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Virulence of an *Escherichia coli* O157:H7 Sorbitol-Positive Mutant

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Virulence and pathogenicity of an *Escherichia coli* O157:H7 sorbitol-positive mutant were investigated with an infant rabbit animal model as well as a battery of in vitro assays. Total cell lysate protein profiles, outer membrane protein profiles, plasmid profiles, and levels of cytotoxic activity against Vero cells were similar in the wild-type and mutant strains. Both adhered to intestinal epithelial cells in culture and reacted with fluorescein isothiocyanate-labeled antiserum against *E. coli* O157:H7. The mutant appeared to be similar to the wild type in all respects except in its ability to ferment sorbitol. [^{14}C]sorbitol uptake and sorbitol-6-phosphate dehydrogenase activities were notably increased in the mutant strain. Diarrhea developed in rabbits administered the wild-type strain and in those fed the sorbitol-positive mutant. There was greater bacterial attachment and mucosal damage in the cecum and large intestine than in the small intestine. Scanning electron microscopy revealed bacteria adhering as single cells and as aggregates closely associated with mucus. Mucosal lesions consisted of areas of tissue necrosis with sloughing of epithelial cells. By transmission electron microscopy, electron-dense necrotic epithelial cells were visible in areas where bacteria were present, and epithelial cell debris containing bacteria was observed between the villar luminal surfaces. Light microscopy of epithelial cells of intestinal sections of infected rabbits revealed noticeable vacuolation and spherical, pyknotic nuclei. These data indicate that the sorbitol-negative phenotype is not associated with the pathogenicity of *E. coli* O157:H7.

Enterohemorrhagic *Escherichia coli* (EHEC), particularly serotypes O157:H7 and O157:NM (nonmotile), is a major cause of food-borne illness in the United States, Canada, and Europe (3, 5, 6, 20, 29, 33, 35). Unlike most strains of *E. coli*, typical clinical isolates of *E. coli* O157:H7 and *E. coli* O157:NM do not ferment sorbitol within 24 h (29, 30), lack β-glucuronidase activity (8, 29, 30), and do not grow at 45.5°C (8). Many screening methods used for the isolation of EHEC from food products and stool specimens utilize sorbitol MacConkey agar (SMA) as the primary isolation medium (5, 10, 18, 21, 22, 25). Colonies which are sorbitol negative are selected and confirmed as *E. coli* O157:H7 by biochemical, serological, and Vero cell cytotoxicity assays.

It appears that some strains of *E. coli* O157:H7 and O157:NM will ferment sorbitol within 24 h or mutate to a sorbitol-positive phenotype under certain conditions (10). Gunzer et al. (16) recently reported that 17 of 44 strains of *E. coli* O157 isolated from patients with diarrhea or hemolytic-uremic syndrome (sequela of hemorrhagic colitis) fermented sorbitol within 24 h of incubation. Bitzan et al. (2) isolated sorbitol-fermenting *E. coli* O157:H$^-$ from stools of children in Central Europe with hemolytic-uremic syndrome. No research has been performed to determine whether a sorbitol-negative phenotype is related to the pathogenicity of the serovar. Potentially, pathogenic sorbitol-positive variants would not be isolated with protocols that rely on the use of sorbitol-containing medium.

Sorbitol is widely used in food manufacture in the United States. Some common food applications of sorbitol include its use as a sweetener, humectant, and cryoprotectant in soft ice cream and in fish products (31) and in cured pork products to reduce charring (11). Although *E. coli* O157:H7 is mainly associated with consumption of contaminated ground beef, the organism has also been isolated from pork and from other types of fresh meats and poultry (9). If food products containing sorbitol become contaminated with *E. coli* O157:H7, the microorganism may mutate to the sorbitol-positive phenotype.

The present study describes the production of an *E. coli* O157:H7 sorbitol-positive mutant and assesses the significance of this phenotype in relation to virulence of the organism. The study included an array of in vitro tests and use of an experimental *E. coli* O157:H7 infant rabbit infection model to study virulence.

MATERIALS AND METHODS

Production of sorbitol-positive mutant. *E. coli* O157:H7, strain A9124-1, was obtained from the Centers for Disease Control and Prevention. The sorbitol-positive mutant was selected by growth of the wild-type strain at 37°C in purple broth base (Difco, Detroit, Mich.) containing 1% sorbitol (Sigma, St. Louis, Mo.). After several overnight transfers into fresh medium, a portion of the culture was plated onto SMA. A sorbitol-positive colony was selected and streaked again onto SMA. No sorbitol-negative colonies were formed, and the phenotype was stable.

Plasmid analysis. Plasmid isolation was performed by the method of Flamm et al. (12), and the plasmid profiles of the wild-type (wt) and mutant strains were analyzed by agarose (0.9%) gel electrophoresis. Plasmids were visualized under UV illumination following ethidium bromide staining.

Cytotoxicity assay. Culture supernatants and bacterial cell lysates of the wt and mutant strains were assayed in Vero cells (ATCC CCL 81) for the presence of Shiga-like toxin activity (32). The Vero cells were grown to confluent monolayers in microtiter plates. One-hundred-microliter portions

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of bacterial lysates (0.8 mg of protein) or culture supernatants were added, and serial 1:2 dilutions were made in cell culture medium (minimal essential medium [ICN Biomedicals, Inc., Flow Laboratories, Costa Mesa, Calif.] containing 5% fetal bovine serum). The monolayers were incubated at 37°C for 72 h under 5% CO_2, and the cells were examined daily for cytotoxicity by light microscopy.

Cell lysate and outer membrane protein (OMP) preparations. Bacterial cell lysates were prepared by sonication of cultures (24) grown for 18 h at 37°C in brain heart infusion broth (BHI) (Difco). For isolation of OMP, bacteria were cultured for 18 h at 37°C in BHI, and OMPs were prepared according to the method of Portnoy et al. (27). The detergent-insoluble OMP fraction was suspended in 1% sodium dodecyl sulfate (SDS) and stored at −20°C. Bacterial lysate and OMP samples were mixed with an equal volume of electrophoresis sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (14) (4% acrylamide in stacking gel, 12.6% acrylamide in separating gel).

Adherence assay. The wt and mutant strains were cultured in BHI for 18 h at 37°C with agitation, washed with phosphate-buffered saline (PBS) (pH 7.2), and resuspended in PBS, and 100 μl of the bacterial suspensions (2×10^8 bacteria) was added to monolayers of HEp-2 (ATCC CCL 23) and Intestine 407 (ATCC CCL 6) cells grown on circular glass slides. Following a 2-h incubation at 37°C under 5% CO_2, the monolayers were washed with PBS and then incubated with fluorescein isothiocyanate-labeled antiserum against *E. coli* O157:H7 (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) for 30 min at 37°C. The slides were washed and examined by fluorescence microscopy.

Enzyme and uptake assays. Sorbitol-6-phosphate dehydrogenase and [^{14}C]sorbitol uptake assays were performed as described by Lengeler and Lin (17). For the enzyme assays, the bacteria were grown in mineral medium containing 0.2% glucose and 1% casein hydrolysate. The wt and mutant strains were also grown for 18 h in tryptic soy broth (TSB) without glucose (Difco), and then a portion was transferred to either TSB without glucose or TSB containing 0.25% sorbitol or 0.25% glucose and grown for an additional hour. The activity of sorbitol-6-phosphate dehydrogenase was measured by reduction of a tetrazolium dye. The reaction was started by the addition of sonic extract of the wt or mutant strains, and the optical density change at 570 nm was monitored. The enzyme activity is expressed in micromoles per minute per milligram of protein. For the uptake assays, bacteria were grown in mineral medium containing 0.2% glucose and 1% casein hydrolysate. Exponentially growing cells were harvested by centrifugation, resuspended in medium without glucose, and incubated for 30 min at room temperature. The bacteria were washed with medium without glucose and resuspended to a density of 10^8 bacteria per ml. One hundred microliters of D-[U-^{14}C]sorbitol (Amersham, Arlington Heights, Ill.) (12.5 Ci/mol, 2.5×10^{-5} M) was added to 0.9 ml of the bacterial suspensions. After 1-, 15-, and 30-min incubations at 37°C, the bacteria were collected on membrane filters and washed. The amount of radioactivity on the dried filters was measured in a scintillation counter. [^{14}C]sorbitol uptake activity is expressed in nanomoles per minute per milligram of protein. Protein concentration was determined by the method of Bradford (4).

Infection of infant rabbits. Pregnant New Zealand White rabbits (NZR Ventures, Manchester, Md.) were obtained approximately 2 weeks prior to the expected parturition date. The rabbits were housed in individual cages with their litters throughout the duration of the experiment. Rectal swabs of infant rabbits were obtained for culture prior to inoculations. Four days after parturition, the infant rabbits (five to seven per group) were inoculated with either the wt or mutant strains. Bacteria were grown in BHI for 18 h. A total of 10 ml was transferred to 100 ml of BHI, and the cultures were grown for an additional 4 h. The bacteria were harvested by centrifugation, washed twice with PBS, and resuspended in 10% $NaHCO_3$. The rabbits were inoculated with 5×10^7 bacteria (0.5 ml) with an animal feeding needle (20 gauge, straight, 1.5 in. [ca. 3.8 cm] in length) placed above the tongue, close to the throat to allow the rabbits to swallow the suspensions slowly. The animals were then given 0.5 ml of 0.85% NaCl. The rabbits in the control group were given 0.5 ml of 10% $NaHCO_3$ and 0.5 ml of 0.85% NaCl.

The rabbits were observed for onset of diarrhea detectable by fecal staining of the perinea or hind legs or for any other sign of illness. One rabbit from each group including the control group was sacrificed by CO_2-induced hypoxia 24 h postinoculation. When the animals developed diarrhea, they were sacrificed. Rabbits that died spontaneously and those that were sacrificed were prepared for necroscopy after rectal swabs were taken for culture. Rectal swabs were placed into 0.5 ml of BHI, and a portion was streaked onto MacConkey agar, SMA, and BHI agar. The kidneys, spleens, and sections of liver were removed and frozen at −20°C. They were homogenized in 10 ml of 0.1% peptone solution, and a portion of each sample (100 μl) was plated onto MacConkey agar, SMA, and BHI agar. The plates were incubated overnight at 37°C. Sections of intestine (proximal small, distal small, cecum, proximal large, and distal large) were cut, and a loopful of contents was obtained from each site for culture on MacConkey agar, SMA, and BHI agar plates. Colonies were confirmed as *E. coli* O157:H7 by direct immunofluorescence with fluorescein isothiocyanate-labeled antiserum against *E. coli* O157:H7 (Kirkegaard & Perry Laboratories, Inc.). The intestinal sections were placed in fixative (23) (4% formaldehyde–1% glutaraldehyde) for several days at 4°C prior to preparation for microscopy.

Microscopy of intestinal sections. For scanning and transmission electron microscopy (SEM and TEM), glutaraldehyde-formaldehyde-fixed sections were fixed in an OsO_4 solution, dehydrated in a graded ethanol series, critical point dried from CO_2, and coated with a thin layer of gold by direct current sputtering. TEM sections were embedded in an epoxy resin mixture, and thin sections were stained with uranyl acetate and lead citrate. Resin-embedded thick sections were stained with methylene blue and examined by light microscopy. Sections for fluorescence microscopy were washed with 0.1 M Tris-HCl, pH 7.6, to quench reactive aldehyde groups and were embedded in CRYOform embedding medium (International Equipment Co., Needham, Mass.). Frozen sections were cut to a thickness of 10 μm with a cryostat microtome, attached to coverslips with Mayer's albumin, and kept at 4°C. The sections were incubated with monoclonal antibody 8-9H in PBS containing 5% fetal bovine serum for 30 min at room temperature. Monoclonal antibody 8-9H is highly specific for *E. coli* O157 and was kindly provided by Rebecca Durham, Organon Teknika, Rockville, Md. The samples were washed with PBS and incubated with fluorescein isothiocyanate-labeled sheep anti-mouse immunoglobulin G (Sigma Immunochemicals), for 30 min at room temperature. They were washed with PBS, mounted on glass slides, and photographed with Kodak TMAX-100 film.

TABLE 1. [^{14}C]sorbitol uptake and sorbitol-6-phosphate dehydrogenase activities

E. coli strain	Time[a] (min)	Sorbitol uptake[b] (nM/min/mg of protein)	Sorbitol-6-P-dehydrogenase activity[c] (μM/min/mg of protein)
A9124-1 (wt)	1	2.47	0.16
	15	0.27	
	30	0.10	
A9124-1 (mutant)	1	4.70	1.25
	15	1.39	
	30	1.65	

[a] Times indicate the length of incubation of the wt and mutant with [^{14}C]sorbitol prior to collection of the bacteria on membrane filters.
[b] Similar results were obtained from three separate experiments.
[c] Similar results were obtained from two separate experiments.

RESULTS

Comparison of wt and mutant strains. After four overnight transfers into purple broth base medium containing sorbitol, no sorbitol-positive colonies were visible. After 9 transfers, approximately 50% of the colonies were sorbitol positive on SMA, and by 12 transfers, 100% were sorbitol positive. To determine the nature of the mutation which occurred in the sorbitol-positive mutant, enzyme and sorbitol uptake levels in the wt and mutant strains were compared. Sorbitol-6-phosphate dehydrogenase is an essential enzyme for the utilization of sorbitol. There was a 7.8-fold higher level of sorbitol-6-phosphate dehydrogenase activity in the mutant strain compared with the wt strain (Table 1). The difference in enzyme activity between the wt and mutant strains was similar when they were grown in TSB and transferred to TSB, TSB with glucose, or TSB with sorbitol. The mutant showed increased uptake activity when wt and mutant strains were sampled after different incubation periods for measurement of their ability to take up radioactive sorbitol (Table 1). After 1 min, there was an approximately 1.9-fold increase in uptake activity, and following 15- and 30-min incubations, there were 5- and 16.5-fold increases, respectively.

Analysis by SDS-polyacrylamide gel electrophoresis showed that the wt and mutant strains had similar total protein and OMP profiles. They also showed similar plasmid profiles, both harboring one plasmid of approximately 60 MDa in size (data not shown). The 50% cytotoxic dose values (amount of toxin [reciprocal of dilution of the sample] that caused a cytopathic effect in 50% of a Vero cell monolayer) for the cell lysates of the wt and the mutant against Vero cells were equivalent, as were cytotoxic activities of the culture supernatants. Values for the 50% cytotoxic dose were 524,288 and 65,576 for the cell lysates and culture supernatants, respectively.

The wt and mutant strains showed similar patterns of adherence to HEp-2 and Intestine 407 cells, with overall adherence being greater with Intestine 407 cells than with HEp-2 cells. Bacteria adhered as single cells to the Intestine 407 monolayers and also formed apparent microcolonies (data not shown). There were no apparent differences in the level of piliation in the wt and mutant strains. Both were sparsely piliated (data not shown).

Infection of infant rabbits with the wt and mutant strains. Pathogenicity of the wt and mutant strains was investigated for infant rabbits. The animals were susceptible to infection, with diarrhea developing in both groups. By 24 h, three of seven rabbits inoculated with the mutant developed diarrhea, and one died spontaneously. Two of five rabbits inoculated with the wt strain had diarrhea by 24 h, and one died spontaneously. All of the rabbits were either sacrificed or dead by 4 days postinoculation. At necroscopy, there was evidence of fluid and gas distension of the abdominal area and in the large intestine in some of the rabbits. The animals in the control group (five rabbits) remained healthy throughout the duration of the experiment.

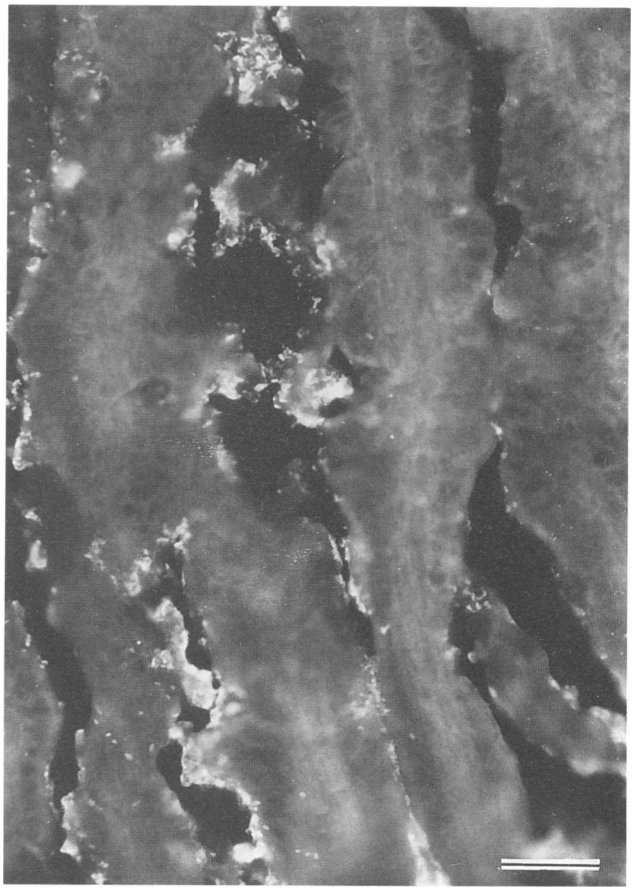

FIG. 1. Fluorescence micrograph showing brightly fluorescent bacteria attached to the mucosal surface of the cecum of an infant rabbit infected with A9124-1 wt (2 days postinoculation). Bar = 50 μm.

Cultures of rectal swabs and intestinal contents yielded sorbitol-negative and sorbitol-positive *E. coli* in the groups inoculated with the wt and mutant strains, respectively. All rabbits were culture positive for either the wt or mutant strains in at least one intestinal site by 1 day postinoculation. By 2 to 4 days, all sites were culture positive with the exception of the small intestine in some rabbits. Cultures of rectal swabs and of intestinal contents of the rabbits in the control group were negative. There were no visible gross pathologic abnormalities of the spleens, livers, and kidneys of infected or control animals, and no bacteria were recovered from cultures of organ homogenates.

Histopathologic examination of segments of the intestinal tract. Immunofluorescence microscopy of intestinal sections treated with monoclonal antibody against *E. coli* O157:H7 revealed brightly fluorescent bacteria intimately attached to the mucosal surface as single cells and as aggregates (Fig. 1).

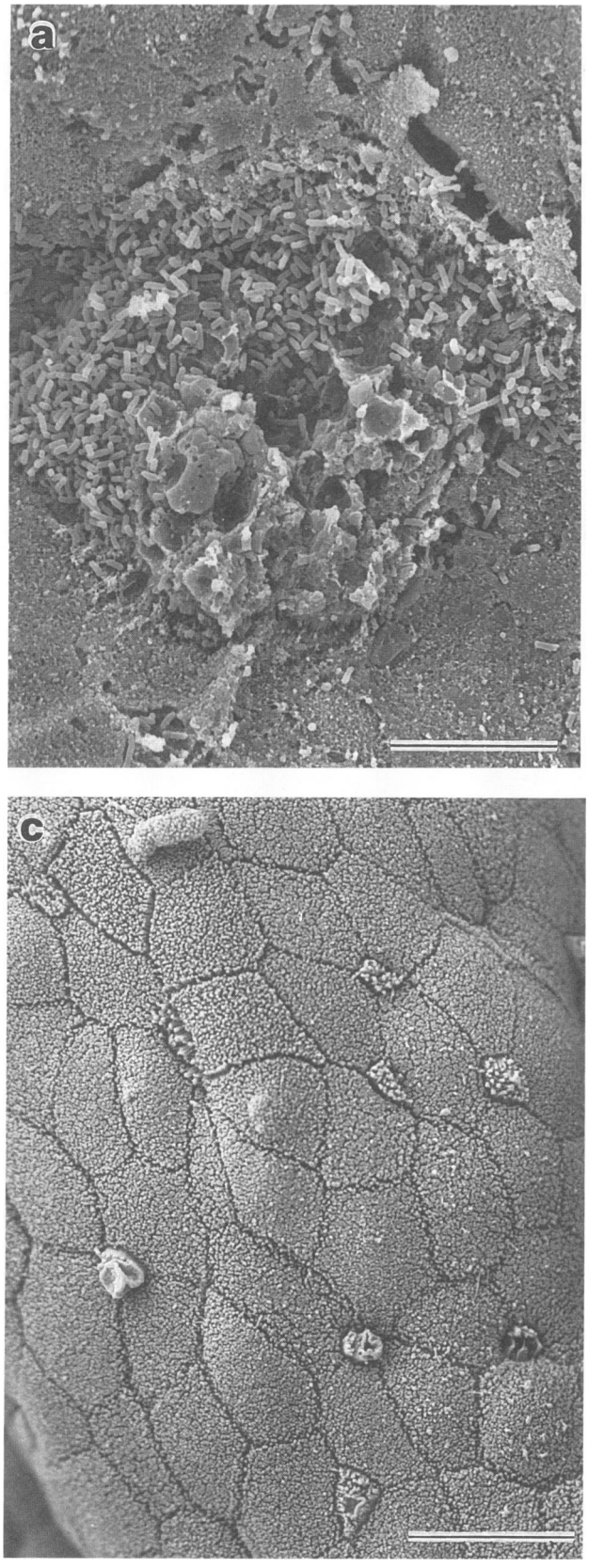

FIG. 2. SEM view of sections of the proximal large intestine of an infant rabbit infected with A9124-1 wt (2 days postinoculation) (a), a rabbit infected with A9124-1 mutant (4 days postinoculation) (b), and a noninfected control rabbit (c). Bar = 10 μm.

By SEM and TEM, no major differences were noted between rabbits inoculated with the wt and those inoculated with mutant strains. The epithelial lesions were more severe in rabbits studied 2 to 4 days postinoculation compared with those examined after 24 h. The largest numbers of organisms were present in the proximal and distal large intestine. Lower numbers of organisms were seen in the cecum, and very few bacteria were present in the proximal and distal small intestine. By 2 days postinoculation, SEM revealed bacteria adhering singly and in clusters throughout the epithelium, particularly in the large intestine (Fig. 2a). Mucus was visible throughout the surface epithelium and was closely associated with aggregates of bacteria. Some bacteria were beginning to burrow through the epithelium. By 4 days postinoculation, mucus-bacterial aggregates covered a large portion of the villous surface, and there was damage to the epithelium in foci in which bacteria were adherent (Fig. 2b). The lesions consisted of areas of tissue necrosis with sloughing of epithelial cells. The intestinal epithelium of the animals in the control group appeared healthy with intact cells and microvilli (Fig. 2c).

TEM revealed many electron-dense damaged cells devoid of microvilli in areas where bacteria were present (Fig. 3). Bacteria penetrated necrotic cells and were present in the cytoplasm. Debris from necrotic epithelial cells was present between the luminal surfaces and contained many bacteria.

Light microscopy of methylene blue-stained thick sections showed that marked vacuolation occurred in epithelial cells of intestinal sections of infected rabbits (Fig. 4a and b).

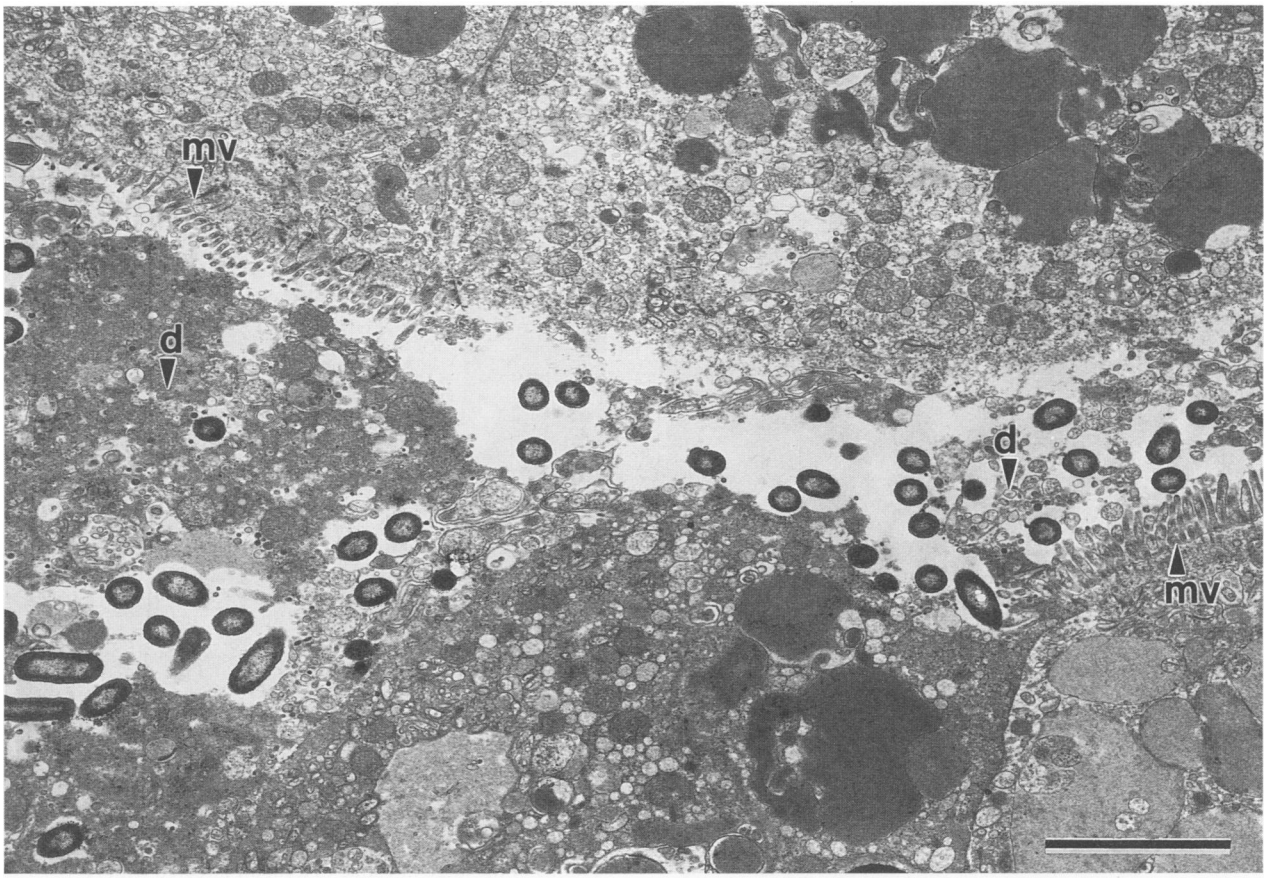

FIG. 3. TEM view of a section of the large intestine of a rabbit infected with A9124-1 wt (2 days postinoculation). mv, microvilli; d, debris. Bar = 2 μm.

Vacuolation resulted in spherical, pyknotic nuclei being forced to the base of the cells. Cells from intestinal sections taken from rabbits in the control group showed typical columnar cells with elongated nuclei and intact microvilli (Fig. 4c).

DISCUSSION

In view of the emergence of *E. coli* as an important food-borne pathogen, reliable methods for its detection in food samples and in patient specimens are urgently needed. Screening methods incorporating the use of SMA may not be sufficient for detection of all *E. coli* organisms of serotype O157:H7 and O157:NM since some strains may be sorbitol fermenters or the organism may mutate to a sorbitol-positive phenotype either in food products or during culture in sorbitol-containing media. Furthermore, use of SMA is not useful for detection of other EHEC organisms, for example, serotype O26:H11, that are sorbitol positive.

The infant rabbit animal model has been used previously to study the pathogenesis of the disease caused by *E. coli* O157:H7 (26, 28). The present studies showed that both the wt and mutant strains were pathogenic, causing similar clinical symptoms and mucosal damage in infant rabbits. Greater bacterial attachment and mucosal injury were observed in sections of the large intestine and cecum compared with sections of small intestine. This is in agreement with other studies which also demonstrated that the most extensive damage occurred in the proximal and distal colon, cecum, and appendix (26, 28).

Several reports have shown that EHEC is capable of producing attaching and effacing lesions along the apical epithelial cell membranes in animal models (13, 34). This involves the bacterial cells becoming intimately attached to cuplike projections formed on the columnar epithelial cell membrane. In the present study, there were areas of disruption or loss of microvilli in areas where bacteria were adherent. However, although bacteria were present in close proximity to the epithelial cell membrane, there was no evidence of the production of attaching and effacing lesions. Instead, SEM of rabbit intestinal sections revealed foci of mucus-bacterial aggregates and also single cells covering the villous surface. Further, the in vitro adherence studies using intestinal cells also showed that bacteria adhered as single cells and formed microcolonies without attaching and effacing lesions. This is in accordance with a report by Winsor et al. (36), who studied adherence of EHEC with a human colonic epithelial cell line as an in vitro model. They found that bacteria formed microcolonies in areas of tight junctions and were also present as single cells. They also did not observe typical attaching and effacing lesions. This suggests that there may be strain-related differences in the mechanisms of adherence and cellular damage among EHEC strains.

Epithelial cell vacuolation and nuclear pyknosis were evident by light microscopy, indicating that the cells were

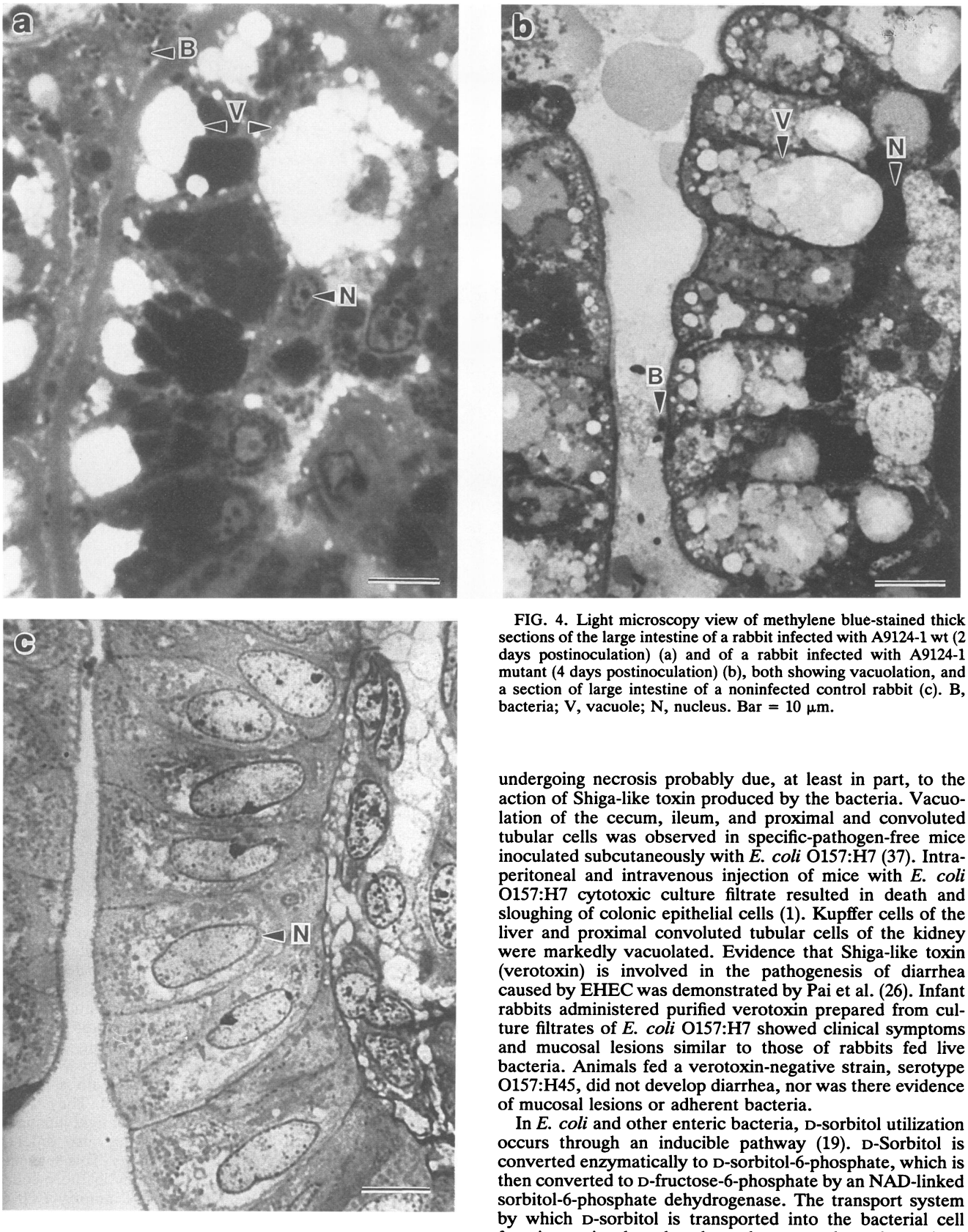

FIG. 4. Light microscopy view of methylene blue-stained thick sections of the large intestine of a rabbit infected with A9124-1 wt (2 days postinoculation) (a) and of a rabbit infected with A9124-1 mutant (4 days postinoculation) (b), both showing vacuolation, and a section of large intestine of a noninfected control rabbit (c). B, bacteria; V, vacuole; N, nucleus. Bar = 10 μm.

undergoing necrosis probably due, at least in part, to the action of Shiga-like toxin produced by the bacteria. Vacuolation of the cecum, ileum, and proximal and convoluted tubular cells was observed in specific-pathogen-free mice inoculated subcutaneously with *E. coli* O157:H7 (37). Intraperitoneal and intravenous injection of mice with *E. coli* O157:H7 cytotoxic culture filtrate resulted in death and sloughing of colonic epithelial cells (1). Kupffer cells of the liver and proximal convoluted tubular cells of the kidney were markedly vacuolated. Evidence that Shiga-like toxin (verotoxin) is involved in the pathogenesis of diarrhea caused by EHEC was demonstrated by Pai et al. (26). Infant rabbits administered purified verotoxin prepared from culture filtrates of *E. coli* O157:H7 showed clinical symptoms and mucosal lesions similar to those of rabbits fed live bacteria. Animals fed a verotoxin-negative strain, serotype O157:H45, did not develop diarrhea, nor was there evidence of mucosal lesions or adherent bacteria.

In *E. coli* and other enteric bacteria, D-sorbitol utilization occurs through an inducible pathway (19). D-Sorbitol is converted enzymatically to D-sorbitol-6-phosphate, which is then converted to D-fructose-6-phosphate by an NAD-linked sorbitol-6-phosphate dehydrogenase. The transport system by which D-sorbitol is transported into the bacterial cell functions via the phosphoenolpyruvate-dependent phosphotransferase system (PTS). Several soluble and mem-

brane-bound enzymes involved in this system catalyze the transfer of the phosphoryl group of phosphoenolpyruvate to D-sorbitol. Enzyme I (phosphoenolpyruvate:HPr phosphotransferase) and HPr (phosphorylcarrier protein) are general cytoplasmic proteins required for phosphorylation of all sugars which are phosphorylated via the PTS. Mutations in the genes coding for the synthesis of these proteins result in inhibition of transport of all PTS sugars. Mutations in a promoter-like sequence which is part of the *pts* operon result in reduced expression of the genes encoding enzyme I and HPr (7). The levels of these two proteins are increased by growth on a PTS sugar substrate. HPr (*ptsH*) and enzyme I (*ptsI*) together with the promoter region (*ptsHp*) form the *pts* operon.

The D-sorbitol PTS requires intermediate phosphorylation of the D-sorbitol-specific enzyme II (integral membrane enzyme) and enzyme III (peripheral membrane enzyme) (15). Cytoplasmic D-sorbitol-6-phosphate is then oxidized by sorbitol-6-phosphate dehydrogenase. The D-sorbitol (D-glucitol) operon consists of *gutC* (regulatory gene), *gutA* (enzyme II), *gutB* (enzyme III), and *gutD* (sorbitol-6-phosphate dehydrogenase). A repressor of the *gut* operon (*gutR*), which is encoded in a region downstream from the operon, recognizes the major substrate of enzyme II as an inducer. Mutations in this gene probably result in constitutive expression of the *gut* operon genes. Grenier et al. (15) found that growth in the presence of sorbitol resulted in induction of enzyme II, enzyme III, and sorbitol-6-phosphate dehydrogenase. A mutant constitutive for the sorbitol operon synthesized enzyme II, enzyme III, and sorbitol-6-phosphate dehydrogenase at high levels.

In the present study, uptake of sorbitol and levels of sorbitol-6-phosphate dehydrogenase were greatly increased in the mutant; therefore, it is possible that a mutation may have occurred in a regulatory gene. Lengeler and Lin (17) reported that when wild-type *E. coli* was grown in the presence of 0.2% glucose, sorbitol uptake activity was 2.5 nM/min/mg after 30 s of exposure to [^{14}C]sorbitol. This compares with uptake activity of the wt, which was 2.47 nM/min/mg, whereas that of the mutant was 4.70 nM/min/mg (Table 1). When cells were grown in the presence of 0.2% sorbitol, sorbitol-6-phosphate dehydrogenase activity of wild-type *E. coli* was 0.42 μM/min/mg (17), whereas enzymatic activity of the sorbitol-positive mutant tested in the present study was somewhat higher at 1.25 μM/min/mg (Table 1).

The mutant is maintained in BHI medium, which contains glucose, and has retained the sorbitol-positive phenotype after approximately 15 bimonthly passages into fresh BHI medium, indicating that it is a stable mutation. Two other *E. coli* O157:H7 strains were cultured in sorbitol-containing medium under the same conditions as strain A9124-1, and we were unsuccessful in isolating sorbitol-positive mutants; therefore, not all strains of *E. coli* O157:H7 are susceptible to the type of mutation undergone by strain A9124-1. Perhaps, the length of incubation and number of transfers in sorbitol-containing medium were insufficient for selection of sorbitol-positive mutants of the other two strains.

The present results demonstrate that *E. coli* O157:H7 expressing a sorbitol-positive phenotype can be selected under certain conditions. The wt and mutant strains had equally high levels of Shiga-like toxin activity and caused similar clinical symptoms and mucosal lesions when administered to infant rabbits, indicating that sorbitol utilization does not correlate with pathogenicity. EHEC detection methods, such as protocols which incorporate SMA, should not rely on the inability of the serovar to ferment sorbitol since sorbitol-positive *E. coli* O157:H7 and O157:NM have been isolated, and sorbitol-positive mutants can be produced as demonstrated in the present study.

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