Attaching and effacing lesions in vivo and adhesion to tissue culture cells of Vero-cytotoxin-producing *Escherichia coli* belonging to serogroups O5 and O103

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Certain isolates of *Escherichia coli* from humans and animals with enteric disease attach to enterocytes and cause 'attaching and effacing' (AE) lesions. *E. coli* strain S22-1, serotype O103 : H2, isolated from a child with diarrhoea, contained two plasmids; one of these (pDEP12) hybridized with the CVD419 DNA probe derived from a plasmid found in *E. coli* O157 : H7 and associated with expression of fimbriae and ability to adhere to Intestine 407 cells. Strain S102-9, serotype O5 : H-, isolated from a calf with dysentery, contained six plasmids, one of which also hybridized with the CVD419 probe. Loss of pDEP12 coincided with reduced adhesion to HEp-2 or Intestine 407 cells cultured in vitro; reintroduction of this plasmid restored adhesiveness. Loss of the plasmid in strain S102-9 that hybridized with the CVD419 probe did not cause a decrease in adhesion. Accumulations of actin were seen in vitro in the fluorescence actin staining (FAS) test of strains S22-1, S102-9 and their derivatives, irrespective of the plasmid content of these strains or the prevalence of attached bacteria. Strain S22-1 and its plasmidless derivative caused AE lesions of equal severity in experimentally infected gnotobiotic piglets; piglets inoculated with an isolate from a healthy human or pig did not develop these lesions. These results indicate that the CVD419 probe is not specific for genes conferring the ability to adhere to HEp-2 or Intestine 407 cells by these *E. coli* and that the adhesins detected in vitro, or plasmid-encoded properties, are not required for strain S22-1 to cause AE lesions in gnotobiotic pigs or to cause the accumulation of actin in cells in vitro.

**Introduction**

Certain isolates of *Escherichia coli* from human and animal disease produce cytotoxins which act on Vero cells (Konowalchuk et al., 1977); they produce one or two distinct Vero cytotoxins, VT1 and VT2 (Scotland et al., 1985b), also called SLT I and SLT II (Strockbine et al., 1986). Some *E. coli* strains, including Vero-cytotoxin-producing *E. coli* (VTEC) attach to the apical surfaces of enterocytes in their natural host and in gnotobiotic pigs where they produce 'attaching and effacing' (AE) lesions (Moon et al., 1983; Tzipori et al., 1986; Hall et al., 1988).

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Abbreviations: AE, attaching and effacing; EAF, EPEC adherence factor; EPEC, enteropathogenic *E. coli*; FAS, fluorescence actin staining; SLT, Shiga-like toxin; VT, Vero cytotoxin; VTEC, Vero-cytotoxin-producing *E. coli*.

they also possess attachment mechanisms detectable by in vitro tests (Karch et al., 1987; Sherman et al., 1987; Dorn et al., 1989). Serotypes of particular interest which possess these characteristics are O157 : H7, the predominant isolate in human outbreaks of haemorrhagic colitis (Riley et al., 1983; Pai et al., 1984; Smith et al., 1987), and serotype O5 : H-, isolated from calves with dysentery (Chanter et al., 1984; Hall et al., 1985; Moxley & Francis, 1986).

The toxins are not the cause of AE lesions (Tzipori et al., 1987; Hall et al., 1988), although they are thought to be involved in the pathogenesis of disease (Fontaine et al., 1988); the role of attachment mechanisms detectable in vitro is not clear. The O5 : H- calf strain (S102-9) and three human VTEC strains (serotypes O55 : H7, O103 : H2 and O111 : ac : H1) possess the ability to adhere to HEp-2 cells and Intestine 407 cells in vitro (Dorn et al., 1989). These strains did not hybridize with the entero-pathogenic *E. coli* (EPEC) adherence factor (EAF) DNA.
Methods

Bacterial strains. Strain S22-1, serotype O103:H2, was isolated from a child hospitalized with diarrhoea in Canada (Gurwith & Williams, 1977). Strain S102-9, serotype O157:H7, was isolated from a calf with enteritis from a herd with this illness in England (Chanter et al., 1984). Strain E1541/68, serotype O154:H4, was isolated from a healthy human and strain E53181, serotype O8:H9, was isolated from a healthy pig; both were used as controls for in vivo and in vitro experiments. Additional information about the properties of these four wild-type strains is provided in Table 1.

S22-1 carries two plasmids designated pDEP12 and pDEP13. Derivatives lacking pDEP12, pDEP13 or both plasmids, as listed in Table 1, were obtained. Strain S22-1-5B was isolated following heat inactivation of the plasmid pDEP12 resulting from a 46 MDa plasmid that hybridized with the CVD419 probe; an isolate lacking this plasmid was designated S22-1-5B.

Plasmid DNA preparation, transformation, agarose gel electrophoresis and Southern hybridization. Plasmid DNA was prepared by the alkaline extraction method (Birnboim & Doly, 1979). Purified plasmid DNA of pDEP12: Tn801 was prepared by the alkaline-SDS lysis method followed by centrifugation in cesium chloride/ethidium bromide density gradients. Transformation with plasmid DNA was done by the method of Kushner (1978). The plasmid DNA extracts were run on 0.6% (w/v) agarose gels in TB buffer containing 89 mM-Tris base, 89 mM-boric acid and 2.5 mM-EDTA (Willshaw et al., 1979). Each strain was run at least twice. Each observed plasmid band was assigned a corresponding molecular mass in MDa using seven molecular mass standards. After treatment of gels with 0.25% HCl, plasmid DNA was transferred to Hybond N membrane by the method of Southern (1975) and hybridized with the CVD419 DNA probe as described above.

HEp-2 cell and Intestine 407 cell adherence. The wild-type strains and their derivative strains were tested for ability to adhere to HEp-2 cells and Intestine 407 cells in the presence of D-mannose by the method of Chanter et al. (1985) using a 6 h incubation period. Bacterial adherence to these cells was classified according to Scalesky et al. (1984). Localized adherence was observed and recorded as the percentage of HEp-2 or Intestine 407 cells with 10 or more adherent bacteria. Strains which adhered to less than 10% of the HEp-2 or Intestine 407 cells were considered nonadherent for that cell line.

Fluorescence actin staining (FAS) test. At the end of a 6 h adherence test HEp-2 cells were fixed in formalin (3%, v/v), washed, and treated to show accumulations of filamentous actin beneath adherent bacteria using the FAS test (Knutton et al., 1989). Briefly, cells were permeabilized with Triton X-100 (0.1% in phosphate-buffered saline [PBS, NaCl, 8.0 g l⁻¹; KCl, 0.2 g l⁻¹; Na₂HPO₄, 1.5 g l⁻¹; KH₂PO₄, 0.2 g l⁻¹]), and stained with fluorescein isothiocyanate-phalolini (5 µg ml⁻¹ in PBS; Sigma). The same stained cell monolayer was used to visualize adherent bacteria by phase contrast microscopy and concentrations of actin by incident light fluorescence.

Animals. Sixteen gnotobiotic piglets were obtained from two litters (Tavernor et al., 1971). At 3-d-old they were inoculated orally after the morning feed with 5 ml sterilized canned milk (Carnation) containing 9.6 × 10⁶ to 5.6 × 10⁷ c.f.u. of E. coli grown on bovine blood agar at 37°C for 18 h; piglets were inspected daily for signs of diarrhoea and killed 4 d later. The pathogenicity of each strain (S22-1, 62R75, E1541/68, E53181) was assessed by inoculating two piglets from each litter.

Bacteriology. To monitor for bacterial contaminants in gnotobiotic piglets’ faeces were collected prior to inoculation and at necropsy and cultured aerobically and anaerobically on blood agar and in thioglycolate broth. Two piglets inoculated with strain S22-1 were contaminated with a Streptococcus sp. at the time of necropsy. E. coli were enumerated as described previously (Chanter et al., 1984) in scrapings of mucus from the ileum, caecum, colon and rectum, in faeces, and in intestinal contents. The mucus was washed, scraped off with a glass slide and ground to make 10⁻¹ suspensions in saline (0.85%; NaCl). Intestinal contents, faeces or mucosal suspensions (1 g) were serially diluted tenfold in saline and 0.1 ml of appropriate dilutions spread onto MacConkey agar plates (Mackie & McCartney, 1953) in triplicate
before incubation at 37 °C for 18 h. The numbers of E. coli per gram were enumerated according to the dilution of sample and the number of lactose-fermenting colonies per sample.

Necropsy procedures. Tissues for microscopy were removed under halothane anaesthesia (Fluothane, ICI). Short lengths (approximately 5 cm) of small and large intestine were ligated to produce pairs of loops at three sites; adjacent to the ileocaecal junction (lower ileum) and in the proximal and distal halves of the spiral colon. One loop of each pair was filled with mercuroformal and the other with 3% (v/v) glutaraldehyde in 0.1 M-sodium phosphate buffer. Samples from the blind end of the caecum and from the mid rectum were excised under anaesthesia and immersed in fixative. Piglets were killed by intracardiac injection of pentobarbitone sodium (Euthatal; RMB Animal Health). Ligated loops of intestine were removed and immersed in fixative. Brains were removed and immersed in mercuroformal together with samples of liver and kidney. Samples of intestinal walls and contents were saved for bacteriological examination. Fixed tissues were processed and examined for the presence of lesions by light microscopy and transmission electron microscopy to confirm the nature of lesions detected by light and scanning electron microscopy.

Results

Relationship between plasmids and adherence to cells in vitro

Strain S22-1 possessed two plasmids, pDEP12 and pDEP13, of which pDEP12 (44 MDa) hybridized with the CVD419 DNA probe; strain S102-9 possessed six plasmids, one of which (46 MDa) also hybridized with the CVD419 probe (Table 1); both isolates were adherent to cells in vitro. Loss of the CVD419-hybridizing plasmid (pDEP12) from S22-1 coincided with reduction in the ability of derivatives S22-1-5B, 62R64 and 62R75 to adhere to cells in vitro. Transformation of pDEP12 into the plasmidless derivative 62R75 produced a further derivative, 62R140, in which adherence to cells in vitro was restored. The derivative (62R70) which had lost pDEP13 but retained pDEP12 was still adherent. The derivative of S102-9, which lacked the CVD-419-hybridizing plasmid, was, however, as adherent as the parent strain or strain S22-1. Strains S22-1 and S102-9 were positive in the FAS test. All localized clusters of bacteria were associated with intense spots of fluorescence of the HEp-2 cells. Although these areas were related to attached bacteria, not every adherent bacterium was associated with fluorescence. Similar results were obtained with some strains by Knutton et al. (1989). All the derivatives of S22-1 and S102-9, even those which gave only rare clusters of adherent bacteria, were similarly positive in the FAS test and caused distinct concentrations of fluorescence (Table 1).

The control strains E1541/68 and E53181 possessed plasmids which did not hybridize with the CVD419 probe; these strains did not adhere to cells in vitro nor did they react in the FAS test.

Clinical observations

Liquid faeces were passed by piglets inoculated with strain 62R75 and at necropsy the perineum of these piglets was soiled with adherent faeces (Table 1). The faeces of piglets inoculated with strain 62R75 were soft or very soft, although they were liquid in one piglet on the third

### Table 1. Properties of the strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Plasmids (MDa)*</th>
<th>DNA probes</th>
<th>Adherence tests</th>
<th>Effects on gnotobiotic piglets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>VTI CVD419 HEp-2† Intestine 40′† FAS‡</td>
<td>Diarrhoea</td>
<td>Colonization</td>
</tr>
<tr>
<td>S22-1</td>
<td>O103: H2</td>
<td>pDEP13, pDEP12</td>
<td>+</td>
<td>22</td>
<td>+</td>
</tr>
<tr>
<td>S22-1-5B</td>
<td>O103: H2</td>
<td>pDEP13</td>
<td>-</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>62R64</td>
<td>O103: H2</td>
<td>pDEP13</td>
<td>+</td>
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<td>NT</td>
</tr>
<tr>
<td>62R70</td>
<td>O103: H2</td>
<td>pDEP12</td>
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<td>NT</td>
<td>NT</td>
</tr>
<tr>
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<td>NT</td>
</tr>
<tr>
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<td>+</td>
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<td>NT</td>
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<tr>
<td>S102-9</td>
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<td>NT</td>
<td>NT</td>
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<td>62R15</td>
<td>O5: H+</td>
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<tr>
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<td>-</td>
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</tr>
<tr>
<td>E53181</td>
<td>O8: H9</td>
<td>None</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT, Not tested.

* pDEP13, 51 MDa; pDEP12, 44 MDa. Other plasmids are identified according to their molecular mass; plasmids which hybridize with the CVD419 probe are in **bold** type.

† Number of cells with 10 or more locally adherent bacteria per 100 cells; all tests were done at least twice.

‡ For a positive test, 10% or more of 200 cells had 10 or more locally adherent bacteria that were associated with concentrations of fluorescence; all tests were done at least twice.
and fourth days after inoculation. The faeces of piglets inoculated with strains E1541 (human commensal) and E53181 (pig commensal) were soft, except that two piglets inoculated with E53181 passed liquid faeces on the third and fourth days after inoculation. One piglet from the first litter, inoculated with S22-1 and contaminated with a *Streptococcus* sp., collapsed in lateral recumbency and developed paddling movement of all limbs immediately before necropsy. A piglet inoculated with 62R75 died during the night and was discarded from the study because the intestinal tissues were autolysed.

**Gross pathology**

Gross lesions were not detected. Watery contents were noted in the large intestines of piglets inoculated with strain S22-1. In the piglet which collapsed in lateral recumbency prior to necropsy, the renal pelvices were dilated and contained white floccular material and there were yellow foci, 0.5 mm in diameter, in the renal papillae. The brain and spinal cord of this pig were coloured pale yellow.

**Light microscopy**

Lesions were not detected in the kidneys, small intestines or brains of any piglet except that the meninges of the piglet contaminated with a *Streptococcus* sp., which was found in lateral recumbency prior to necropsy, were infiltrated markedly with neutrophils. Lesions were detected in the livers of eight piglets. These were not correlated with inoculation of any particular strain of *E. coli* because they were detected in at least one piglet from each group. Hepatocytes in the centres of the lobules were swollen, the cytoplasm was rarefied and vacuolated and nuclei were enlarged; increased numbers of hepatocytes in mitosis were detected.

Lesions were detected in the large intestines of all piglets inoculated with strains S22-1 and 62R75 (Table 1); the caecum and ascending and descending colon were always affected but lesions were not always detected in the rectum. Enterocytes located midway between crypt orifices were infected most frequently with bacteria; infection occasionally extended into crypt orifices and rarely to the bottom of crypts. The epithelium was damaged at the sites of infection and enterocytes, many

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**Fig. 1.** Scanning electron micrograph of the colonic mucosa of a piglet inoculated with the plasmidless *E. coli* strain 62R75. The surface is extensively colonized (triple arrows) between crypt orifices (double arrows). Orifices of goblet cells are visible (single arrow). Bar, 50 μm.
Colonization and adhesion of VTEC

Fig. 2. Scanning electron micrograph of the caecal mucosa of a piglet inoculated with E. coli strain S22-1. Microvilli are effaced on infected cells and a bacterium is visible by a pedestal (arrow). Bar, 1 μm.

which were sloughed, were swollen and contained intracellular lipid; nuclei were irregularly shaped and often swollen. Foci of neutrophil infiltration were seen in the lamina propria and occasionally in the epithelium, the neutrophils having apparently emigrated through the epithelium. Lesions were not detected in the large intestines of piglets inoculated with strains E1541/68 and E53181 (Table 1).

Scanning electron microscopy

Lesions were not seen in the small intestines of any piglet. They were detected in the large intestines of all piglets inoculated with strains S22-1 and 62R75, but not in piglets inoculated with other strains. The enterocytes located between crypt orifices were infected most frequently (Fig. 1) and abnormal microvilli were seen on these infected cells. Tufts of abnormally long microvilli occurred on some cells whereas they were absent from other cells (Fig. 2). Many enterocytes appeared to be sloughing. Bacteria were often located in depressions on the enterocyte surface and occasionally on cytoplasmic pedestals (Fig. 2).

Transmission electron microscopy

Transmission electron microscopy of the large intestines of piglets inoculated with strains S22-1 and 62R75 revealed close apposition between bacterial and enterocyte surfaces and effacement of microvilli (Fig. 3); bacteria were frequently located in cup-shaped depressions on the surface but cytoplasmic pedestals were not seen frequently by transmission electron microscopy.

Discussion

The loss of pDEP12 from strain S22-1 correlated with the reduction in adherence to both HEp-2 cells and to Intestine 407 cells. This plasmid hybridized with the CVD419 DNA probe, so the observation is consistent with the finding of Karch et al. (1987) that curing of a 60 MDa plasmid, which also hybridized with the CVD419 probe, correlated with loss of adherence to Intestine 407 cells.

The other VTEC examined in this study, strain S102-9, did not lose its ability to adhere to HEp-2 cells
and Intestine 407 cells when it lost the 46 MDa plasmid which hybridized with the CVD419 probe. The inconsistency of these observations, that strain S102-9 does not have adherence-encoding genes on the CVD-419-hybridizing plasmid, indicates that the genetic basis for adherence to epithelial cells in tissue culture for some non-O157:H7 VT-producing E. coli may be different from that reported for strains of serotype O157:H7 (Karch et al., 1987). The inability of serotype O157:H7 strains to adhere to HEp-2 cells (Karch et al., 1987) also differs from observations that VTEC strains of serogroups O5, O55, O103 and O111 have the ability to adhere to both HEp-2 cells and to Intestine 407 cells (Dorn et al., 1989). Future investigations may reveal involvement of outer-membrane proteins, capsule or other cellular components in the adherence mechanisms of VTEC.

Knutton et al. (1989) have proposed that the in vitro FAS test can be used to identify strains of E. coli that are able to cause AE lesions in intestinal mucosa. They showed that EPEC and VTEC, for example strains of serogroup O157, were both positive in the FAS test. The two VTEC in the present study, S22-1 and S102-9, were positive in the FAS test and the ability of strain S22-1 to cause AE lesions was confirmed in the gnotobiotic piglet.

Although the FAS test is dependent on initial attachment of a strain to tissue culture cells, Knutton et al. (1989) showed that fluorescence can be clearly demonstrated even when the proportion of cells with localized attaching bacteria is very small. This was confirmed in the present work with derivatives that had reduced attaching ability, due to the loss of a plasmid, but were still positive in the FAS test. Loss of a 60 MDa plasmid, pMAR2, from the enteropathogenic strain E2348/69 also caused a decrease in attachment (Knutton et al., 1987; McConnell et al., 1989) but did not cause a loss of the ability of the strain to cause fluorescence in the FAS test (Knutton et al., 1989). This may indicate another adhesive mechanism where the frequency of occurrence of bacterial and/or cell culture receptors is low in vitro. However, the loss of the plasmid pMAR2 was accompanied by a decrease in the ability to cause diarrhoea in adult volunteers compared to the parent strain E2348/69 (Levine et al., 1985).
Gnotobiotic piglets inoculated with *E. coli* of serotype O103:H2 (strain S22-1), isolated from a child with diarrhoea, produced liquid faeces, whilst diarrhoea was not seen consistently in piglets inoculated with the plasmidless variant. This may have been the result of studying a small group of piglets, and killing them early in the disease process before diarrhoea developed. It was not due to differences in the ability of the two strains to infect the colon and cause lesions, because both strains colonized the surface of the large intestine equally well and caused lesions identical to AE lesions described previously in piglets inoculated with enterohaemorrhagic *E. coli* of serotype O157:H7 (Tzipori et al., 1986) and VTEC of other serogroups (Tzipori et al., 1989).

In piglets inoculated with VTEC, the severity of disease and distribution of infection and lesions was variable (Tzipori et al., 1987, 1989). Infection and lesions were usually absent from the small intestine and extensive in the large intestine, where there was little or no inflammation. EPEC also caused AE lesions but the distribution of infection was slightly different and inflammatory changes more prominent (Tzipori et al., 1989). *E. coli* which cause AE lesions in gnotobiotic piglets may do so by disrupting and modifying the cytoskeleton and epithelial cell membrane (Knutton et al., 1987) so that bacteria appear to be attached in cup-shaped depressions on the enterocyte surface or on cytoplasmic pedestals or more complex cytoplasmic protrusions (Moon et al., 1983; Tzipori et al., 1986; Hall et al., 1988). In this study, bacteria were detected in cup-shaped depressions on the enterocyte surface but disruption of the enterocyte surface into pedestals or more complex protrusions was uncommon and seen less frequently than in previous studies of *E. coli* S102-9 in gnotobiotic calves and piglets (Hall et al., 1985, 1988); the reason for these differences is unclear.

Lesions were detected in the livers of several piglets, but they were not caused by any particular strain because at least one abnormal liver was detected in each experimental group. The cause of the liver lesions was not investigated, but they could have been the result of endotoxin liberated by the large numbers of Gram-negative bacteria in the intestines or because the piglets were exposed to halothane for approximately 30 min prior to death. One piglet inoculated with strain S22-1 developed clinical signs suggestive of central nervous system pathology and a purulent meningitis was detected *post mortem*; this piglet had apparently developed streptococcal meningitis immediately prior to necropsy.

The results of inoculating these piglets indicate that the genes encoding for factors involved in the development of AE lesions are located in the chromosome of strain S22-1. The present results, obtained using *E. coli* of serotype O103:H2, are in agreement with earlier obser-

vations of piglets inoculated with *E. coli* of serotype O155:H7 (Tzipori et al., 1987, 1989). These earlier studies, which had shown that a particular 60 MDa plasmid which hybridized with the CVD419 DNA probe was not essential for expression of AE lesions in gnotobiotic pigs, did not comment on other plasmids which might encode factors involved in the development of AE lesions. The present study confirms that a plasmid which hybridized with the CVD419 DNA probe was not required for production of AE lesions and shows additionally that the lesions were produced by the plasmid-free derivative.

A comparison of the results of the in vitro adhesion tests with those from inoculating piglets shows that the adhesion test gave no guide to pathogenicity. The derivative of S22-1 which lacked pDEP12 and adhered poorly to HEp-2 cells or Intestine 407 cells was capable of inducing actin accumulation in vitro, colonizing the porcine large intestine and causing AE lesions.

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References


