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Identification of a Family of Intimins Common to *Escherichia coli* Causing Attaching-Effacing Lesions in Rabbits, Humans, and Swine

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Intimin, an outer membrane protein encoded by *eaeA* that mediates close attachment of enteropathogenic bacteria to apical surfaces of epithelial cells, is required for formation of the attaching-effacing lesions and for full pathogenesis of the bacteria. Analysis of the *eaeA* sequence indicates that there is a high degree of homology at the N termini but less at the C termini of intimins. Antisera specific for the C-terminal third of RDEC-1 intimin, used to screen outer membrane proteins from 50 rabbit enteropathogenic *Escherichia coli* (EPEC), human EPEC, and human enterohemorrhagic *E. coli* (EHEC) strains, identified cross-reactive intimins from 24 isolates. Sequence analysis of the *eaeA* genes from human EPEC O111 and EHEC O26 isolates indicates that their intimins have C termini nearly identical to that of RDEC-1 intimin. Our results suggest that there are at least three families of related intimins and that the presence of intimin similar to that of RDEC-1 is not restricted by serogroup or host specificity.

A number of enteropathogenic bacteria, including enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *Hafnia alvei* (4), and *Citrobacter rodentium* (formerly *Citrobacter freundii* biotype 4320) (41), cause attaching-effacing (AE) lesions in the gut mucosa. These bacteria have been isolated from humans and a number of animal species, including rabbits, pigs, mice, cows, sheep, and dogs (6, 8, 13, 20, 21, 29, 35, 37, 43, 49). AE bacteria generally infect the small or large intestine and cause diarrhea (as with EPEC and *H. alvei*) (30) or bloody diarrhea (38) (and in certain cases hemolytic uremic syndrome [26]). A strain of *C. rodentium*, a murine enteropathogen, infects the large intestine and causes transmissible murine colonic hyperplasia in young mice (40). Although there are a wide variety of hosts and clinical symptoms, the presence of the AE lesion appears to be associated with fluid secretion and diarrhea that are characteristic of infection by all of these bacteria (30).

The AE lesion, first reported by Staley et al. (43), has a characteristic pattern of effacement of microvilli, pedestal formation, and intimate adherence of bacteria to the epithelial cell membrane as observed by electron microscopy. The induction of AE lesions has been described in the three-stage model of EPEC pathogenesis, presented by Donnenberg and Kaper (11), involving initial attachment of the bacteria to epithelial cells, signal transduction and phosphorylation of host proteins, and intimate attachment of the bacteria to the apical surfaces of the epithelial cells. Host specificity appears to be determined by the presence of plasmid-encoded pili, such as bundle-forming pili in human strains (12) and AF/R1 pili in the rabbit EPEC (REPEC) strain RDEC-1 (47), which mediate the initial attachment of the bacteria to host cells. All AE bacteria have a chromosomal pathogenicity cassette, called LEE (for locus of enterocyte effacement), which is a 35-kb region of DNA that encodes the gene products necessary for formation of the AE lesion; these products include proteins that mediate attachment, proteins similar to those found in type III secretory pathways, and proteins involved in phosphorylation of host epithelial cell proteins (33, 39).

The components of LEE (15, 22, 27, 28, 33) and their role in pathogenesis have been examined for human EPEC E2348/69 (O127:H6) and EHEC O157:H7 strains. Research in our laboratory has focused on the use of RDEC-1 as a model of AE pathogenesis. RDEC-1, a REPEC strain, causes AE lesions in rabbits (44) that are indistinguishable from those produced by human EPEC (43). In addition, RDEC-1 induces AE lesions in human cultured cells if it expresses adhesins that mediate initial attachment (9, 23). The RDEC-1 strain lysogenized with bacteriophage-encoded Shiga-like toxin I produces an EHEC-like disease in rabbits (42). This animal model of diarrhea provides an opportunity to study the pathogenesis of AE bacteria.

One of the factors that promotes intimate adherence is the product of *eaeA*, a 94- to 97-kDa outer membrane protein (OMP) known as intimin. Recently, Rosenshine et al. (39) have identified the host epithelial cell receptor, a tyrosine-phosphorylated membrane protein called Hp90, that associates directly with intimin. The *eaeA* gene, located on the bacterial chromosome as part of LEE (33), has been cloned and sequenced from human EPEC (24), human EHEC (5, 48), and *H. alvei* (C terminus only) (17), as well as from *C. rodentium* (40). We have cloned RDEC-1 *eaeA* (3) and have shown it to have greater than 80% DNA sequence homology to the EPEC, EHEC, and *C. rodentium eaeA* genes. Amino acid sequence comparisons of intimins show greater than 90% homology over the first 659 amino acid residues but less homology over the remaining 280 residues at the C terminus of the protein. Using an intimin fusion protein and an in vitro binding assay, Frankel et al. (18) have demonstrated that the binding activity of EPEC intimin is located at the C terminus of the protein. Experimental evidence (13, 17, 25) indicates that changes in the amino acid sequence among intimins represent antigenic variation, not functional differences. It is not known if these antigenic differences represent individual variation or if there is a discrete number of families of antigenically related intimins.

In this study, we describe the immunological cross-reactivi-

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TABLE 1. Immunological cross-reactivities of intimins

Strain or species	Host (class)	Serotype[a]	Reactivity[b]
RDEC-1	Rabbit (REPEC)	O15:H−	+
933	Human (EHEC)	O157:H7	−
H19	Human (EHEC)	O26:H11	+
8:84	Human (EHEC)	O157:H7	−
3213-86	Human (EHEC)	O157:H7	−
B6914-M51	Human (EHEC)	O157:H7	−
H30	Human (EHEC)	O26	−
A9619-C2	Human (EHEC)	O45:H2	−
B1545	Human (EHEC)	NA	−
3119-86	Human (EHEC)	NA	−
B2387	Human (EHEC)	NA	−
3124-85	Human (EHEC)	NA	−
G85	Human (EHEC)	O26:B6	+
G667	Human (EPEC)	O128	−
E2348/69	Human (EPEC)	O127:H6	−
E2362/75	Human (EPEC)	O55:NM	−
E2430/78	Human (EPEC)	O111:NM	+
E2450/80	Human (EPEC)	O119:H6	+
E851/71	Human (EPEC)	O142:H6	−
E128010	Human (EPEC)	O114	−
Chile 38	Human (EPEC)	O86	−
G87 (Germany)	Human (EPEC)	O111:B4	+
G88 (Mexico)	Human (EPEC)	O111:B4	−
C. rodentium	Mouse (colonic hyperplasia)	NA	−
DBS 364	Rabbit (REPEC)	NA	−
DBS 369	Rabbit (REPEC)	NA	+
DBS 387	Rabbit (REPEC)	NA	+
E232	Rabbit (REPEC)	O109:H2	+
U82/123	Rabbit (REPEC)	O109:H2	−
U82/160	Rabbit (REPEC)	O109:H2	−
U82/215	Rabbit (REPEC)	O109:H2	−
U82/72	Rabbit (REPEC)	O128:H−	−
U82/80	Rabbit (REPEC)	O132:H sp.agg	−
U82/90	Rabbit (REPEC)	O132:H2	+
U82/146	Rabbit (REPEC)	O132:H sp.agg	+
U82/150	Rabbit (REPEC)	O132:H sp.agg	+
U82/168	Rabbit (REPEC)	O132:H2	Weak
U82/183	Rabbit (REPEC)	O128:H2	Weak
U83/4	Rabbit (REPEC)	O132:H2	Weak
U82/260	Rabbit (REPEC)	O20:H7	Weak
U83/45	Rabbit (REPEC)	O128:H2	−
U82/158	Rabbit (REPEC)	O sp.agg:H7	Weak
U82/85	Rabbit (REPEC)	O15:H−	+
U82/95	Rabbit (REPEC)	O109:H2	+
U82/207	Rabbit (REPEC)	O109:H sp.agg	+
U82/172	Rabbit (REPEC)	O109:H2	−
U83/11	Rabbit (REPEC)	O15:H−	+
U83/12	Rabbit (REPEC)	O15:H−	+
U83/39	Rabbit (REPEC)	O15:H−	+
U83/40	Rabbit (REPEC)	O15:H−	+
U83/68	Rabbit (REPEC)	O15:H−	+

[a] Serotype information was provided by the strain source or was contained in culture collection records at WRAIR. NA, serogroup information not available; sp.agg, spontaneous agglutination.

[b] Cross-reactivity was measured by immunoblotting with anti-RDEC intimin at a 1:5,000 dilution. Reactivity was measured as positive (+), weakly positive (Weak), or negative (−).

ties of intimins isolated from human (EPEC and EHEC) and rabbit (REPEC) strains with antisera specific for RDEC-1 intimin. Sequence analyses of intimins from three human EPEC and EHEC strains (O111 and O26 serogroups) indicate that these bacteria possess intimins nearly identical to RDEC-1 intimin.

The bacterial strains used in this study are listed in Table 1. REPEC strains were kindly provided by Johan Peeters and have been described in detail elsewhere (37). *C. rodentium* and additional REPEC strains were gifts from David Schauer at the Massachusetts Institute of Technology. RDEC-1, human EPEC, and human EHEC strains (gifts from J. Robert Cantey, Ed Boedeker, Sam Formal, David Maneval, and John Newland) are part of the culture collection at the Walter Reed Army Institute of Research (WRAIR). Bacteria were cultured at 37°C on Luria-Bertani (LB) agar and Maconkey agar (Difco,

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Detroit, Mich.) or in LB broth and Antibiotic Medium 3 broth (Penassay broth; Difco) as required.

An RDEC-1 intimin fusion protein was generated by methods described by Frankel et al. (17). A region of RDEC-1 *eaeA* corresponding to the terminal 282 amino acid residues was amplified by PCR and cloned in the same translational reading frame as the *malE* gene of *Escherichia coli*. The forward primer, including an added *Eco*RI restriction linker (5′CTGA ATTCGCCAGTATTACTGAGATTAAG), and the reverse primer, including an added *Xba*I restriction linker (5′-CTTCT AGAATTATTTACACAAACAGAG), were synthesized by using PCR Mate DNA Synthesizer model 391 (Applied Biosystems, Foster City, Calif.) and purified by using PD10 Sephadex G-25 columns (Pharmacia Biotech, Uppsula, Sweden). Cloned RDEC-1 *eaeA* (p357-1) was used as a template for PCR (GeneAmp PCR Kit and DNA Thermal Cycler; Applied Biosystems), and the reactions were performed for 25 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The 862-bp PCR fragment was purified, digested with *Eco*RI and *Xba*I, and ligated to the pMAL-p2 expression vector (New England Biolabs, Beverly, Mass.) by using T4 ligase (Gibco). The plasmid construct was transformed into competent *E. coli* DH5α, and the intimin fusion protein was isolated from the bacterial periplasim according to the manufacturer's instructions (New England Biolabs). Antisera against the fusion protein were raised in New Zealand White rabbits (Hazelton Biotechnologies, Vienna, Va.) by intramuscular injection of 500 mg of fusion protein in complete Freund's adjuvant (Difco) followed by a second injection 3 weeks later with 300 mg of protein in incomplete Freund's adjuvant (Difco). Antisera were collected 14 days later and tested for reactivity with the fusion protein and maltose binding protein by immunoblotting. The antisera were absorbed against whole cells of *E. coli* DH5α and maltose binding protein (to remove nonspecific antibodies) by incubating sera and bacteria at room temperature for 4 to 8 h with constant shaking. The suspension was centrifuged to remove bacteria, and the antisera were transferred to clean tubes. Antisera were stored at 4°C in 0.02% sodium azide (Sigma) until used.

OMPs were prepared by using a modification of the procedure of Fedorka-Cray et al. (16). Cells were grown overnight at 37°C in Penassay broth (Difco) and centrifuged at 1,200 × *g* and 4°C. Bacterial pellets were resuspended in 1 ml of 10 mM HEPES (pH 7.4) and maintained on ice for the remainder of the procedure. Bacteria were disrupted with a Sonifer Cell Disruptor (model W140; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) fitted with a microtip (Misonic Incorporated, Farmingdale, N.Y.) for 2 min at 20% relative output. Cellular debris were removed by centrifugation for 10 min at 9,700 × *g* at 4°C; bacterial cell membranes were collected by centrifugation of the supernatants for 30 min at 15,800 × *g*. The membrane pellets were resuspended in 400 μl of 10 mM HEPES (pH 7.4)–1% *N*-lauroylsarcosine (Sigma) and incubated at room temperature with shaking for 30 min. OMPs were collected by centrifugation for 30 min at 15,800 × *g*, and the pellets were washed once (without mixing) in HEPES. The pellets, containing OMPs, were resuspended in 100 μl of HEPES and stored at −20°C until analyzed. OMPs were separated on 10% Tricine gels (Novex, San Diego, Calif.) for 1.5 h at 125 V. Proteins were visualized by staining with Rapid Coomassie stain (Diversified Biotech, Boston, Mass.) according to the manufacturer's instructions. When immunoblots were required, duplicate gels were prepared, one for protein visualization and one for transfer to nitrocellulose. Proteins were transferred to nitrocellulose (0.2-μm-pore-size Protran Nitrocellulose; Schleicher and Schuell, Keene, N.H.) according to the manufacturer's instructions (Novex). Nonspecific binding of proteins was blocked by incubating the nitrocellulose in 7% nonfat dry milk in 10 mM Tris-HCl–150 mM NaCl–0.05% Tween 20 (TBST) at room temperature for 1 h. Primary and secondary antisera were diluted to appropriate concentrations in 2% nonfat dry milk in TBST. Membranes were washed three times with TBST before and after incubation with antisera. Anti-intimin rabbit serum was diluted at 1:5,000 for specific detection of intimin. Immunoblots were developed with peroxidase-labeled goat anti-rabbit immunoglobulin G (1:30,000 dilution) and TMB peroxidase reagent with membrane enhancer according to manufacturer's instructions (Kirkegaard and Perry Laboratories, Gaithersburg, Md.).

TABLE 2. Comparison of the variable regions of intimins to that of RDEC-1 intimin

Organism[a]	% Identity[b]	
	DNA	Amino acid
EPEC E2430/78 (O111a,b:NM)[c]	99.6	99.6
EHEC H19 (O26:H11)[c]	99.8	100
EHEC G85 (O26:B6)[c]	99.8	100
REPEC 84/110/1 (O103)	99.9	99.9
C. rodentium 4280	84.0	80.5
EPEC E2348/69 (O127:H6)	60.9	54.4
EHEC 933 (O157:H7)	60.1	55.9
EHEC CL8 (O157:H7)	59.8	55.1
H. alvei	56.3	52.4

[a] See text for GenBank accession numbers.
[b] DNA (849 bp) and amino acid (282 residues) sequence comparisons of the *eaeA* variable regions (C termini) of intimins from the indicated organisms and RDEC-1 intimin were performed with the DNAStar alignment program.
[c] Data are from this study.

OMP preparations from 50 bacterial isolates, including 10 human EPEC, 12 human EHEC, and 27 REPEC strains, were screened for intimins that are recognized by antisera to RDEC-1 intimin (Table 1). The strains are from the culture collection at WRAIR and represent a variety of serotypes isolated from a wide range of geographic locations, including North America, Great Britain, and Europe. All strains produced 94- to 97-kDa proteins, a molecular mass range which is similar to that for intimin. Most REPEC strains (19 of 27), belonging to a variety of serotypes, produced a cross-reactive OMP similar to one produced by RDEC-1. Five of the REPEC strains have an O15:H− serotype like RDEC-1 but, unlike RDEC-1, did not possess the AF/R1 adhesin. Of the 22 human EPEC and EHEC isolates screened, 5 strains possessed OMPs that reacted with antisera specific for RDEC-1 intimin. These strains included E2430/78 (O111), EHEC H19 and G85 (O26), and EPEC 2450/80 (O119) (Table 1). This cross-reactivity did not appear to be serogroup specific, as other EPEC O111 and EHEC O26 isolates screened did not possess intimins that react with the RDEC-1 antisera. Intimins from EPEC E2348/69 (O127:H6) and EHEC 933 (O157:H7), which show only 54 and 56% identity at the C terminus, respectively (Table 2), did not cross-react with RDEC-1 antisera. Figure 1 shows a representative sodium dodecyl sulfate (SDS)-polyacrylamide gel (left panel) and immunoblot (right panel) demonstrating RDEC-1-cross-reactive intimins. The antisera did not recognize 94- to 97-kDa OMPs isolated from DH5α or rabbit commensal *E. coli* strains (data not shown). Consistent with these results, in previous experiments (1) we found that sera from RDEC-infected rabbits recognized intimins from E2430/78 (O111) and EHEC H19 and G85 (O26), but not from EPEC E2348/69 (O127:H6) or EHEC 933 (O157:H7).

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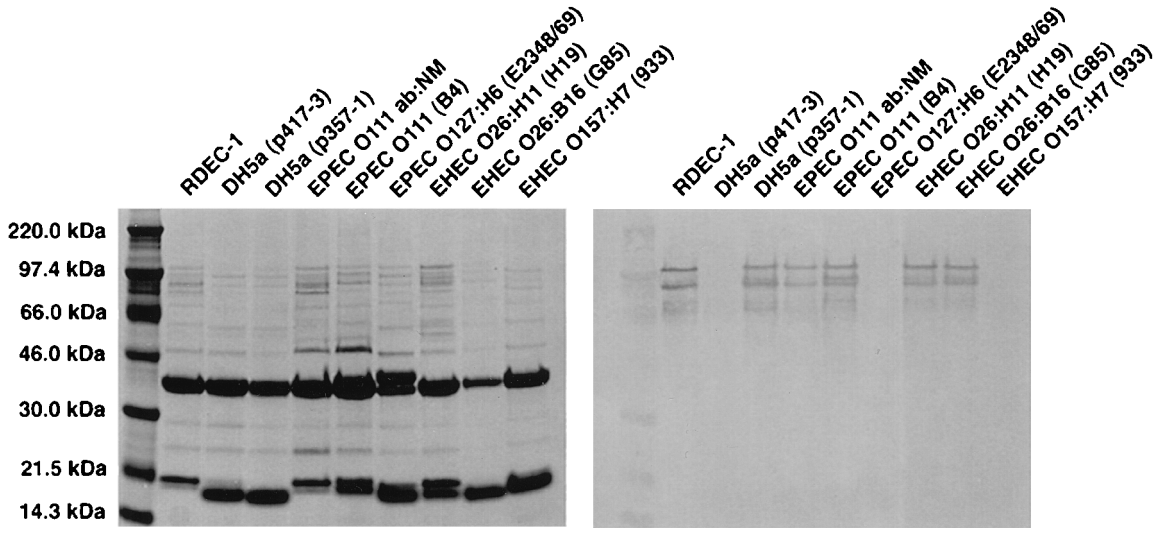

FIG. 1. Cross-reactivities of intimins with antisera specific for RDEC-1 intimin. OMPs were prepared by sonication and detergent extraction and separated by SDS-polyacrylamide gel electrophoresis. (Left) Coomassie blue staining; (right) immunoblot developed with RDEC-1 intimin antisera. The bacterial strains used are indicated. DH5α transformed with p357-1 was the positive control (cloned RDEC-1 *eaeA*), and DH5α transformed with p417-3 was the negative control (*eaeA* deletion derivative of p357-1). Molecular mass markers are in the first lane of each panel, with their apparent molecular masses indicated.

Immunoblots reveal two major protein bands that react with antisera to RDEC-1 intimin, one at approximately 97.5 kDa and a smaller band at approximately 80 to 90 kDa (Fig. 1). N-terminal amino acid sequencing of human EPEC E2348/69 (19) and porcine EPEC 91-19-172 (O45) (50) indicates that intimin is posttranslationally modified so that the N-terminal residue of the mature protein corresponds to the 40th amino acid residue of the predicted amino acid sequence. N-terminal sequence analysis of the larger, 97.5-kDa band observed in RDEC-1 OMP preparations confirms that this band is the mature (posttranslationally modified) protein (starting at residue 40) (1). It is likely that the smaller reactive protein(s) observed on the immunoblots represents proteolytic degradation of intimin during the membrane preparation procedure. We have observed this smaller band in immunoblots of sonicated OMP preparations, but only the 97.5-kDa band is observed in whole-cell preparations probed with antisera specific for RDEC-1 intimin (1). Similar cross-reactive bands were observed by Zhu et al. (50) in their outer membrane preparations, and the larger (97-kDa) protein was also determined to be mature intimin.

To determine the extent of homology among the cross-reactive intimins, we sequenced DNA corresponding to the C termini of the *eaeA* products from three human *E. coli* strains. Primers to the RDEC-1 *eaeA* sequence were used to generate a series of independent PCR products for sequencing the C termini of EPEC E2430/78, EHEC H19, and EHEC G85 intimins. Bacteria were grown overnight in LB broth, washed three times in phosphate-buffered saline, and resuspended in sterile water to a density of $>10^8$ cells/ml. PCR was as described above but with 1 μl of whole cells as the template. PCR products were purified by using STE Select G-50 columns (5 Prime-3 Prime, Boulder, Colo.) or the Wizard PCR Purification System (Promega Corporation, Madison, Wis.) and used as templates in sequencing reactions. Primers used to generate the RDEC-1 intimin fusion protein were used to amplify DNA corresponding to the carboxyl-terminal 282 amino acids of EPEC E2430/78 (O111), EHEC H19 (O26), and EHEC G85 (O26). The resulting PCR products were purified, digested with *Eco*RI and *Xba*I, ligated to the pUC19 vector, and transformed into *E. coli* DH5α. Successful cloning was confirmed by using restriction enzyme digestion and PCR (with RDEC-1 primers). Purified plasmids containing cloned DNA were used as templates for DNA sequencing.

Templates for DNA sequencing were prepared by PCR (as described above) or with the JETSTAR Midi plasmid kit (Genomed INC., Research Triangle Park, N.C.) and sequenced by AmpliTaq cycle sequencing with fluorescent-dye-labeled chain termination chemistry and an ABI 373A DNA sequencer (Applied Biosystems/Perkin Elmer). Electropherograms were analyzed and edited by using Sequencher 3.0 Analysis Software (Gene Codes, Ann Arbor, Mich.). Comparisons of *eaeA* sequences were performed by using the database at the National Center for Biotechnology Information (NCBI) (National Institutes of Health, Bethesda, Md.) via the BLAST search algorithm and by the DNASTAR alignment software program (DNASTAR, Madison, Wis.). The following strains and sequences (accession numbers are in parentheses) were used in comparisons: RDEC-1 (U60002), REPEC 84/110/1 (U59502), EPEC E2348/69 (M34051), EHEC 933 (Z11541), *H. alvei* (L29509), and *C. rodentium* (L11691).

Comparison of the sequence from the *C. rodentium* intimin variable region indicates that it is closely related to RDEC-1 intimin, yet antiserum to RDEC-1 intimin does not cross-react with *C. rodentium* intimin (Table 1). This suggests that the intimins isolated from the cross-reactive REPEC and human EPEC and EHEC strains are greater than 80% homologous to RDEC-1 intimin, while those that did not react either did not produce intimin or express an antigenically distinct intimin. In order to determine the extent of homology of the variable regions of the intimins that are cross-reactive with RDEC-1 intimin, we sequenced 849 bp of DNA corresponding to the C termini of the intimins. The DNA sequences from EPEC E2430/78 (O111) and EHEC H19 and EHEC G85 (O26) were derived from multiple independently generated PCR fragments. The use of PCR products from independent reactions confirmed that similarities (or differences) in the sequence were not a result of errors introduced during a single PCR amplification.

The DNA sequence of 849 bp from EPEC E2430/78 (O111)

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eaeA indicated there were three base differences and 99.6% sequence homology compared with *eaeA* from RDEC-1. The predicted amino acid sequence from EPEC E2430/78 intimin indicates 99.6% amino acid homology compared with the predicted amino acid sequence of RDEC-1 intimin. The amino acid sequence from EPEC E2430/78 shows that there is a single amino acid residue difference (residue 932) compared with the protein sequence of RDEC-1 intimin.

Two different EHEC O26 *eaeA* sequences were obtained, and they were identical. Comparison of these *eaeA* sequences with *eaeA* from RDEC-1 shows there are two base differences in the EHEC sequences and 99.8% DNA homology. The two base differences result in no amino acid residue differences, indicating there is 100% protein sequence homology over this region of intimin.

The *eaeA* gene from RDEC-1 and its predicted amino acid sequence at the C terminus of intimin have a high degree of sequence variation compared with the five intimin sequences in the NCBI database (3). In this study, we have identified cross-reactive OMPs isolated from a variety of REPEC strains and human EPEC and EHEC strains. The C termini of intimins from three cross-reactive human EPEC and EHEC isolates were cloned, and their sequences showed they are nearly identical to RDEC-1 intimin. Table 2 summarizes the DNA and amino acid sequence comparisons of the variable regions of the intimins from this study and from the NCBI database.

Five human AE bacterial isolates possessed intimins that cross-reacted with antisera specific to RDEC-1 intimin, and the relationship of EHEC H19, EHEC G85, and EPEC E2430/78 intimins to RDEC-1 intimin was supported by DNA sequence analysis. The results indicate that these strains belong to a family of enteropathogens that possess antigenically related intimins. Jerse and Kaper (25) found evidence of a second distinct family of intimins by using antisera against EPEC E2348/69 (O127:H6) intimin. The antiserum used in these experiments was raised against an alkaline phosphatase fusion protein that contained most of the intimin protein (24). In their study (25), they screened 22 EPEC and EHEC isolates belonging to the O127, O55, O86, O111, O114, O119, O142, O26, and O157 serogroups and RDEC-1 for intimins that cross-react with antisera to EPEC E2348/69 intimin. They found that 14 of 22 isolates expressed intimins that cross-react with EPEC E2348/69 antisera (including O127, O55, O86, O111, O114, O1119, O142, and O26 serogroups), while 8 of 22 isolates (O15, O127, O114, O26, and O157 serogroups) did not express cross-reactive intimins. EPEC E2348/69 antisera did not recognize RDEC-1 or EHEC 933 intimin (25). Most of the isolates in that study were not identified by individual strain designations, so it is not known if any are identical to isolates used in our study. On the basis of our data and data presented by Jerse and Kaper (25), there appear to be at least three families of antigenically related intimins, i.e., those similar to intimins from RDEC-1, EPEC E2348/69, and EHEC 933, respectively. Taken together, the identification of antigenically related intimin families and functional studies in vitro (17) and in vivo (13) indicate that the C termini of intimins are antigenically, but not functionally, different. Our studies indicate that a group of *E. coli* strains isolated from different hosts and from a wide range of geographic locations has related intimins. This suggests that these strains are closely related in the evolution of their pathogenicity or at least in their acquisition of LEE.

It was not surprising that most of the REPEC isolates screened possessed intimins that cross-react with antisera specific for RDEC-1 intimin. The discovery of an intact, conserved locus in a group of diverse bacteria is consistent with a group of virulence genes that are spread by horizontal transfer during recent evolution (33). If there is horizontal transfer of LEE between bacteria, this may readily occur in strains that can infect the same host species. It was surprising that some of the rabbit isolates did not react or gave a weak reaction with antisera to RDEC-1 intimin. These strains all produced proteins observed by SDS-polyacrylamide gel electrophoresis to be in the size range reported for intimin. The weak reaction or absence of reaction with the antisera does not appear to be serogroup specific. This suggests these rabbit strains have intimins antigenically distinct from that of RDEC-1, possibly belonging to one of the other intimin families.

The DNA sequence from a REPEC strain was recently submitted to the NCBI database. Sequence comparison of *eaeA* from REPEC strain 84/110/1 (O103) (accession number U59502) to RDEC-1 *eaeA* indicates that these genes are nearly identical. In addition, intimin from this strain reacts with our antisera (45). Zhu et al. (50) have described intimin from a porcine AE *E. coli* 91-19-172 (O45) isolate. Antisera against formalin-treated EPEC 91-19-172 and RDEC-1 reacted with intimins from either strain but not with intimin from human EPEC E2348/69 (O127:H6). Taken together with the cross-reactivity data and sequence comparisons presented here, this suggests that the porcine EPEC 91-19-172 and REPEC 84/100/1 isolates also possesses intimins that belong to the RDEC-1 intimin family. The sequences of intimins from AE bacteria isolated from other animal hosts have not been determined.

The evolutionary relationships among AE *E. coli* strains have been studied by multilocus enzyme electrophoresis (to characterize chromosomal genotypes) and with gene probes and adherence assays (to characterize virulence properties) (7, 36, 46). These studies suggest that most of the diarrheal outbreaks and sporadic cases are caused by a small proportion of related *E. coli* clones. Using multilocus enzyme electrophoresis, Whittam et al. (46), have established that EHEC O157:H7 (associated with hemolytic colitis and hemolytic uremic syndrome) is most closely related to O55:H7 strains (associated with infantile diarrhea) and only distantly related to O157 strains (associated with enteric infections in animals). As the *eaeA* gene is chromosomally encoded, its sequence could also be used as an indicator of clonal relatedness. Indeed, Louie et al. (32), using DNA sequencing of PCR templates derived from the EPEC O55:H7 *eaeA* gene, have shown that the C terminus of EPEC O55:H7 *eaeA* is 98% similar to that of EHEC O157:H7 *eaeA*. On the basis of this sequence data, EPEC O55:H7 possesses an intimin that belongs to the O157:H7 intimin family. The grouping of AE bacteria based on their electrophoretic types (ET), as suggested by Whittam et al. (46), reflects differences in virulence factors (7) and is not restricted by serotype or disease class (7, 46). It is likely the *eaeA* gene, since it has diverged along with the rest of the chromosomal background, will be similar in members of ET clusters (46). It is unknown if RDEC-1, EPEC E2430/78, EHEC H19, and EHEC G85 belong to similar ET clusters; however, we have demonstrated that these strains, belonging to O15, O26, and O111 serotypes and EPEC and EHEC disease classes, possess identical intimins.

The presence of related cross-reactive intimins may have implications in the prevention of disease. The role of intimin in EPEC pathogenesis and its identification as a virulence factor has been described by Donnenberg et al. (14). In volunteer studies, they challenged individuals with EPEC E2348/69 or an isogenic *eaeA* deletion mutant of EPEC E2348/69 and demonstrated that intimin was required for full pathogenicity. Volunteer studies by Levine et al. (31) have shown that a single

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subject who had preexisting anti-intimin antibodies was protected from disease following challenge with EPEC E2348/69. We have detected specific anti-intimin secretory and humoral immune responses in rabbits following oral challenge and have some evidence of protection from RDEC-1 challenge in rabbits with preexisting antibodies to intimin (2). The specific role of intimin as a protective antigen has not been studied in detail in vivo; however, studies in vitro (10, 34) have demonstrated that antibody to intimin prevents attachment of the bacteria in tissue culture. If anti-intimin antibodies are protective, it may be possible to identify the major groups of intimins and use them in a polyvalent vaccine cocktail to prevent EPEC and EHEC infections in humans and livestock. Our RDEC-1 intimin fusion protein and RDEC-1 model of EPEC and EHEC infection provide the tools necessary to address these questions in greater detail.

Nucleotide sequence accession numbers. The nucleotide sequences for EPEC E2430/78 (O111), EHEC H19 (O26), and EHEC G85 (O26) have been submitted to GenBank and assigned accession numbers U62655, U62656, and U62657, respectively.

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