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Heterogeneity in Phenotypic and Genotypic Characteristics among Strains of *Hafnia alvei*

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***Hafnia alvei* is an emerging human pathogen associated with sporadic cases and outbreaks of diarrhea. Bangladeshi isolates of *H. alvei* possess the *Escherichia coli* attaching and effacing (*eaeA*) gene and demonstrate an attaching and effacing phenotype. In the present study we examined 11 Canadian *H. alvei* isolates and strain 19982 from Bangladesh to determine if the formation of attaching and effacing lesions is a property shared among multiple isolates. Attaching and effacing lesions were detected by induction of tyrosine kinase protein phosphorylation and cytoskeletal rearrangements in infected tissue culture epithelial cells with immunofluorescence microscopy and by the examination of infected cells with transmission electron microscopy. The presence of the *eaeA* gene was examined by PCR and colony blot hybridization. Profiles of outer membrane protein extracts, chromosomal macrorestriction fragments, and plasmids were also examined. Accumulation of host phosphotyrosine proteins and rearrangement of the cytoskeletal protein α-actinin were both observed in HEp-2 cells infected with *H. alvei* 19982. In contrast, none of the other 11 clinical *H. alvei* isolates demonstrated either of these responses, nor did they form attaching and effacing lesions under electron microscopy. Consistent with the absence of the attaching and effacing phenotype, these clinical isolates did not possess the *eaeA* gene. The outer membrane protein profiles of all of the Canadian isolates were identical but differed from that of *H. alvei* 19982. Pulsed-field gel electrophoresis and plasmid profile analyses of the clinical *H. alvei* isolates differed substantially from those of the Bangladeshi strain. These results indicate that there is heterogeneity among *H. alvei* strains with respect to signal transduction, attaching and effacing adhesion, outer membrane constituents, and genotype. Epidemiological studies on enteropathogenic *H. alvei* thus need to go beyond simple species designations and require specific identification of the virulent clones.**

Hafnia alvei is a member of the family *Enterobacteriaceae* that has recently been associated with sporadic cases of diarrhea in humans (2, 3). It has also been implicated in at least one outbreak of diarrheal illness in a hospital setting (21). Long before these reports, Sakazaki (26) showed that *Hafnia* species can cause fluid accumulation in rabbit ligated ileal loops. The ability of *Hafnia* species to cause attaching and effacing (AE) lesions in host epithelial cells has now been identified as a virulence factor (2).

The AE phenotype was first described for strains of enteropathogenic *Escherichia coli* (EPEC) infecting both rabbits and humans (20). AE lesion formation is recognized as an important virulence factor for EPEC (6). The AE lesion is characterized by focal destruction of the microvilli and intimate contact between the bacteria and the host plasma membrane with concomitant rearrangement of various cytoskeletal elements, including filamentous actin (F-actin), α-actinin, talin, and ezrin, at sites of bacterial attachment (11, 17). This rearrangement of cytoskeletal proteins gives rise to electron-dense adhesion pedestals which are a characteristic feature of AE pathogens (6). Rosenshine et al. (25) showed that in AE lesions formed following EPEC infection, there is also accumulation of tyrosine-phosphorylated proteins.

The AE phenotype is dependent on the presence of a chromosomal gene, designated the *E. coli* AE (or *eaeA*) gene (15). The *eaeA* gene was present in all *H. alvei* strains isolated from stool samples of humans with acute diarrhea in Bangladesh, including the reference strain, 19982 (2, 3). The *H. alvei* reference strain 19982 shares genotypic and phenotypic features with all of the other isolates from Bangladesh described to date (2, 3). These strains also cause AE lesions following infection of eukaryotic cells both in vitro and in vivo in the rabbit ileum (2, 3).

The purpose of the present study was to determine whether AE lesion formation is a general feature of enteric *H. alvei* isolates. In addition, the similarity between the *H. alvei* reference strain from Bangladesh and the Canadian isolates with respect to outer membrane proteins (OMP), DNA macrorestriction, and plasmid profiles was also determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Nine isolates of *H. alvei*, designated H1 to H9, cultured from stool samples obtained from children presenting with diarrhea at The Hospital for Sick Children in Toronto, Ontario, Canada, were employed in this study. Two isolates of *H. alvei* (strains D-46-NF and D-67-NF) from an outbreak of diarrheal illness in a hospital in Newfoundland were also included (21). The reference strain employed was *H. alvei* 19982, which was originally isolated from a 9-month-old girl with vomiting and diarrhea by M. J. Albert (International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh) (2) and kindly provided to us by R. Robins-Browne (Royal Children's Hospital, Parkville, Australia). The AE strains of EPEC (strain E2348/69), vero-cytotoxin-producing *E. coli* (VTEC) (strains CL8 and CL56) (6, 10), and *Citrobacter freundii* biotype 4280 (kindly provided by D. B. Schauer, Massachusetts Institute of Technology, Cambridge, Mass.) (28) were included as positive controls.

Bacteria were grown overnight in static, nonaerated Penassay (Difco Laboratories, Detroit, Mich.) broth at 37°C. Cultures were harvested by centrifugation

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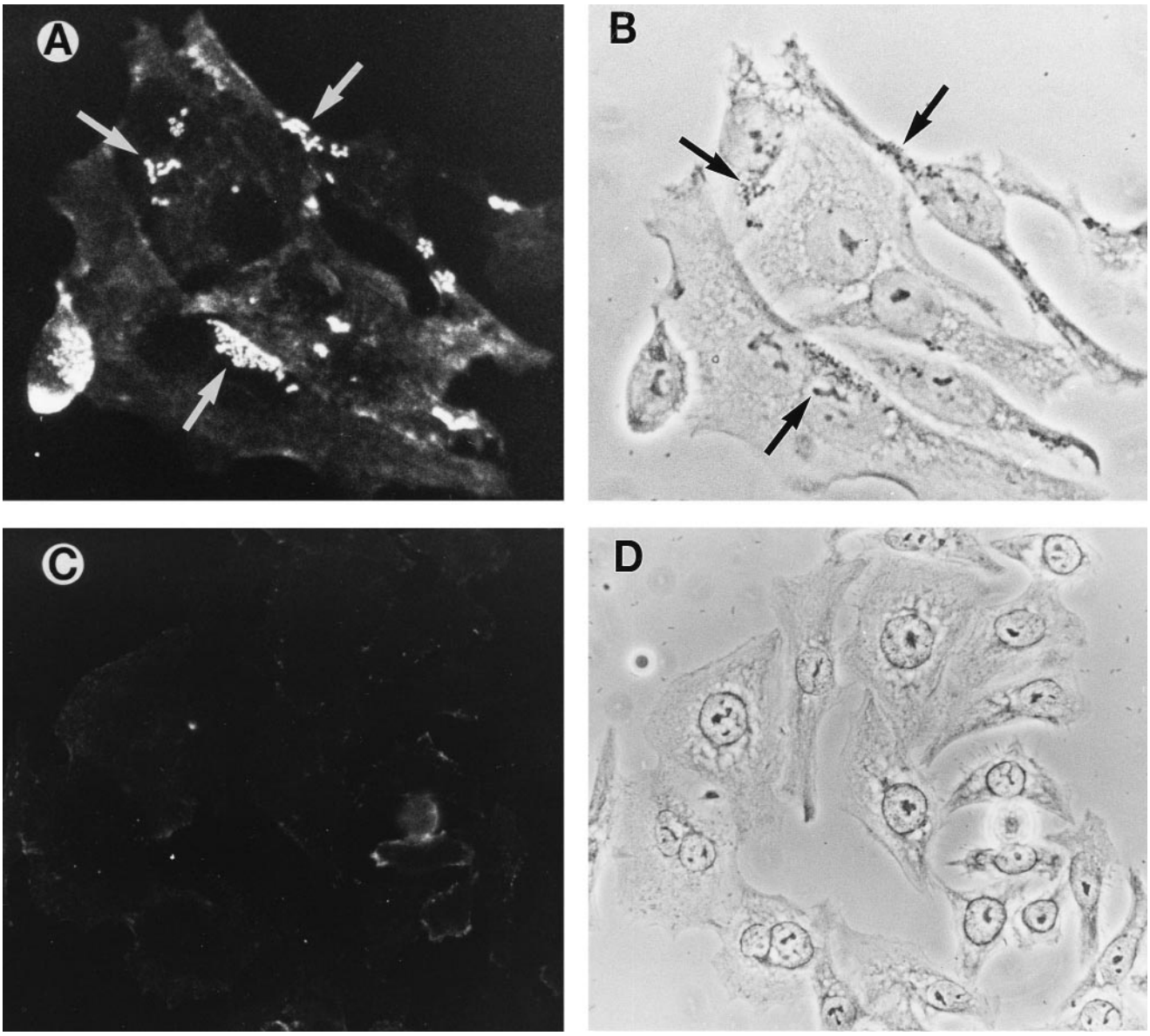

FIG. 1. α-Actinin immunofluorescence (A and C) and phase-contrast microscopy (B and D) of HEp-2 cells infected for 6 h with *H. alvei* 19982 (A and B) and *H. alvei* H1 (C and D). Intense foci of α-actinin were seen in cells infected with *H. alvei* 19982, which corresponded to foci of adherent bacteria observed by phase-contrast microscopy (arrows). In contrast, cells infected with *H. alvei* H1 did not show α-actinin fluorescence. Approximate magnification, ×300.

at 2,500 × *g* for 15 min, and the pellets were resuspended in sterile phosphate-buffered saline (PBS) (pH 7.4) at a concentration of 10^{10} CFU/ml.

Eukaryotic cell cultures. The human epithelial cell line HEp-2 (ATCC CCL23; American Type Culture Collection, Rockville, Md.) was maintained as monolayers in 75-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.) in minimum essential medium (Life Technologies, Grand Island, N.Y.) containing 15% (vol/vol) heat-inactivated fetal calf serum (Cansera International Inc., Rexdale, Ontario, Canada), 0.5% L-glutamine (ICN Biomedicals Inc., Costa Mesa, Calif.), 0.1% sodium bicarbonate (ICN), 2% penicillin-streptomycin (ICN), and 1% amphotericin B (ICN) (13) in 5% CO_2 at 37°C.

Immunofluorescence detection of α-actinin. The rearrangement of α-actinin in epithelial cells following bacterial infection was determined by the method described by Finlay et al. (11), with minor modifications (14). Briefly, HEp-2 cells were seeded into two-well Lab-Tek (Nunc Inc., Naperville, Ill.) chamber slides and grown overnight to subconfluence. The monolayers were inoculated with 5 × 10^7 bacteria and incubated for 6 h at 37°C, with a change of medium midway through the infection period. The cell cultures were then washed six times with PBS, fixed in 100% methanol for 10 min at 25°C, and washed three times with PBS. A 1:100 dilution of murine monoclonal anti-α-actinin (Sigma Chemical Company, St. Louis, Mo.) was added to the monolayers and incubated for 1 h at 37°C with continuous gentle agitation. After six washes in PBS, monolayers were incubated in the dark at room temperature with a 1:200 dilution of fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa.). The cell monolayers were washed six times in PBS and dried, and the slides were mounted in Slow-Fade (Molecular Probes Inc., Eugene, Oreg.) antifade reagent. The slides were then examined by fluorescence and phase-contrast microscopy (Leitz Dialux 22; Leica Canada Inc., Willowdale, Ontario, Canada) to detect foci of fluorescence corresponding to regions of bacterial attachment.

Immunofluorescence detection of tyrosine-phosphorylated proteins. Rearrangement of tyrosine-phosphorylated proteins underneath the adherent bacteria in infected HEp-2 cells was evaluated by the method described by Rosenshine et al. (25). Briefly, HEp-2 cells were grown overnight to subconfluence, inoculated with the test strains, and incubated for 3 h in tissue culture medium. After the infection period, eukaryotic cell monolayers were washed six times with PBS to remove the nonadherent bacteria. The cells were then fixed in 2% paraformaldehyde for 15 min at 25°C, washed three times with PBS, and permeabilized with 0.1% Triton X-100 for 5 min. Accumulation of tyrosine-phosphorylated proteins was detected by incubation of cells with a 1:50 dilution of murine monoclonal antiphosphotyrosine immunoglobulin G (Upstate Biotechnology

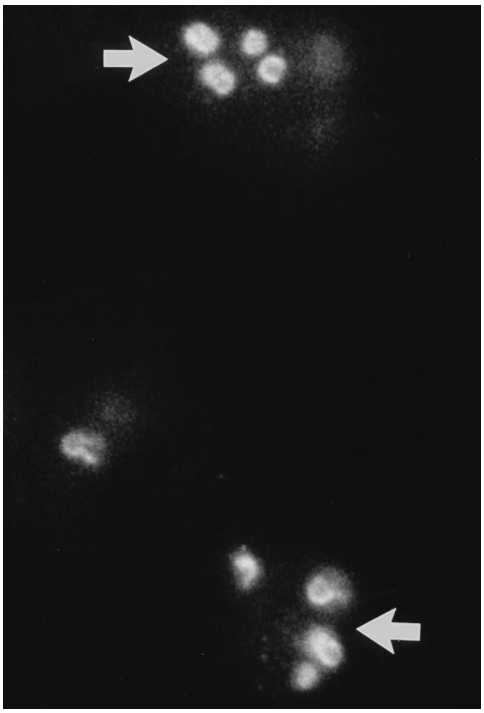

FIG. 2. Immunofluorescence of phosphotyrosine proteins in HEp-2 cells infected for 3 h with *H. alvei* 19982. Intense foci of tyrosine-phosphorylated proteins were seen (arrows) and corresponded to adherent bacteria when observed by phase-contrast microscopy. No such foci of fluorescence were seen with any of the Canadian clinical isolates of *H. alvei* (data not shown). Approximate magnification, ×1,000.

Inc., Lake Placid, N.Y.) for 1 h at 37°C (13). Following six washes in PBS, the cells were stained with a 1:50 dilution of fluorescein isothiocyanate-conjugated anti-mouse rabbit immunoglobulin G for 1 h at 37°C. The slides were washed, mounted, and examined for the accumulation of tyrosine-phosphorylated proteins, as described above for the α-actinin assay.

Electron microscopy. HEp-2 cells were grown to confluence on 75-cm^2 tissue culture flasks. Monolayers were washed in Hanks balanced salt solution (ICN), inoculated with approximately 10^8 bacteria in antibiotic-free tissue culture medium, and incubated for 6 h at 37°C with one medium change midway through the incubation period. After being washed six times with PBS, cells with adherent bacteria were gently removed from the plastic surface by trypsinization with 0.25% trypsin (ICN) at 37°C for 5 min. The cells were pelleted by centrifugation in 25% fetal calf serum, fixed in 2% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), postfixed in 2% osmium tetroxide, and dehydrated through a series of graded acetone washes. The samples were embedded in Epon, and ultrathin sections were cut, placed on copper grids, and stained with uranyl acetate and lead salts (29). The grids were examined for the presence of both adherent bacteria and AE lesions with a Philips 300 transmission electron microscope at an accelerating voltage of 60 kV.

PCR. Test strains were grown on Columbia agar containing 5% sheep blood (PML Microbiologicals, Mississauga, Ontario, Canada). Single colonies were resuspended in 0.05 ml of 1× PCR buffer (Perkin-Elmer Cetus, Norwalk, Conn.) and boiled for 10 min prior to use as template DNA in PCR assays. Primers (C1E [5′-TCGTCACAGTTGCAGGCCTGGT-3′] and C2E [5′-CGAAGTCTTATCC GCCGTAAAGT-3′]) based on the central region of the *eaeA* gene of VTEC strain CL8 have been described previously (19). Amplification reactions in 0.05 ml used 1 mM deoxynucleoside triphosphates, 1× PCR buffer, 1 U of *Taq* polymerase (GeneAmp; Perkin-Elmer Cetus), 20 pmol of each primer, and 0.002 ml of boiled bacterial suspension as a template. Amplification was carried out with an initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min with a GeneAmp PCR System 9600 (Perkin-Elmer Cetus). After amplification, 0.01 ml of the reaction mixture was analyzed by agarose gel electrophoresis. VTEC strain CL8 (serotype O157:H7) and EPEC strain E2348/69 were included as positive controls, and a sample containing no DNA was included as a negative control.

Colony blot hybridization. A 1.1-kb *eaeA* probe, C1-C2, was made by PCR amplification with the primers described above and the EPEC strain E2348/69 as a template. Strains were grown overnight in duplicate at 37°C on nylon filters overlaid onto a Columbia agar plate. Probe labeling and colony blot hybridization were performed by using the ECL direct nucleic acid labeling and detection system (RPN 3000; Amersham Life Science, Buckinghamshire, England). Hybridizations were performed overnight at 42°C, and subsequent steps were carried out according to the manufacturer's instructions.

Extraction of OMP and SDS-PAGE. OMP were extracted by Sarkosyl precipitation according to the method of Achtman et al. (1). In brief, overnight cultures of *H. alvei* broth were centrifuged at 2,500 × *g* for 15 min, and the pellet was suspended in 10 mM Tris buffer (pH 8). The suspension was chilled and then sonicated with intermittent cooling until the culture went from cloudy to translucent. The sonicate was centrifuged as before to remove any unbroken bacteria. The resulting supernatant was then centrifuged at 20,000 rpm in an SS34 rotor (Sorvall Instruments, Wilmington, Del.) for 1 h at 4°C. The pellet was resuspended in distilled water, and the OMP were extracted from this suspension by incubation with 8 volumes of 1.67% (wt/vol) *N*-lauroyl sarcosine (Sigma) in 11.1 mM Tris buffer (pH 7.6) for 20 min at 25°C. The OMP extracts were collected by centrifugation at 20,000 rpm in the SS34 rotor for 1.5 h at 20°C. The resulting OMP pellets were suspended in distilled water and stored at −20°C.

The extracts were mixed with loading buffer and heated for 5 min prior to resolution by gel electrophoresis in 10% polyacrylamide. The samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 45 min at a constant voltage of 200 V as described by Laemmli (18), and the gel was stained with Coomassie brilliant blue.

PFGE. DNA samples were prepared as described previously (5) with the minor modifications noted below. Overnight broth cultures of *H. alvei* were pelleted, washed, and resuspended in 10 mM Tris-Cl (pH 7.6)–1 M NaCl wash solution. An equal volume of 1.3% low-melting-point agarose (Bethesda Research Laboratories, Gaithersburg, Md.) was added to the bacterial cells, mixed, aliquoted to pulsed-field gel electrophoresis (PFGE) plug molds (Pharmacia, Uppsala, Sweden), and allowed to solidify. The plugs were lysed in 5 ml of lysis solution (6 mM Tris-Cl [pH 7.6], 1 M NaCl, 100 mM EDTA [pH 7.5], 0.5% Brij 58 [Sigma], 0.2% deoxycholate [Sigma], 0.5% Sarkosyl) containing fresh hen egg white lysozyme (1 mg/ml) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and 20 μl of bovine pancreatic RNase (Boehringer Mannheim) per ml. The plugs were incubated in this solution for 16 h at 37°C and then incubated in an equal volume of deproteination solution (0.5 M EDTA [pH 9 to 9.5], 1% lauryl sarcosine, 1 mg of proteinase K [Boehringer Mannheim] per ml) for 48 h at 50°C with gentle shaking.

Prior to digestion with restriction enzymes, the plugs were washed four times in 10-ml aliquots of 10 mM Tris-Cl (pH 7.5)–1 mM EDTA (TE) at 37°C for a minimum of 2 h. Following initial experiments to identify suitable rare-cutting restriction enzymes, genomic DNA from each of the isolates was digested with the rare-cutting restriction enzymes I-*Ceu*I (TAACTATAACGGTCCTAA/GG TAGCGA) (New England Biolabs, Mississauga, Ontario, Canada) and *Sfi*I (GG CCN_4/NGGCC) and *Not*I (GC/GGCCGC) (Boehringer Mannheim). Restriction enzyme digestion was performed in a total volume of 0.3 ml containing 20 to 30 U of enzyme per plug. *Sfi*I restriction digests were incubated overnight at 50°C, while *Not*I and I-*Ceu*I restriction digests were incubated for the same period of time at 37°C.

Electrophoresis was performed with a contour-clamped homogenous electric field apparatus (LKB 2015; Pharmacia LKB Biotechnology, Uppsala, Sweden) (5). Plugs were loaded into agarose wells and electrophoresed in 0.5× TBE buffer (0.045 M Tris-borate, 0.001 M EDTA [pH 8.0]) at 14°C. To achieve optimal resolution across a wide range of molecular sizes, a variety of electrophoresis conditions were utilized to separate I-*Ceu*I restriction fragments. Lambda concatemers (Promega Corporation, Madison, Wis.) and the chromosomes of *Saccharomyces cerevisiae* YPH149 (16) were used as size markers. Gels were stained for 30 min in ethidium bromide, destained overnight, and photographed under UV light.

Data analysis. DNA fragment patterns were compared by direct visualization. Similarities between strains were scored by using the Dice coefficient, also known as the coefficient of similarity (24). This coefficient, *F*, scores the number of DNA bands shared between two species and is calculated from the formula $F = 2n_{xy}/(n_x + n_y)$, where n_x is the total number of DNA bands generated from isolate *x*, n_y is the total number of bands from isolate *y*, and n_{xy} is the total number of bands common to both isolates (24).

Extraction of plasmid DNA. Plasmid extraction was performed according to the alkaline lysis method (27). Briefly, a 2-ml overnight broth culture of *H. alvei* was centrifuged at 14,000 rpm in a Microfuge. The medium was removed, the pellet was resuspended in 0.1 ml of 50 mM glucose–25 mM Tris-Cl (pH 8.0)–10 mM EDTA (pH 8.0), 0.2 ml of 0.2 M NaOH–1% SDS was added, and the mixture was incubated at room temperature. After 10 min, 0.2 ml of 3 M potassium acetate–5 M glacial acetic acid was added, and the tube was incubated on ice for 10 min. The tube was then centrifuged at 14,000 rpm for 5 min, the supernatant was transferred to a fresh tube for extraction with phenol and precipitated with 2 volumes of ethanol, and the DNA was redissolved in TE. Plasmid samples were resolved in 0.7% agarose with lambda *Hin*dIII fragments as size markers.

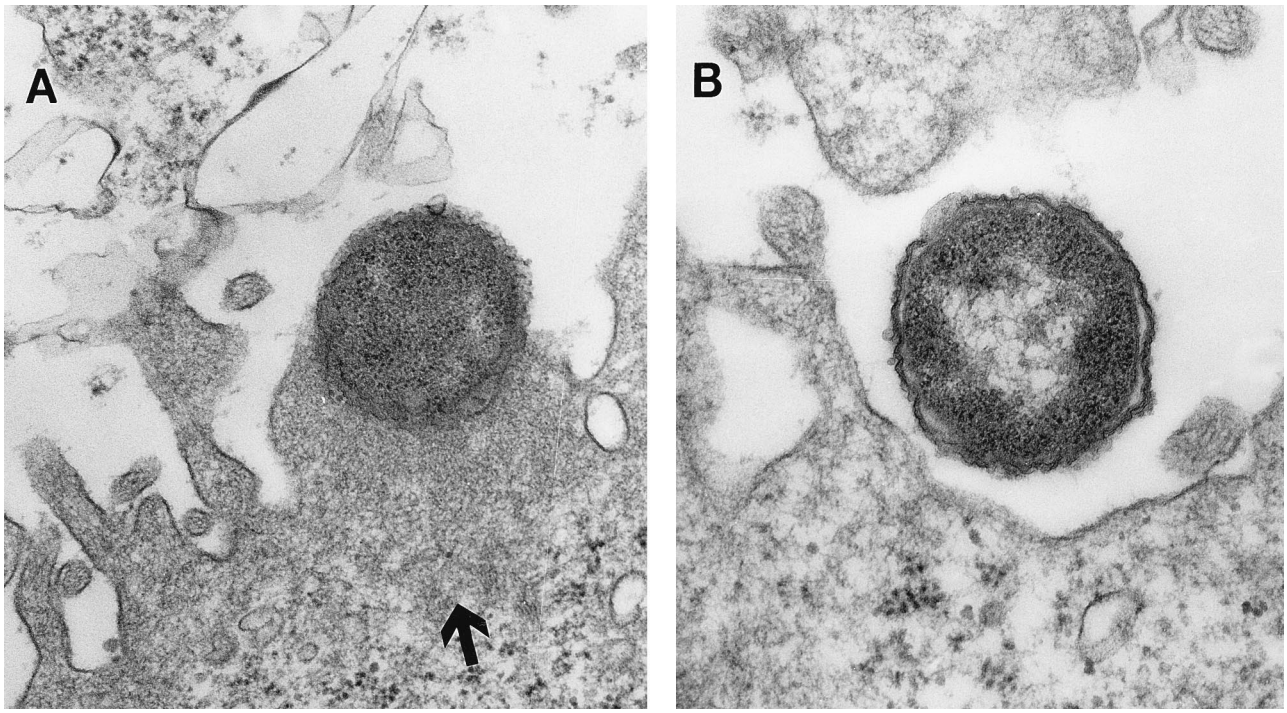

FIG. 3. Transmission electron micrographs of HEp-2 cells infected with *H. alvei* 19982 (A) and *H. alvei* H1 (B) after 6 h. AE electron-dense adhesion pedestals were seen in cells infected with *H. alvei* 19982 (the arrow in panel A points to one such adhesion pedestal). In contrast, *H. alvei* H1 adhered to the cells and effaced microvilli but did not form AE lesions. Approximate magnification, ×50,000.

RESULTS

α-actinin detection. Intense foci of α-actinin fluorescence, comparable to those seen for the AE-positive control strain E2348/69, were observed in regions corresponding to areas of bacterial attachment in HEp-2 cells infected with *H. alvei* 19982 from Bangladesh (Fig. 1). In contrast, none of the 11 Canadian *H. alvei* isolates induced an α-actinin response in HEp-2 cells (Fig. 1). The fluorescence intensity observed for the cells infected with these *H. alvei* strains was similar to that observed for cells infected with the *E. coli* laboratory strain HB101 and for uninfected epithelial cells (data not shown).

Phosphotyrosine responses. Intense foci of fluorescence corresponding to tyrosine-phosphorylated proteins were detected under the adherent bacteria in HEp-2 cells infected with the AE-positive *H. alvei* strain 19982 (Fig. 2). In contrast, when two of the nine isolates of *H. alvei* from Toronto (H1 and H8) and the two isolates from Newfoundland were tested, they failed to induce any detectable tyrosine phosphorylation (Fig. 2); the fluorescence intensity of the cells was similar to that of cells infected with the AE-negative *E. coli* strain HB101 and cells left uninfected (data not shown).

In vitro adhesion of *H. alvei*. Of the three strains (H1, D-46-NF, and 19982) examined by transmission electron microscopy for the AE phenotype, only the α-actinin-positive *H. alvei*

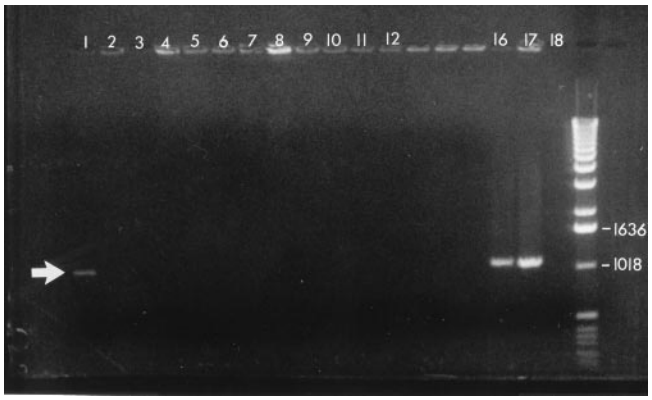

FIG. 4. PCR fragments amplified from *H. alvei* isolates by using VTEC-specific primers derived from the 1.1-kb central fragment of the *eaeA* gene. *H. alvei* 19982 from Bangladesh (lane 1) amplified a PCR product of approximately 1 kb that matched in size with the *eaeA* gene fragment amplified from the positive controls, VTEC strain CL8 (lane 16) and EPEC strain E2348/69 (lane 17). In contrast, each of the 11 *H. alvei* isolates from Canada (lanes 2 to 12) failed to amplify an *eaeA* PCR product. Numbers on the right are sizes in base pairs.

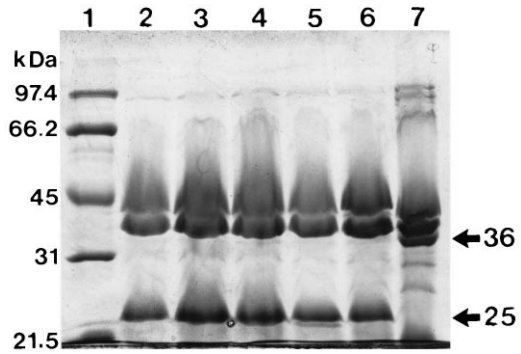

FIG. 5. Coomassie blue-stained outer membrane extracts from *H. alvei* 19982 (lane 7), selected Toronto isolates (H2 to H5 [lanes 2 to 5, respectively]), and one isolate from Newfoundland (D67-NF [lane 6]) run in parallel with low-molecular-mass reference standards (lane 1). The 36-kDa OmpA band was present only in strain 19982 (arrow), while the 25-kDa band was present in all of the other isolates (arrow).

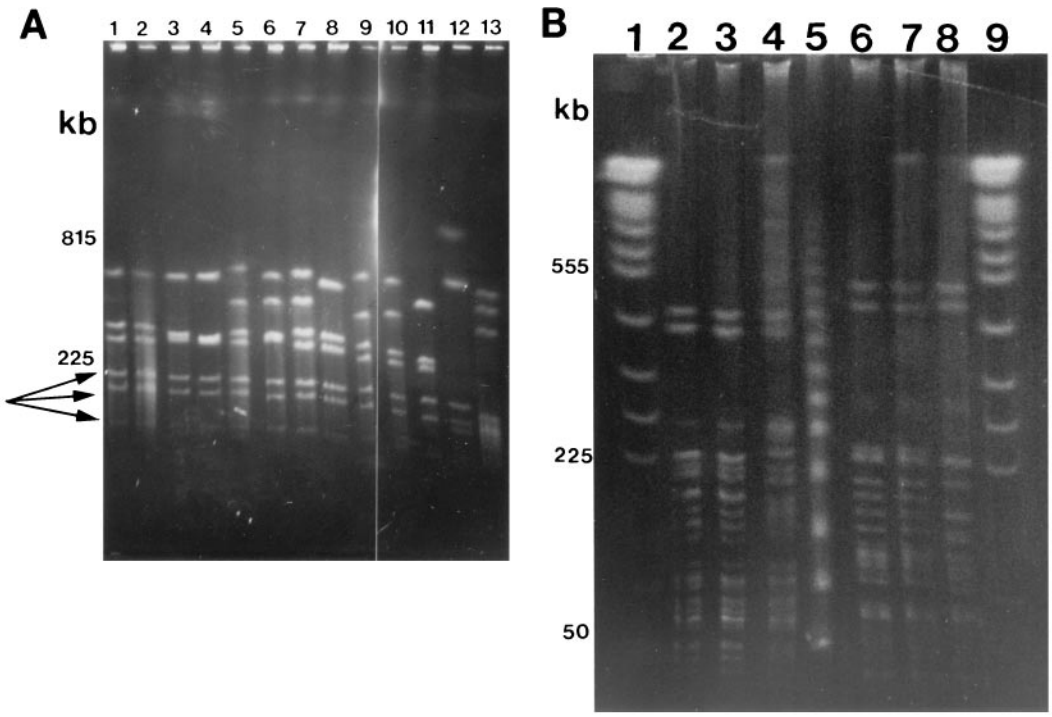

FIG. 6. (A) I-*Ceu*I-generated macrorestriction profiles for *H. alvei* isolates. A 1% agarose gel was run for 24 h with a pulse time ramped from 40 to 80 s in a field strength of 10 V/cm. Molecular sizes are indicated to the left of the gel. Lanes 1 to 9, *H. alvei* H1 to H9, respectively; lanes 10 and 11, strains D46-NF and D67-NF, respectively; lanes 12 and 13, strain 19982 and EPEC strain E2348/69, respectively. Arrows indicate restriction bands common to all isolates tested. (B) *Sfi*I- and *Not*I-generated macrorestriction profiles for *H. alvei* H1 to H3. A 1% agarose gel was run for 24 h with a pulse time ramped from 10 to 60 s in a field strength of 10 V/cm. Lambda concatemers (lane 5) and YPH 149 yeast chromosomes (lanes 1 and 9) were used as size markers. Molecular sizes are indicated to the left of the gel. *H. alvei* H1 to H3 were digested with *Sfi*I (lanes 2 to 4, respectively) and *Not*I (lanes 6 to 8, respectively).

strain 19982 demonstrated intimate attachment to HEp-2 epithelial cells. Classical electron-dense AE pedestals, comparable to those observed with EPEC strain E2348/69 (20), were observed (Fig. 3). In contrast, neither H1 nor D-46-NF, which were α-actinin negative, showed intimate attachment to epithelial cells (Fig. 3). The bacteria adhered closely and effaced microvilli at several points, but classical AE lesions were not observed.

Screening for the *eaeA* gene. The presence of the *eaeA* gene was analyzed by using both PCR and colony blot hybridization. Primers C1E and C2E from the conserved central portion of the *eaeA* gene were used to amplify a 1.1-kb fragment (19). Only the *H. alvei* reference strain 19982 from Bangladesh generated an amplified PCR product of the expected size. The size of this amplified fragment matched that of the fragment generated from the positive controls VTEC strain CL8 and EPEC strain E2348/69 (Fig. 4). In contrast, none of the nine isolates from Toronto or the two isolates from Newfoundland amplified an *eaeA* fragment of the predicted size (Fig. 4). Results obtained by PCR analysis for the *eaeA* gene were confirmed by colony blot hybridization, which showed only *H. alvei* 19982 hybridizing with the *eaeA* gene probe (data not shown).

OMP profiles. The OMP profile of the *H. alvei* reference strain 19982 was different from that of each of the 11 *H. alvei* isolates from Canada. Similar to the case for VTEC strain CL56 (10), two major bands with apparent molecular masses of 40 and 36 kDa were present in strain 19982, while the 36-kDa band was missing in each of the Canadian isolates of *H. alvei* (Fig. 5). The 40- and 36-kDa bands have been previously shown to be the porins OmpF and OmpA, respectively, in VTEC strain CL56 (10). Since the 36-kDa protein was present only in *H. alvei* 19982, its N-terminal amino acid sequence was determined. There was a 100% match over 11 amino acids at the N terminus (Ala-Pro-Lys-Asp-Asn-Thr-Trp-Tyr-Thr-Gly-Ala) (PIR database, release 46.0) between this protein and the OmpA precursor proteins of *E. coli*, *Shigella dysenteriae*, and *Serratia marcescens*. The OMP profiles of all the other *H. alvei* isolates, although from two different sources (Toronto and Newfoundland), were similar. All of the Canadian isolates showed the presence of a major OMP band with an apparent molecular mass of 25 kDa (Fig. 5). Of note, this OMP was absent in *H. alvei* 19982 (Fig. 5) and the VTEC comparison strain (data not shown). When the N-terminal amino acid sequence of this band from one of the Canadian isolates (H1) was determined, it revealed a novel peptide sequence (Ala-Asn-Ser-Asp-Asn-Thr-Gly-Tyr-Tyr-Ala-Gly-Ala-Lys-Leu-Gly) (PIR database, release 46.0).

PFGE. The novel extremely rare-cutting, intron-encoding restriction enzyme I-*Ceu*I was the only enzyme which cleaved the genes of these organisms with sufficient rarity to allow for the determination of similarity coefficients. I-*Ceu*I restriction enzyme digestion generated seven or eight restriction fragments from each of the isolates studied (Fig. 6A). The high-molecular-mass restriction bands seen in each lane remained unresolved; therefore, meaningful comparisons of the sizes of these bands could not be made. However, all 12 *H. alvei* strains (including the reference strain 19982) shared three common restriction bands of between 50 and 180 kb in size (Fig. 6A, arrows). In addition, all 11 clinical isolates from Canada shared either one (strains 4 and 6) or two restriction bands of between 375 and 450 kb. These bands were absent from the reference strain. In addition, the reference strain harbored a large,

900-kb band that was not seen in any of the Canadian strains (Fig. 6A).

The restriction enzymes *Not*I and *Sfi*I generated >20 restriction fragments from each isolate. The restriction patterns obtained with these enzymes confirmed the identical I-*Ceu*I-generated genomic profiles observed for strains H1 and H2 (Fig. 6B). In addition, strain H3 was shown to be closely related to H1 and H2. However, these more frequently cutting restriction enzymes failed to demonstrate any significant degree of similarity between any of the other isolates (data not shown). It is interesting that while H1 and H2 have identical restriction patterns, they were from different patients with no apparent contact between them. In contrast, H1 and H3 were from the same patient, and their restriction patterns were similar but not identical.

With I-*Ceu*I, similarity coefficients among the 12 clinical strains varied between 0.6 and 1.0. Three pairs of isolates, i.e., strains H1 and H2, strains H5 and H7, and strains H9 and D46-NF, were identical when I-*Ceu*I was used. I-*Ceu*I macrorestriction patterns for the reference strain 19982 demonstrated greater divergence from all of the Canadian *H. alvei* strains. The coefficient of similarity between strain 19982 and other *H. alvei* strains studied did not exceed 0.66.

Plasmid profiles. Plasmids were identified in 7 of the 12 isolates (data not shown). Strains H1 to H4, H8, and D67-NF harbored a plasmid of approximately 3 kb. The *H. alvei* reference strain contained a 1-kb plasmid which was not present in any of the other strains. No correlation was observed between plasmid profiles and other genotypic or phenotypic features of the strains studied.

DISCUSSION

H. alvei strains isolated in Bangladesh from children with diarrhea have been shown to adhere to epithelial cells in an AE pattern and to possess the *eaeA* gene (2, 3), which is considered to be an underlying virulence factor. In the present study, we examined clinical isolates of *H. alvei* from two different Canadian centers to determine their abilities to demonstrate the AE phenotype and whether the *eaeA* gene might be the basis of their virulence.

The rearrangement of cytoskeletal elements, including F-actin and α-actinin, underneath foci of bacterial attachment is a hallmark of AE pathogens (6, 11, 17). We have previously shown that α-actinin rearrangement in infected epithelial cells is a reliable method for determining the ability of putative AE pathogens to rearrange cytoskeletal elements and their potential to form AE lesions (14). Therefore, we tested the Canadian isolates of *H. alvei* for their ability to induce α-actinin rearrangement in infected HEp-2 epithelial cells. The Bangladeshi *H. alvei* strain 19982 and the AE-positive EPEC strain E2348/69 showed intense foci of α-actinin fluorescence in regions subjacent to areas of bacterial attachment. These results are in agreement with the original observations of Albert et al. (3), who showed a positive fluorescent actin staining test for *H. alvei* 19982. In contrast, none of the 11 clinical isolates from Canada induced an α-actinin response, indicating that they are not AE organisms.

To confirm the lack of an AE phenotype, selected strains of *H. alvei* were examined by transmission electron microscopy to detect the formation of AE lesions on infected HEp-2 cells. Consistent with the α-actinin assay, only the α-actinin-positive *H. alvei* strain 19982 showed electron-dense adhesion pedestals typical of AE pathogens. In contrast, the two *H. alvei* test strains, while adhering to cells and effacing microvilli, failed to demonstrate an AE adhesion pattern. As this finding was similar to that obtained previously with an *eaeA*-negative VTEC strain of serotype O113:H21 (9), we also examined whether the Canadian *H. alvei* isolates possessed the *eaeA* gene. The strains were shown, by both PCR and colony blot hybridization analyses, to be deficient in the *eaeA* gene. These results indicate that the adhesion mechanisms of *H. alvei* isolated in Canada are phenotypically and genotypically distinct from those of *H. alvei* isolated in Bangladesh. This indicates that there is heterogeneity in the virulence properties of *H. alvei* strains found in different geographical regions.

Heterogeneity among *H. alvei* strains with respect to the presence of the *eaeA* gene may have a number of explanations. For instance, the Canadian strains tested in our study could have spontaneously lost the *eaeA* gene. This possibility is unlikely, however, because the *eaeA* gene is chromosomally encoded (6), and spontaneous loss of *eaeA* is uncommon in *eaeA*-positive EPEC and VTEC. Alternatively, some *H. alvei* strains, such as strain 19982, may have acquired this gene by horizontal transfer from EPEC or VTEC.

H. alvei strains with and without the *eaeA* gene may represent separate taxons. Earlier studies have shown that there is only 50 to 55% DNA homology between different strains of *H. alvei* (30), although those studies did not involve examination of the *eaeA*-positive and -negative strains. More recently, Ridell et al. (22) reported that *eaeA*-positive *H. alvei* strains show certain unique biochemical reactions that are not demonstrated by the *eaeA*-negative *Hafnia* isolates. In addition, by randomly amplified polymorphic DNA-PCR, the *eaeA*-positive *H. alvei* strains had identical profiles that differed from those of the *eaeA*-negative strains. Partial sequencing of the 16S rRNA showed that *eaeA*-positive *H. alvei* strains had greater homology with EPEC than did the *eaeA*-negative *H. alvei* strains (22). In the present study, we demonstrated that OMP profiles of Canadian *eaeA*-negative *H. alvei* isolates were identical to each other but differed from that of *H. alvei* 19982 from Bangladesh. Moreover, the OMP profile of the *eaeA*-positive strain 19982 more closely resembled that of VTEC than that of any of the other *eaeA*-negative *H. alvei* isolates. Analysis of macrorestriction profiles generated by PFGE demonstrated a clear difference between the genomic DNA profiles of strain 19982 and the Canadian isolates tested. Strain 19982 also contains a unique 1-kb plasmid. In light of these data, the present study suggests that the current taxonomic placement of *H. alvei* should be reconsidered. Therefore, future epidemiological studies on enteropathogenic *H. alvei* need to go beyond simple species designations and should be adapted to specifically identify the virulent clones.

We also showed in this study that *H. alvei* 19982 is able to induce a protein tyrosine kinase response. This finding is similar to that obtained for EPEC infection, in which activation of a tyrosine kinase signal is essential for subsequent cytoskeletal rearrangement (25). Similar to the case for EPEC, phosphotyrosine proteins are rearranged underneath the adherent *H. alvei* 19982. This suggests that cytoskeletal rearrangement observed upon infection with *H. alvei* 19982 is a consequence of an upstream tyrosine kinase signal, because only strain 19982 shows a positive fluorescent actin staining response (3, 23) and recruitment of α-actinin to form AE lesions.

Most of the Canadian *H. alvei* isolates were from the stools of patients suffering with diarrhea, but their etiological relationship to enteritis is unclear. Since none of the isolates were found to form AE lesions, one possibility is that these strains are transient organisms that were being isolated as part of the commensal flora of the intestine. However, in a recent study from Finland, Ridell et al. (23) found a strong epidemiological association of *H. alvei* with diarrhea, in that they isolated this

organism only from patients with diarrhea. The isolates from Finland were also negative for the AE phenotype by fluorescent actin staining testing (23). Therefore, another possibility is that novel, but as-yet-unidentified, virulence factors distinct from the *eaeA* gene are responsible for the diarrheagenic potential of some strains of *H. alvei*, including those described in this paper. These *eaeA*-negative strains did adhere to epithelial cells and effaced microvilli in vitro when observed by transmission electron microscopy. Potential virulence factors could include the production of as-yet-unidentified enterotoxins and invasion. For instance, the novel 25-kDa OMP that we have shown to be present in the Canadian *H. alvei* isolates but absent from the *eaeA*-positive reference strain might have a role as an adhesin in the *eaeA*-negative *H. alvei* pathogenesis.

Other virulence factors could include bacterial products that induce the activation of intracellular second-messenger molecules in infected eukaryotic cells, including, for example, inositol 1,4,5-triphosphate and intracellular free calcium. Both of these second messengers have been previously implicated to play a role in the pathogenesis of diseases caused by other enteropathogens (4, 8, 12). Increases in intracellular free calcium and other second-messenger molecules lead to diarrhea by the secretion of ions and water in the intestine due to the opening of Ca^{2+}-mediated chloride channels (7). Future studies will therefore examine the ability of the Canadian *eaeA*-negative *H. alvei* isolates to induce signal transduction responses in infected epithelial cells.

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REFERENCES

1. **Achtman, M., A. Mercer, B. Kusecek, A. Pohl, M. Heuzenroeder, W. Aaronson, A. Sutton, and R. P. Silver.** 1983. Six widespread bacterial clones among *Escherichia coli* K1 isolates. Infect. Immun. **39:**315–335.
2. **Albert, M. J., K. Alam, M. Islam, J. Montanaro, A. S. M. H. Rahman, K. Haider, M. A. Hossain, A. K. M. G. Kibriya, and S. Tzipori.** 1991. *Hafnia alvei*, a probable cause of diarrhea in humans. Infect. Immun. **59:**1507–1513.
3. **Albert, M. J., S. M. Faruque, M. Ansaruzzaman, M. M. Islam, K. Haider, K. Alam, I. Kabir, and R. Robins-Browne.** 1992. Sharing of virulence-associated properties at the phenotypic and genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei*. J. Med. Microbiol. **37:**310–314.
4. **Baldwin, T. J., W. Ward, A. Aitken, S. Knutton, and P. H. Williams.** 1991. Elevation of intracellular free calcium levels in HEp-2 cells infected with enteropathogenic *Escherichia coli*. Infect. Immun. **59:**1599–1604.
5. **Bourke, B., P. Sherman, H. Louie, E. Hani, P. Islur, and V. L. Chan.** 1995. Physical and genetic map of the genome of *Campylobacter upsaliensis*. Microbiology **141:**2417–2424.
6. **Donnenberg, M. S., and J. B. Kaper.** 1992. Enteropathogenic *Escherichia coli*. Infect. Immun. **60:**3953–3961.
7. **Donowitz, M., M. E. Cohen, M. Gould, and G. W. G. Sharp.** 1989. Elevated intracellular Ca^{2+} acts through protein kinase C to regulate rabbit ileal NaCl absorption. J. Clin. Invest. **83:**1953–1962.
8. **Dytoc, M., L. Fedorko, and P. M. Sherman.** 1994. Signal transduction in human epithelial cells infected with attaching and effacing *Escherichia coli* in vitro. Gastroenterology **106:**1150–1161.
9. **Dytoc, M., A. Ismaili, D. J. Philpott, R. Soni, J. L. Brunton, and P. M. Sherman.** 1994. Distinct binding properties of *eaeA*-negative verocytotoxin-producing *Escherichia coli* of serotype O113:H21. Infect. Immun. **62:**3494–3505.
10. **Dytoc, M., R. Soni, F. Cockerill III, J. DeAzavedo, M. Louie, J. Brunton, and P. Sherman.** 1993. Multiple determinants of verotoxin-producing *Escherichia coli* O157:H7 attachment-effacement. Infect. Immun. **61:**3382–3391.
11. **Finlay, B. B., I. Rosenshine, M. S. Donnenberg, and J. B. Kaper.** 1992. Cytoskeletal composition of attaching and effacing lesions associated with enteropathogenic *Escherichia coli* adherence to HeLa cells. Infect. Immun. **60:**2541–2543.
12. **Foubister, V., I. Rosenshine, and B. B. Finlay.** 1994. A diarrheal pathogen, enteropathogenic *Escherichia coli* (EPEC), triggers an inositol phosphate flux in infected epithelial cells. J. Exp. Med. **179:**993–998.
13. **Ismaili, A., D. Philpott, M. Dytoc, and P. Sherman.** 1995. Signal transduction responses following adhesion of verocytotoxin-producing *Escherichia coli*. Infect. Immun. **63:**3316–3326.
14. **Ismaili, A., D. J. Philpott, M. Dytoc, R. Soni, S. Ratnam, and P. M. Sherman.** 1995. Alpha-actinin accumulation in epithelial cells infected with attaching and effacing gastrointestinal pathogens. J. Infect. Dis. **172:**1393–1396.
15. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87:**7839–7843.
16. **Kim, N. W., R. Lombardi, H. Bingham, E. Hani, H. Louie, D. Ng, and V. L. Chan.** 1993. Fine mapping of the three rRNA operons on the updated genomic map of *Campylobacter jejuni* TGH9011 (ATCC 43431). J. Bacteriol. **175:**7468–7470.
17. **Knutton, S., T. Baldwin, P. H. Williams, and A. S. McNeish.** 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. Infect. Immun. **57:**1290–1298.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) **227:**680–685.
19. **Louie, M., J. deAzavedo, R. Clarke, A. Borczyk, H. Lior, M. Richter, and J. Brunton.** 1994. Sequence heterogeneity of the *eae* gene and detection of Verotoxin-producing *Escherichia coli* using serotype-specific primers. Epidemiol. Infect. **112:**449–461.
20. **Moon, H. W., S. C. Whipp, R. A. Argenzio, M. M. Levine, and R. A. Giannella.** 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. **1340–1351.**
21. **Ratnam, S., R. W. Butler, S. March, S. Parsons, P. Clarke, A. Bell, and K. Hogan.** 1979. Enterobacter hafniae-associated gatroenteritis. Newfoundland Can. Dis. Weekly Rep. **5:**231–232.
22. **Ridell, J., A. Siitonen, L. Paulin, O. Lindroos, H. Korkeala, and M. J. Albert.** 1995. Characterization of *Hafnia alvei* by biochemical tests, random amplified polymorphic DNA PCR, and partial sequencing of 16S rRNA gene. J. Clin. Microbiol. **33:**2372–2376.
23. **Ridell, J., A. Siitonen, L. Paulin, L. Mattila, H. Korkeala, and M. J. Albert.** 1994. *Hafnia alvei* in stool specimens from patients with diarrhea and healthy controls. J. Clin. Microbiol. **32:**2335–2337.
24. **Romling, U., D. Grothues, T. Heuer, and B. Tummler.** 1992. Physical genome analysis of bacteria. Electrophoresis **13:**626–631.
25. **Rosenshine, I., M. S. Donnenberg, J. B. Kaper, and B. B. Finlay.** 1992. Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phosphorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. EMBO J. **11:**3551–3560.
26. **Sakazaki, R.** 1961. Studies on the Hafnia group of Enterobacteriaceae. Jpn. J. Med. Sci. Biol. **14:**223–241.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Schauer, D. B., and S. Falkow.** 1993. Attaching and effacing locus of a *Citrobacter freundii* biotype that causes transmissible murine colonic hyperplasia. Infect. Immun. **61:**2486–2492.
29. **Sherman, P., R. Soni, M. Petric, and M. Karmali.** 1987. Surface properties of the Vero cytotoxin-producing *Escherichia coli* O157:H7. Infect. Immun. **55:**1824–1829.
30. **Steigerwalt, A. G., G. R. Fanning, M. A. Fife-Asbury, and D. J. Brenner.** 1976. DNA relatedness among species of *Enterobacter* and *Serratia*. Can. J. Microbiol. **22:**121–137.