some of the strains studied produced colanic acid only when grown in the presence of *p*-fluorophenylalanine which, when incorporated into certain proteins, alters their activity. It was suggested that the compound is incorporated into a protein which normally represses the production of colanic acid. Therefore, with an inactive repressor protein, colanic acid could be synthesised. In the current study, only strains that lacked the 60-MDa plasmid produced EPS. Thus, it is possible that the plasmid codes for a repressor protein responsible for inhibiting the production of EPS. It is not clear why strains 45753-35P– and 932P– did not produce visible amounts of EPS. However, these strains possess a 4.5-MDa plasmid which is not present in strains B1409P– or 933P–. Whether there is any interaction between proteins encoded by the 4.5-MDa plasmid and those encoded by the 60-MDa plasmid DNA remains to be determined.

Studies have shown that virtually all *E. coli* O157:H7 strains possess a plasmid of *c.* 60-MDa and a high percentage of the strains also possess one or more smaller plasmids.^{15,38–40} All the strains in the present study possessed the 60-MDa plasmid; 12 (71%) of the 17 strains also possessed one or more smaller plasmids. Four plasmid profile patterns were observed among all the strains. It is not clear why the strains were cured of the 60-MDa plasmid but retained the smaller plasmids. It was observed that not all the *E. coli* strains could be cured readily of the 60-MDa plasmid. Although both strains contain only the 60-MDa plasmid, strain B1409 lost the plasmid after several transfers in medium containing ethidium bromide, whereas we were repeatedly unsuccessful in curing strain A9124-1.

The 60-MDa plasmid does not appear to be essential for adherence to INT 407 or to HEp-2 cells in culture since the plasmid-cured and parent strains adhered in a similar manner and equally well to the epithelial cells. Adherence was mannose-resistant. There was also no difference in the level of adherence between strains K12 C600 and K12 C600-LK3. Strain 932P– adhered better to the epithelial cells than the parent strain, 932 (table). These results are in agreement with those of Junkins and Doyle¹⁸ who also found that strain 932P– had a greater adherent ability than strain 932.

Not all *E. coli* O157:H7 strains have been found to be capable of adherence to INT 407 and HEp-2 cells. Dorn and Angrick⁴¹ found that only 20% of bovine strains and 33% of meat (beef) strains tested adhered to cells in culture. There was no correlation between adherence and the presence of the 60-MDa plasmid as evidenced by hybridisation with the CVD419 DNA probe.^{41,42} This probe hybridises with the 60-MDa plasmid of *E. coli* O157:H7 strains and it also

hybridises with many other verotoxigenic *E. coli* including serotype O26:H11 which is also considered EHEC.⁴³ Wadolowski *et al.*⁴⁴ found that 933cu-rev, a plasmid-cured derivative whose virulence was apparently augmented through inoculation in mice, adhered at higher levels to mouse intestine than the parent strain.

Although few in number, pili were visible on both cured and parent strains. Both strain K12 C600 and strain K12 C600-LK3 were non-pilate. Therefore, these results show that the plasmid is not involved in the production of pili. This is in agreement with studies by Toth *et al.*¹⁶ who demonstrated that there was no correlation between the presence of the 60-MDa plasmid and the production of pili. Since all of the *E.*

coli O157:H7 strains were pilate, these adhesins may be involved in adherence. It is not clear why the bacteria were so sparsely pilate; however, it is possible that after repeated subculture *E. coli* lose the capacity to produce pili abundantly.⁴⁵

In summary, our findings are in agreement with other studies which have demonstrated that adherence of *E. coli* O157:H7 does not correlate with the presence of the 60-MDa plasmid. However, the plasmid may be involved in regulating the production of EPS and involved in the synthesis of a 33-kDa OMP, the function of which will require future research.

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