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Molecular Characterization of the *tia* Invasion Locus from Enterotoxigenic *Escherichia coli*

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Enterotoxigenic *Escherichia coli* (ETEC) shares with other diarrheal pathogens the capacity to invade epithelial cell lines originating from the human ileum or colon, although the role of invasion in ETEC pathogenesis remains undefined. Two distinct loci (*tia* and *tib*) that direct noninvasive *E. coli* to adhere to and invade intestinal epithelial cell lines have previously been isolated from cosmid libraries of the classical ETEC strain H10407. Here, we report the molecular characterization of the *tia* locus. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cellular fractions of *E. coli* DH5α carrying the *tia*-positive cosmids and recombinant plasmid subclones revealed that this locus directs the production of a 25-kDa protein (the Tia protein) that is localized to the outer membrane. The *tia* locus was subcloned to a maximum of 2 kb and mutagenized with bacteriophage Mu*d*. Synthesis of this protein was directly correlated with the ability of subclones and Mu*d* transposon mutants to adhere to and invade epithelial cells. Sequencing of the *tia* locus identified a 756-bp open reading frame. All transposon insertions resulting in an invasion-negative phenotype mapped to this open reading frame. The open reading frame was amplified and directionally cloned behind the *lac* promoter of pHG165. This construct directed DH5α to express a 25-kDa protein and to adhere to and invade epithelial cells. The role of the *tia* gene in directing epithelial adherence and invasion was further assessed by the construction of chromosomal *tia* deletion derivatives of the parent ETEC strain, H10407. These *tia* deletion strains were noninvasive and lacked the ability to adhere to human ileocecal cells. The *tia* gene shares limited homology with the *Yersinia ail* locus and significant homology with the *hra1* agglutinin gene cloned from a porcine ETEC strain. Additionally, *tia* probes hybridized to geographically diverse ETEC strains, as well as some enteropathogenic *E. coli*, enteroaggregative *E. coli*, and *Shigella sonnei* strains.

Diarrheal diseases in developing countries accounted for nearly one-quarter of the estimated 12.9 million deaths among children under the age of 5 in 1990, the last year for which data are available (53). Enterotoxigenic *Escherichia coli* (ETEC) causes a substantial proportion of these illnesses. Each year, this pathogen is responsible for over 600 million cases of diarrheal illness and an estimated 700,000 deaths in children under the age of 5 (28). ETEC remains the most common cause of diarrhea in travelers (7, 15, 37) and in soldiers deployed to developing countries. ETEC was the most common pathogen isolated from soldiers with diarrheal illness during Operation Desert Shield (27). While not a frequent cause of disease in developed countries, sporadic outbreaks continue to be reported (11).

Disease caused by ETEC is initiated by the ingestion of contaminated food or drink. Colonization of the proximal small intestine is a critical element in the pathogenesis of enterotoxigenic disease (22) and is mediated by fimbrial colonization factor antigens (29). Subsequent elaboration of heat-labile and/or heat-stable enterotoxin (LT and ST, respectively) (43) leads to net secretion of fluid and diarrhea (3).

In addition to enterotoxin production, *E. coli* causes diarrhea by a variety of other mechanisms (33, 43). For example, epithelial cell invasion has been demonstrated in enteroinvasive *E. coli* (EIEC), which is associated with dysenteric diarrheal disease. However, the other types of enterovirulent *E. coli* not associated with dysenteric disease, including enteropathogenic *E. coli* (EPEC) (12), ETEC (17), enterohemorrhagic *E. coli* (EHEC) (39), and, more recently, enteroaggregative *E. coli* (EAggEC) (6), have been shown to invade cultured human epithelial cells. The role of epithelial cell invasion in the pathogenesis of ETEC remains undefined. A limited number of biopsies of small-intestinal mucosae obtained during the course of experimental animal or human ETEC infections have failed to demonstrate intracellular organisms in epithelial cells (14, 34). However, human and animal studies suggest that factors in addition to enterotoxin production may be important in the pathogenesis of ETEC infection. Human volunteers developed diarrhea following challenge with ETEC strains that had lost the capacity for toxin production (35). The same strains cause fluid accumulation and diarrhea in an in vivo rabbit model (44, 51).

Elsinghorst et al. demonstrated that a classical ETEC strain (H10407) invades human intestinal cell lines and that the capacity for invasion is located on two separate, chromosomally encoded loci, designated *tia* and *tib* (toxigenic invasion loci A and B) (17, 18). We report here the molecular characterization of the *tia* locus and examine its contribution to epithelial cell invasion by H10407.

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MATERIALS AND METHODS

Bacterial strains, tissue culture cells, and culture conditions. Bacterial strains used in this study and plasmids employed are listed in Table 1. ETEC strain

TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
H10407	ETEC serotype O78:H11; CFA/I	19
H10407S	Spontaneous Sm^r derivative of H10407	18
DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	
POI1734	F$^-$ Mu*d*1 1734 *ara*::(Mu*cts*) Δ(*proAB-argF-lacIPOZYA*)*XIII rpsL*	10
POII1734	F$^-$ Mu*d*II 1734 *ara*::(Mu*cts*) Δ(*proAB-argF-lacIPOZYA*)*XIII rpsL*	10
MC4100	F$^-$ *araD139* Δ(*lacIPOZYA-ARGF*)*U169 rpsL thi*	9
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km	47
Plasmids		
pHG165	pBR322 copy number derivative of pUC8; ColE1 replication origin	49
pHC79	Cosmid cloning vector	26
pET102	Cosmid containing ETEC *tia* invasion locus from H10407	17
pET123	pHC79-based subclone containing 2,944-bp *tia*-positive *Hin*dIII-*Sal*I fragment from pET102	This study
pET124	1,911-bp *Hin*dIII-*Eco*RI fragment from pET123 subcloned into pHG165 *Eco*RI-*Hin*dIII site	This study
pET125	pET123 subclone obtained by *Cla*I digestion, deletion of 919-bp *Cla*I fragment, and religation	This study
pET126	Subcloned from pET125 by *Ava*I digestion and religation	This study
pET127	Subcloned from pET123 by *Eco*RI digestion and religation	This study
pCVD442	*sacB*-containing suicide vector	13
pET168	2,944-bp *Hin*dIII-*Sal*I fragment from pET123, containing *tia*, cloned into pHG165	This study
pET169	Subclone of pET168 obtained by *Cla*I-*Ava*I digestion and religation, resulting in deletion of 549 bp from the 5′ end of *tia*	This study
pET179	1,991-bp *Hpa*I fragment from pET169, containing the last 207 bp of *tia* ligated into pCVD442	This study
pET185	856-bp fragment containing the *tia* locus, amplified by PCR[a] from pET125, directionally cloned into pHG165	This study

[a] Primers used in the amplification of the *tia* locus from pET125 were as follows (*Bam*HI and *Hin*dIII restriction sites are underlined): upstream primer, 5′-TAGCATCAGTGGATCCTTCTCTTTTACCCTGTCTTTTGC 3′; downstream primer, 5′-TAGCATCAGTAAGCTTGCAGGCATGGATAAACATTCTC-3′.

H10407 was the parent strain in cloning experiments. *E. coli* DH5α was used as the recipient for all recombinant plasmids. Organisms were grown in Luria broth (LB) (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter; pH 7.6) at 37°C and 200 rpm unless otherwise indicated. Antibiotics were added to growth media to the following final concentrations (in micrograms per milliliter): ampicillin, 100; kanamycin, 20; and streptomycin, 100.

HCT8 (ATCC CCL 244) human ileocecal epithelial cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 1 mM sodium pyruvate, and 2 mM L-glutamine. HCT8 cells were grown at 37°C in 6% CO_2. Tissue culture media and additives were obtained from GIBCO Laboratories, Grand Island, N.Y. HeLa (ATCC CCL2) human cervical, HEp-2 (ATCC CCL23) human laryngeal, HuTu80 (ATCC HTB40) human duodenal, HCT116 (ATCC CCL247) human colonic, and T84 (ATCC CCL248) human colonic cell lines were maintained in accordance with American Type Culture Collection recommendations.

Membrane fractionation. Membrane fractions were isolated from 500-ml late-log-phase LB cultures by one of two methods. In the first method, inner and outer membranes were isolated by sucrose density gradient ultracentrifugation of French press lysates as previously described (18, 45). Alternatively, cells were resuspended in a hypotonic sucrose solution and lysed by repeated flash freezing in dry ice acetone and thawing at 37°C. Inner membranes were solubilized in Triton X-100, and outer membranes were isolated by ultracentrifugation as described by Schnaitman (46).

SDS-PAGE analysis of proteins. Electrophoresis of membrane fractions was performed using discontinuous gels as described by Laemmli (32). The protein concentrations of various fractions were determined by the Bradford method (8). The amounts of protein added per well for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis were as follows: inner and outer membranes, 50 μg; cytosol, 100 μg. Samples were prepared by being heated in sample buffer at 98°C for 10 min. Gels were run for 16 to 18 h at 40 V and 25°C. Gels were stained with Coomassie blue.

Invasion and adherence assays. Bacterial invasion of epithelial cells was measured as protection from the bactericidal antibiotic gentamicin (30). Invasion and adherence assays were performed as previously described (17). Briefly, approximately 5×10^6 log-phase CFU was added to HCT8 monolayers (approximately 7×10^4 cells in 24-well tissue culture plates), which were then incubated at 37°C for 3 h in a 6% CO_2 atmosphere. The actual inoculum for each experiment was determined by quantitative plate count. After being washed, the infected monolayers were incubated for an additional 2 h in tissue culture medium containing gentamicin. The infected monolayers were washed and then lysed in 0.1% Triton X-100 in deionized water, and the bacteria were quantitated by plate count. Since the results of invasion and adherence assays are variable on a daily basis, the datum points presented in the figures and tables are average values (± standard deviation) from triplicate wells of a single experiment and correlate with values obtained in replicate experiments.

Mu*d* mutagenesis. Plasmid pET125 was used to transform POI1734 and POII1734 to Amp^r, and Mu*d* lysates were produced by heat induction. The lysates were then used to transduce MC4100 to Km^r and Ap^r. Lac$^+$ transductants were identified on MacConkey agar. Plasmid DNA obtained from Lac$^+$ Km^r Ap^r transductants was used to transform DH5α. Transformants were then screened for their capacity for invasion of HCT8 cells and for production of specific outer membrane proteins. The location of the Mu*d* insertions in pET125 was determined by restriction enzyme mapping and by sequencing from defined nucleotides at the left end of the bacteriophage through the point of insertion (23).

DNA sequencing analysis. Plasmid pET125 was used as the template for sequencing of the *tia* locus. Oligonucleotide primers were synthesized by the phosphoramidite method (4) and purified by using prepacked Sephadex PD-10 columns (Pharmacia LKB Biotechnology, Uppsala, Sweden). Sequencing reactions were performed with fluorescence-based dideoxy cycle sequencing reagents (Applied Biosystems) in a 9600 thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, Conn.) and Centri-Sep columns (Princeton Separations, Adelphia, N.J.) were used to remove unincorporated nucleotides. The resulting nucleotide sequence and the predicted peptide sequence were compared with sequences in the National Center for Biotechnology Information databases by using the BLAST network service (1, 21).

Construction of a *tia* deletion mutant of H10407. To generate a *tia* deletion, a 2,944-bp *Hin*dIII-*Sal*I fragment from pET123 containing the *tia* locus was ligated into pHG165, yielding pET168. A 1,656-bp *Cla*I-*Ava*I fragment containing the first 549 bp of the *tia* gene was deleted from pET168 to yield pET169. The insert of pET169 was flanked by *Hpa*I sites present in the vector, and the 1,991-bp *Hpa*I fragment from pET169 (containing the remaining 207 bp of the *tia* gene) was cloned into the *Sma*I site of the positive-selection suicide plasmid vector pCVD442 (13) to yield pET179 (see Fig. 1C).

The suicide plasmid pET179 was used to transform *E. coli* SM10λpir. This plasmid was conjugally transferred from SM10λpir to H10407S by plate mating, selecting for Ap^r and Sm^r resistance. Purified transconjugates were grown to late log phase, diluted, and then used to inoculate sucrose selection plates containing tryptone (10 g/liter), yeast extract (5 g/liter), and sucrose (50 g/liter). Inoculated plates were incubated at 30°C overnight. Purified sucrose-insensitive colonies were then screened for loss of Ap^r. Ampicillin-sensitive strains were then screened for loss of the *tia* sequence by hybridization with probe 1 (see Fig. 1C).

Hybridization analysis. Probe 1 (855 bp) containing the *tia* locus was prepared by PCR amplification using pET125 as the target template with sense primer 242213 (5′-TTCTCTTTTTACCCTGTCTTTTGC-3′) and antisense primer 242840 (5′-CGAGGCATGGATAAACATTCTC-3′) (see Fig. 1C). This probe encompassed the region extending from 53 bp upstream from the beginning of the *tia* open reading frame (ORF) to 1 bp downstream from the end of the *tia* ORF. PCR conditions were as follows: denaturation for 5 min at 95°C; then 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and finally 7 min at 72°C in a thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, Conn.). Amplification

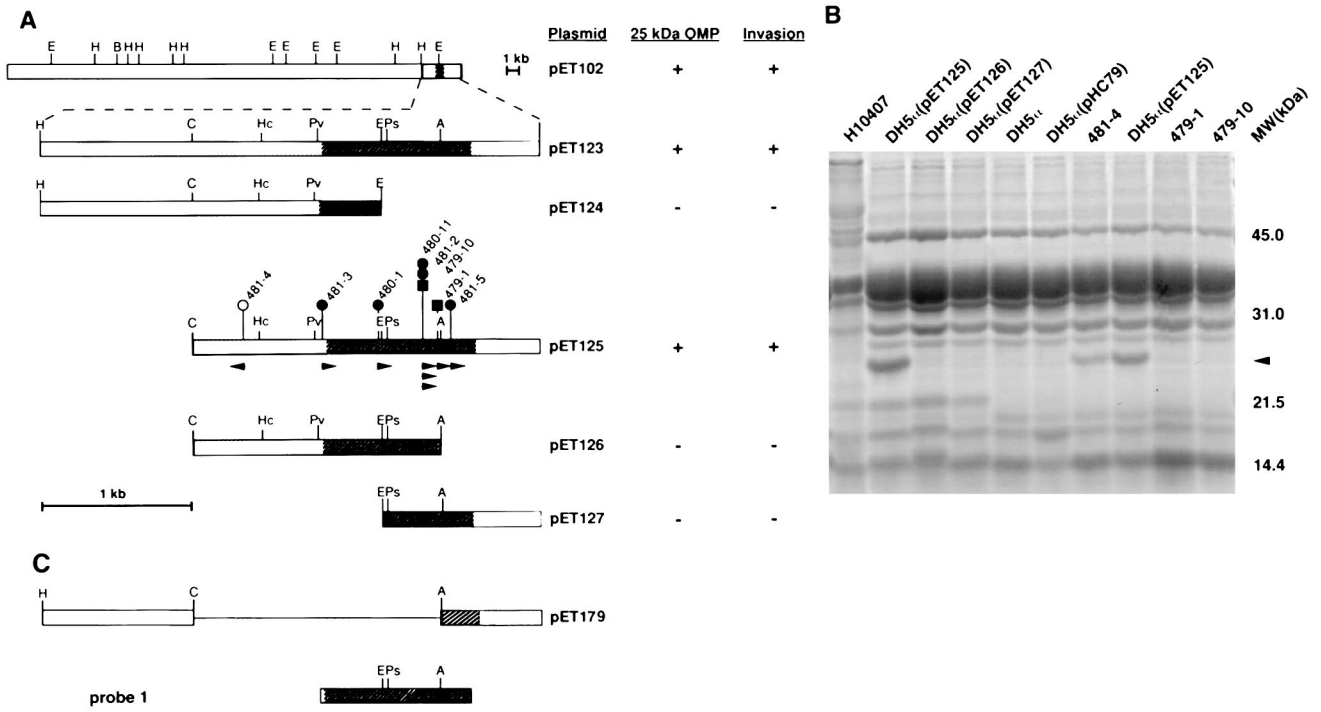

FIG. 1. (A) Subcloning and mutagenesis of the *tia* locus. Cosmid pET102 was the parent plasmid for subcloning. The hatched segments indicate the location of the *tia* ORF. Restriction enzymes: A, *Ava*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; Hc, *Hin*cII; H, *Hin*dIII; Ps, *Pst*I; and Pv, *Pvu*I. The ability of the subclones to direct invasion and production of Tia (25-kDa outer membrane protein [OMP]) is indicated to the right of the restriction maps. Points of Mu*d*I and Mu*d*II transposon insertions are indicated on the map of pET125. The position of the Mu*d*I insertion (481-4) that had no effect on invasion or the production of Tia (open circle), the positions of Mu*d*I and Mu*d*II insertions (closed circles and squares, respectively) that eliminate invasion and the production of Tia and the direction of transcription of the transposon insertions (arrowheads) are shown. (B) Coomassie blue-stained SDS–10% polyacrylamide gel of outer membrane fractions from H10407, recombinant DH5α carrying representative plasmid subclones, and mutagenized pET125. DH5α and DH5α(pHC79) are included as negative controls. 481-4, 479-1, and 479-10, DH5α strains carrying pET125 mutagenized by transposon insertions at positions indicated on the restriction map of pET125 shown in panel A. The migration of Tia (arrowhead) is indicated. Samples in lanes 1 to 4 were prepared on a different day from those in lanes 5 to 11, resulting in alterations in proteins other than Tia (e.g., the additional band migrating at approximately 21.5 kDa in lanes 1 to 4). (C) pET179 positive-selection suicide vector and *tia* locus probe. The portion of pET179 shown is that derived from the original *Hin*dIII-*Sal*I insert region of pET123. The thin line in pET179 indicates the portion of the *Hin*dIII-*Sal*I fragment from pET123 that was deleted in the construction of the suicide plasmid vector.

products were purified by preparative electrophoresis and elution from agarose (Promega Corp., Madison, Wis.) and labeled with either [α-^{32}P]dCTP or digoxigenin-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by random primed DNA labeling.

Genomic DNA was prepared by hexadecyltrimethyl ammonium bromide precipitation of bacterial lysates (52). For hybridization with clinical isolates, approximately 1 μg of DNA was digested with *Bam*HI, electrophoresed through 0.6% Tris-borate-EDTA agarose gels, and transferred to Nytran membranes (Schleicher & Schuell, Keene, N.H.) by the method of Southern (48). The blots were hybridized with either ^{32}P- or digoxigenin-dUTP-labeled *tia* probes. For analysis of Δ*tia* strains, DNA was digested with *Hin*dIII, and hybridization and detection with digoxigenin-labeled *tia* probes were performed in accordance with the manufacturer's protocols (Boehringer Mannheim). Hybridization and stringency washes were done at 40°C. Stringency washes were done with 0.1% SDS–0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

N-terminal sequencing of the Tia protein. Outer membrane protein fractions prepared from DH5α(pET185) were electrophoresed through an SDS–10% polyacrylamide gel and the 25-kDa band was excised. The protein was eluted and sequenced by the Edman degradation method using a Hewlett-Packard G1000-A protein sequencer (16).

Nucleotide sequence accession number. The final nucleotide sequence of the *tia* locus was submitted electronically to GenBank under accession no. U20318 via the Authorin 3.0 program.

RESULTS

ETEC strain H10407 possesses two distinct loci encoding the capacity to invade human epithelial cells. These invasion loci (*tia* and *tib*) confer on *E. coli* HB101 the capacity to adhere to and invade human intestinal epithelial cells. Elsinghorst and Weitz reported that cosmid pET103 carrying *tib* encodes a 104-kDa outer membrane protein and that *tib* mutants of H10407 invade epithelial cells at approximately 15% of the parenteral level (18). We report the characterization of the second invasion locus, *tia*, originally identified on the cosmid pET102.

Subcloning and identification of a 25-kDa outer membrane protein. Cosmid pET102 was subcloned as outlined in Table 1. As shown in Fig. 1A, a gene encoding the expression of a 25-kDa outer membrane protein was localized by plasmid subcloning to the 2,000-bp *Cla*I-*Sal*I insert region of pET125, the smallest subclone demonstrating invasion activity. Phenotypic analysis of DH5α carrying recombinant plasmid subclones of pET102 showed a direct correlation between the production of the 25-kDa outer membrane protein and the capacity for invasion (Fig. 1A and Table 2).

Transposon mutagenesis. Fine genetic mapping by Mu*d* phage mutagenesis of pET125 localized the *tia* gene to the region downstream from the *Pvu*I site in pET125. All of the transposon insertions, except 481-4, which mapped within the insert region of pET125, abrogated the capacity of DH5α to produce the 25-kDa outer membrane protein and invade epithelial cells. DNA sequencing and restriction digest analysis also permitted the determination of the orientation of each of the Mu*d* insertions within the *tia* locus as indicated in Fig. 1A, and hence the direction of transcription of *tia*. This analysis

TABLE 2. Invasion of and adherence to HCT8 (human ileocecal) epithelial cells by *E. coli* H10407 and DH5α carrying *tia* locus subclones

Strain	% Invasion[a]	% Relative invasion[b]	% Adherence[c]	% Relative adherence[d]
DH5α	0.01 ± 0.01	0.6	0.32 ± 0.05	1.7
DH5α(pHC79)	0.03 ± 0.01	1.2	0.31 ± 0.02	1.6
DH5α(pHG165)	0.01 ± 0.01	0.5	0.33 ± 0.04	1.7
DH5α(pET102)	1.55 ± 0.11	70.7	11.79 ± 1.12	62.5
DH5α(pET123)	1.11 ± 0.12	50.7	10.19 ± 0.64	54.0
DH5α(pET124)	0.13 ± 0.07	5.9	0.68 ± 0.06	3.6
DH5α(pET125)	0.89 ± 0.19	40.6	5.87 ± 0.63	31.1
DH5α(pET126)	0.01 ± 0.01	0.2	0.41 ± 0.05	2.2
DH5α(pET127)	0.02 ± 0.02	0.9	0.26 ± 0.03	1.4
DH5α(pET185)	1.14 ± 0.05	52.0	6.34 ± 0.34	33.5
H10407	2.19 ± 0.20	100.0	18.87 ± 2.53	100.0
TY2	3.90 ± 0.27	178.0	15.68 ± 0.76	83.1

[a] Percentage of inoculum resisting treatment with gentamicin.
[b] Invasion relative to *E. coli* H10407 invasion, representing 100%.
[c] Percentage of inoculum remaining on washed monolayers at 3 h (minus the percentage of inoculum resisting gentamicin treatment).
[d] Adherence relative to *E. coli* H10407 adherence, representing 100%.

suggested that the 5′ end of the *tia* gene was located downstream from the *Pvu*I site of pET125.

The *tia* gene encodes a 25-kDa outer membrane protein. Analysis of the DNA sequence from the insert region of pET125 identified a 756-bp ORF. This predicted a protein product of 251 amino acids with a molecular mass of 26.5 kDa. Mu*d* insertions resulting in the invasion-negative Tia-negative phenotype, except for 481-3, mapped within this ORF. The transposon insertion designated 481-3 was located approximately 20 bp upstream from the beginning of the *tia* ORF. To confirm that this ORF encoded *tia*, we designed oligonucleotide primers containing restriction endonuclease sites for the directional cloning of the ORF into pHG165, placing the ORF under the control of the exogenous *lac* promoter. DH5α carrying the resulting plasmid, pET185, expressed the 25-kDa outer membrane protein (Fig. 2) and invaded HCT8 cells (Table 2).

Additional confirmation that the protein was encoded by the ORF was obtained by N-terminal amino acid sequencing, which yielded the sequence DESKTGFYVT (see Fig. 7). This sequence corresponds to amino acids encoded by nucleotides 76 to 106 of the *tia* ORF, indicating that the first 25 amino acids of Tia are cleaved during posttranslational processing. The difference in the predicted molecular mass for Tia (26.5 kDa), based on the translation of the *tia* ORF, and the observed molecular mass on SDS-polyacrylamide gels (25 kDa) is consistent with the removal of approximately 25 amino acids from the Tia precursor molecule during export.

The predicted amino acid sequence for the region preceding the cleavage site possesses features characteristic of bacterial signal peptides. The amino acid sequence alanine-leucine-alanine preceding the cleavage site conforms to the "−3,−1 box" signal peptidase recognition pattern, in which the peptidase recognizes small apolar amino acids at positions −1 and −3 proximal to the cleavage site (41). Secondary structure analysis

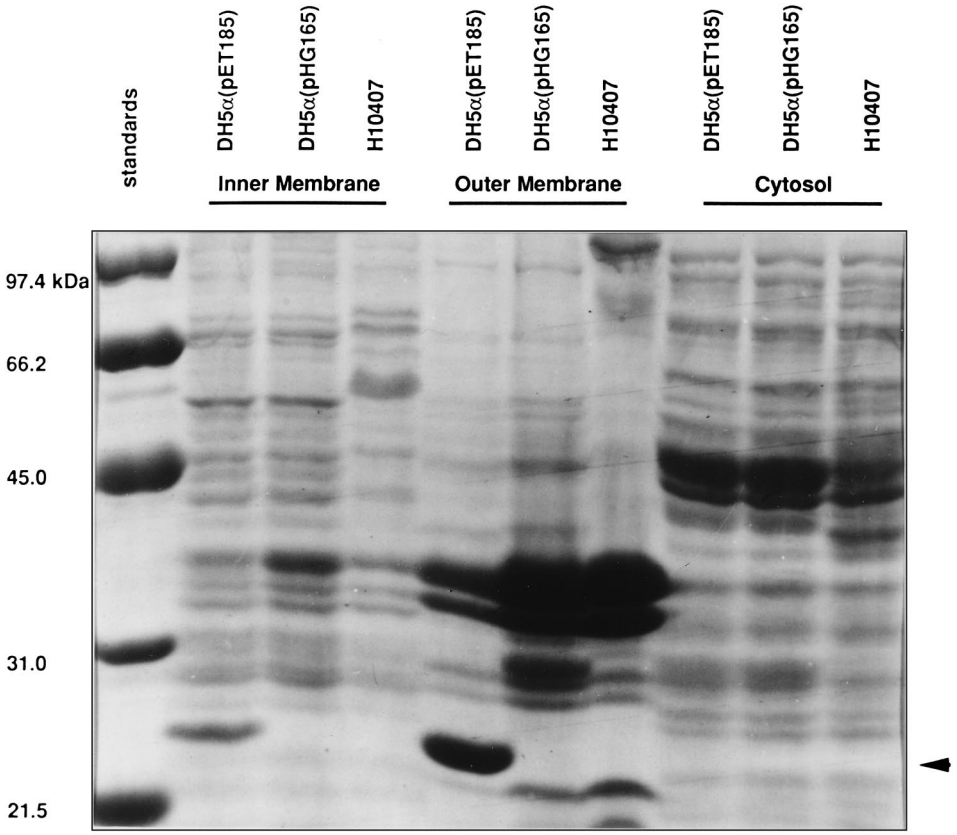

FIG. 2. Coomassie blue-stained SDS–10% polyacrylamide gel of cellular fractions from the parent ETEC strain, H10407, and DH5α(pET185) carrying the amplified *tia* ORF directionally cloned into the plasmid vector pHG165 under the control of the *lac* promoter. DH5α(pHG165) is shown as a negative control. Molecular mass standards are indicated on the left. The migration of Tia is shown on the right (arrowhead).

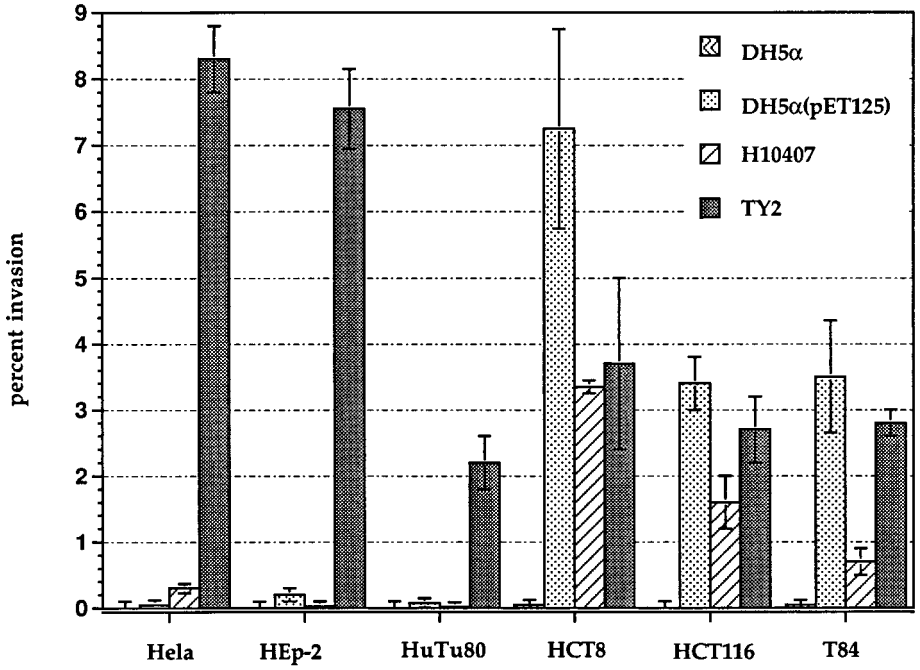

FIG. 3. Invasion of various epithelial cell lines by H10407 and recombinant DH5α expressing the *tia* locus encoded on pET125. Results for *S. typhi* TY2 and *E. coli* DH5α are shown as positive and negative controls, respectively.

(31) of the Tia peptide predicts an alpha-helix configuration for amino acid residues 3 through 23, a feature typical of the hydrophobic core region of bacterial signal peptides (41). Supporting the localization of Tia to the outer membrane is the presence of the amino acids tyrosine-histidine-phenylalanine at the C-terminal end of Tia. This sequence follows the Tyr-X-Phe motif, which is highly conserved among outer membrane proteins and is a putative signal for outer membrane localization (50).

Finally, we isolated subcellular fractions from DH5α (pHG165), DH5α(pET185), and H10407. Figure 2 shows that *tia* is expressed predominantly in the outer membrane of DH5α(pET185). The Tia protein could not be detected in any of the subcellular fractions from the parent strain H10407.

The *tia* locus directs the invasion of specific cultured epithelial cell lines. H10407, the parent ETEC strain from which the *tia* locus was cloned, preferentially invades specific epithelial cell lines (17). In this study, recombinant *E. coli* DH5α containing the *tia* locus invaded epithelial cell lines derived from the ileocecum (HCT8) or colon (HCT116 and T84) (Fig. 3), but not cells derived from the human cervix (HeLa), duodenum (HuTu80), or larynx (HEp-2), paralleling the invasion specificity of the parent strain.

Δ*tia* deletion mutants of H10407 are noninvasive. To assess the contribution of the *tia* locus to epithelial cell invasion by H10407, isogenic mutants containing a deletion in the *tia* locus were constructed (Fig. 1C). *tia* locus probes hybridized with a 5.4-kb band from *Hin*dIII digests of H10407S genomic DNA and with a smaller, 3.9-kb fragment from digests of genomic DNAs from three putative Δ*tia* mutants (Fig. 4). This reduction in size of the hybridization bands reflected the size of the fragment deleted in the construction of the suicide plasmid pET179. To confirm that the *tia* locus had been deleted in these strains, amplifications were performed using primers flanking the *tia* ORF and chromosomal DNA from H10407S and the three putative Δ*tia* mutants. Amplification of H10407S resulted in the anticipated 933-bp product, whereas no amplification product from the putative Δ*tia* mutant strains was observed (data not shown). This result confirmed that the *tia* locus had been deleted from these strains. The Δ*tia* deletion mutants exhibited a marked decrease in the capacity to adhere to or invade HCT8 monolayers (Fig. 5). Unfortunately, we were unable to achieve stable transformation of the H10407 Δ*tia* strains with plasmids bearing the *tia* locus to perform complementation studies.

Hybridization of the *tia* locus with other strains. *Bam*HI-digested genomic DNA from a variety of pathogens was examined for homology to labeled *tia* probes (Table 3). Non-*E. coli* strains tested included the following: *Shigella flexneri*, *Shigella dysenteriae*, *Shigella boydii*, and *Shigella sonnei*; *Salmonella typhimurium* and *Salmonella typhi*; *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*; *Aeromonas hydrophila*;

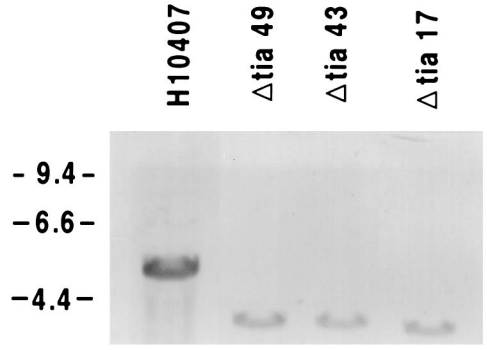

FIG. 4. Southern hybridization of genomic DNA from H10407-S (a streptomycin-resistant mutant of H10407) and H10407-S Δ*tia* deletion constructs with *tia* probe 1. Genomic DNA was digested with *Bam*HI prior to electrophoresis and transfer. Size markers (in kilobases) are shown on the left.

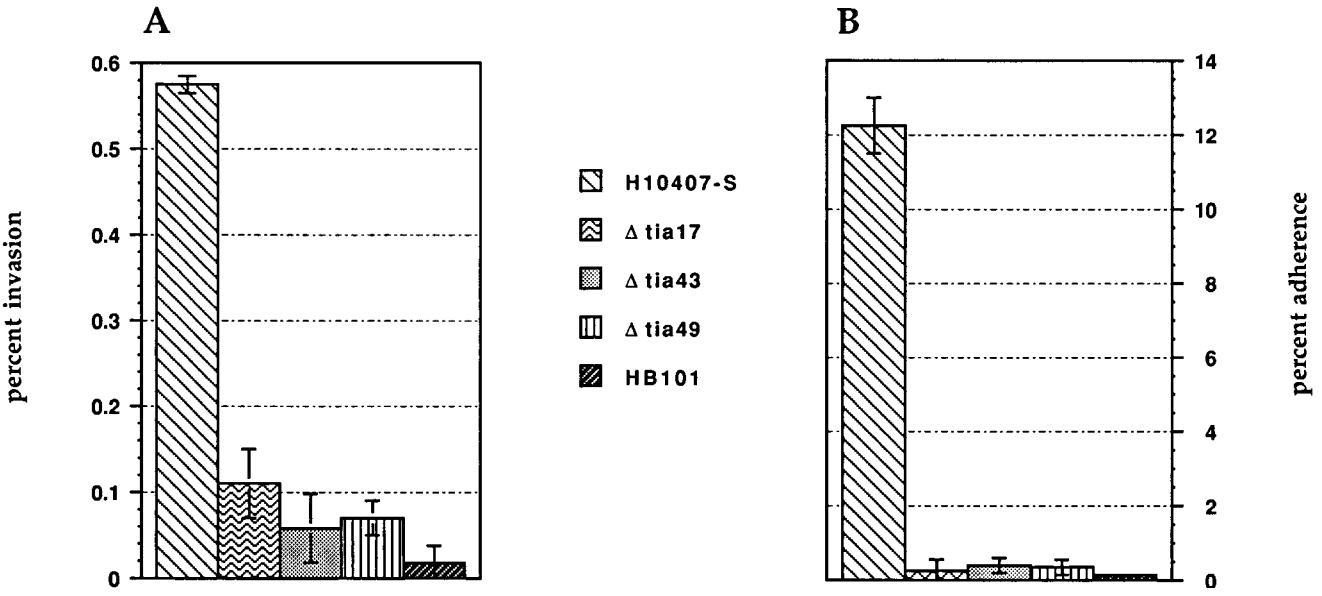

FIG. 5. HCT8 epithelial cell invasion (A) and adherence to HCT8 cells (B) by H10407-S and H10407-S Δ*tia* deletion strains. *E. coli* HB101 is a noninvasive control.

Plesiomonas shigelloides; *Klebsiella pneumoniae*; *Citrobacter freundii*; *Campylobacter jejuni*; *Vibrio cholerae*, *Vibrio vulnificus*, *Vibrio mimicus*, *Vibrio fluvialis*, and *Vibrio hollisae*; and *Bordetella pertussis*. Of these, only *S. sonnei* R071, the only *S. sonnei* strain examined, demonstrated homology. Additionally, 27 geographically diverse ETEC strains comprising a variety of serotypes, colonization factor antigen types, and toxin phenotypes were tested. Of these, 11 strains (41%) (including the parent strain) hybridized with the *tia* probe (Fig. 6). Two strains which hybridized with the *tia* probe also hybridize with *tib* probes (18). Five of the strains which failed to hybridize with *tia* probes had previously been shown to contain the *tib* locus. In addition, the *tia* probe hybridized to genomic DNA from EPEC 607-54 (O55:H6) and EAggEC 042 (O44:H18) (Fig. 6).

The *tia* gene has homology with the *hraI* gene from a porcine ETEC strain. Both the protein and the nucleotide NCBI databases were searched for previously reported sequences with homology to the *tia* gene. This search revealed that the *tia* nucleotide sequence has a limited degree of homology with the *Y. enterocolitica ail* locus but a high degree of homology with the sequence of the *hra-1* gene cloned from the porcine ETEC strain O9:H10:K99 (36). The first 10 N-terminal amino acids of Tia, determined by Edman degradation and sequencing, are identical to the N-terminal amino acid sequence of Hra-1 reported by Lutwyche et al. (36). As shown in Fig. 7, 168 of the 251 amino acids from the predicted translation of *tia* are identical to those of Hra-1.

The *tia* gene also has limited homology with the *ail* gene from *Y. enterocolitica*. Two regions of these molecules show some degree of homology. As indicated in Fig. 7, in the first region, spanning amino acids 78 to 96 of Tia, 11 of 19 amino acids either are identical or represent conserved changes compared with the *ail* gene product. This region in *ail* was predicted by Beer and Miller (5) to be a cell surface domain. The second region of homology, from amino acids 194 to 214 of Tia, was predicted to be a transmembrane portion of the molecule.

DISCUSSION

The *tia* locus, cloned from ETEC strain H10407, has been shown to encode a 756-bp *tia* gene that directs the synthesis of a 25-kDa outer membrane protein. Subcloning and transposon mutagenesis experiments show a direct correlation between production of this protein and the ability of recombinant DH5α to invade and adhere to cultured human intestinal epithelial cells. DNA sequencing identified a 756-bp ORF for the *tia* gene. This isolated ORF directs the production of the 25-kDa outer membrane protein and invasion of cultured human intestinal epithelial cells.

H10407 possesses two loci, *tia* and *tib*, encoding proteins required for the invasion of intestinal epithelial cells. Δ*tia* mutants of H10407 demonstrated marked (≥75%) reductions in the capacity to invade HCT8 cells compared with that of the wild type. Either the *tia* or the *tib* locus can independently direct DH5α to invade, and Δ*tib* deletion mutants also demonstrate ≥75% reductions in invasiveness. The large reduction in invasion capacity achieved by deleting either locus suggests that expression of these loci may be interdependent in the parent strain. Membrane preparations of H10407 indicate that the Tia protein is expressed poorly when this strain is grown under laboratory conditions, again suggesting that its production is tightly regulated in the parent strain. Tia synthesis in DH5α suggests that the *tia* gene is deregulated in this strain and that *tia* expression is repressed in H10407. Therefore, genes responsible for this repression do not appear to be encoded on pET101 or pET102, the original *tia* cosmids.

The *tia* gene appears to encode a unique invasion system. The lack of hybridization of *tia* locus probes with genomic DNA from most invasive enteric pathogens studied suggests that the *tia* locus is distinct from other genes shown to promote uptake of organisms into nonprofessional phagocytes. A search of nucleotide and peptide sequence databases (1) revealed that the amino acid sequence of the *tia* gene has small regions of homology with the *ail* locus from *Y. enterocolitica*. One of these areas of homology was predicted by Beer and Miller to be a

TABLE 3. Summary of *tia* hybridization experiments with *E. coli* strains

Strain	CFA (CS) produced[a]	Serotype	Response to *tia* probe 1[b]	Source[c]
ETEC				
DS244-1	I	O6:H16	−	WRAIR
M633C1	I	O20:Nm	+	CVD
15758a	I	O78:H10	−	CVD
H10407	I	O78:H11	+	WRAIR
Tx1	I	O78:H12	−	AFRIMS
DS229-1	I	O128:H12	−	WRAIR
DS67-1	I	O153:Nm	+	WRAIR
DS99-5	I	O153:H10	+	WRAIR
H410C1	I	Rough:Nm	−	CVD
M109C2	I	Rough:H12	+	CVD
M424C1	II (1,3)	O6:H16	+	WRAIR
DS7-3	II (3)	O8:H9	+	WRAIR
DS220-4	II (2,3)	O11:H33	−	WRAIR
DS373-2	II (2,3)	O18:Nm	−	WRAIR
DS207-2	II (1,3)	O22:Nm	+	WRAIR
DS300-1	IV (4,6)	O8:Nm	−	WRAIR
B4106-1	IV (6)	O27:H7	−	AFRIMS
DS39-5	IV (6)	O115:H35	−	WRAIR
DS349-1	IV (6)	O128:Nm	+	WRAIR
B7A	IV (6)	O148:H28	−	WRAIR
DS2-1	IV (6)	O159:Nm	+	WRAIR
E17018	IV (5,6)	O167:H5	+	WRAIR
M414C1	Non-I	O2:Nm	−	CVD
E2539-C1	Non-I	O25:Nm	−	AFRIMS
10614c	Non-I	O78:Nm	−	CVD
Scott	Non-I	O78:K80	−	CVD
EDL903	Non-I	O88:H25	−	AFRIMS
EPEC				
607-54		O55:H6	+	AFRIMS
B170		O111:Nm	−	AFRIMS
833-90		O119:H6	−	AFRIMS
3336-54		O127:Nm	−	AFRIMS
E2348		O127:H6	−	WRAIR
RDEC-1			−	WRAIR
EIEC (2 strains)			−	AFRIMS
EHEC (7 strains)		O157:H7	−	WRAIR/AFRIMS
EAggEC				
17-2		O3:H2	−	CVD
JM221		O93:H33	−	CVD
309-1-1		O130:H27	−	CVD
103-1-1		O148:H28	−	CVD
042		O44:H18	+	CVD
Normal intestinal isolates of *E. coli* (4 strains)			−	CVD
Other *E. coli* strains				
HB101			−	WRAIR
DH5α			−	WRAIR

[a] Production of CFA and *E. coli* surface (CS) antigen by ETEC strains.
[b] +, hybridization to probe 1 (see Fig. 1C and Fig. 6); −, no hybridization.
[c] AFRIMS, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; CVD, Center for Vaccine Development, Baltimore, Md.; WRAIR, Walter Reed Army Institute of Research.

cell surface domain (5), but it is not clear if the epitopes shared by the *tia* and *ail* loci are critical for invasion.

The *tia* gene shows approximately 60% homology with the nucleotide sequence of the *hra1* gene from *E. coli* O9:H10:K99, an ETEC strain which causes enteric disease in animals (36). The predicted peptides encoded on these genes are approximately 67% homologous. The *hra1* gene appears to encode an outer membrane protein which functions as a nonfimbrial adhesin and which promotes the agglutination of human and animal erythrocytes and human colonic adenocarcinoma (COLO 201) cells. Epithelial cell invasion directed by the *hra1* locus has not yet been studied.

The *tia* gene was found primarily in ETEC, hybridizing with approximately 41% of the strains tested. Hybridization of *tia* probes with genomic DNA from a limited number of strains of other enteric pathogens, including *S. sonnei*, EAggEC, and

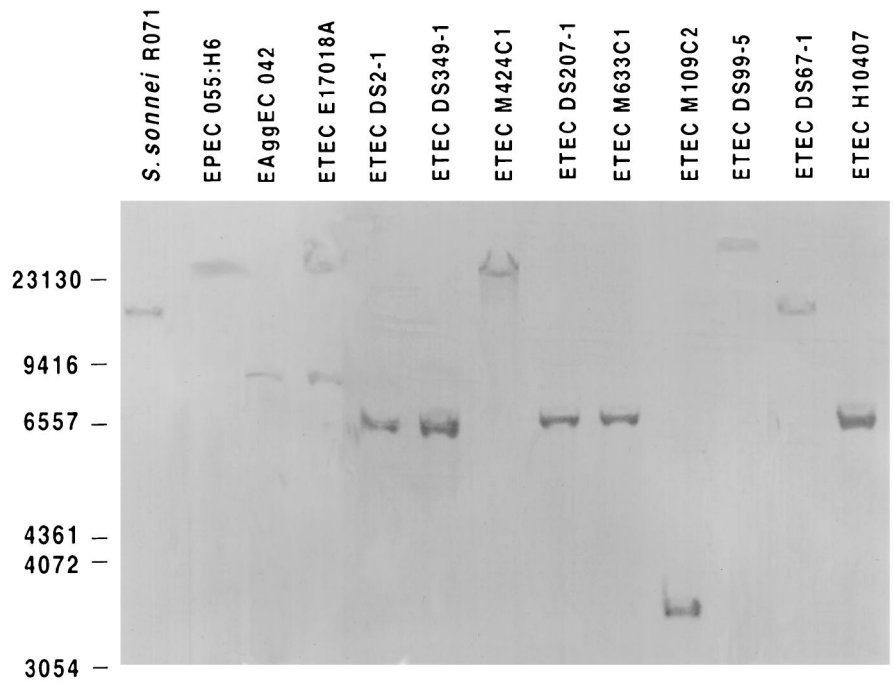

FIG. 6. Southern hybridization of genomic DNA from clinical isolates of various enteric pathogens probed with *tia* probe 1. Genomic DNA was digested with *Bam*HI prior to electrophoresis and transfer. Molecular size markers (in base pairs) are shown on the left.

EPEC, suggests that the *tia* locus has homology with yet-to-be-characterized loci in these strains. EAggEC strains have recently been shown to penetrate HeLa cells, a phenotype that is not shared by H10407 or any of the recombinant clones of the *tia* locus, suggesting either that more than one invasion locus exists in EAggEC or that the *tia* gene has only partial homology with invasion loci of this pathogen.

The Tia protein is expressed predominately in the outer membrane. The Tia protein was identified solely in outer membrane preparations of the pHC79-based *tia* recombinants and

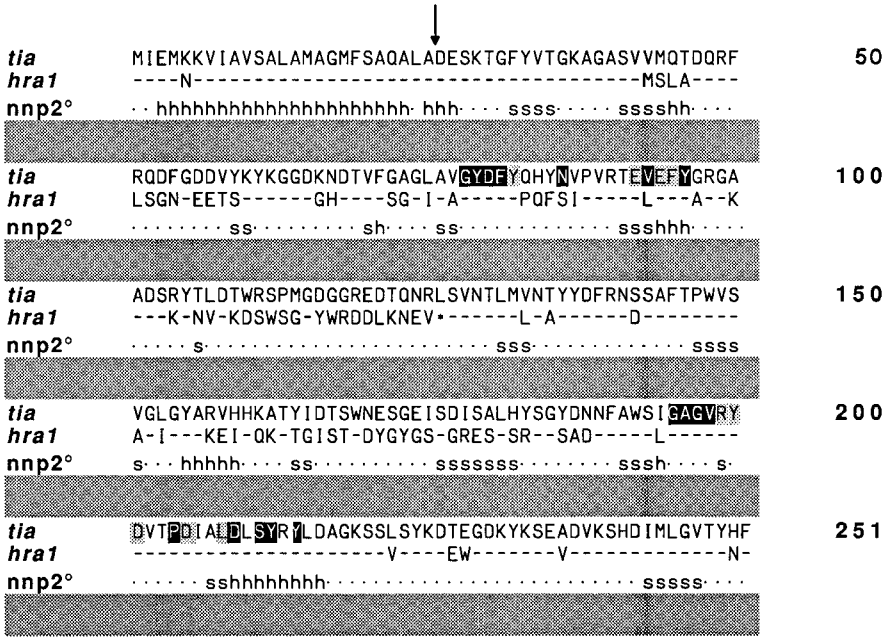

FIG. 7. Alignment of translations of *tia* and *hra1* (from *E. coli* O9:H10:K99), regions of *tia-ail* homology, and predicted secondary structure for Tia. Optimal alignment of the *tia* and *hra1* sequences was done by using the ALIGN program algorithm (38). Identities between corresponding amino acid residues (dashes) and a gap inserted to maximize identity between sequences (asterisk) are indicated. Secondary structure prediction (nnp2°) was calculated by the nearest-neighbor method using the nnpredict program (31). h, helical; s, strand; ., no prediction. Regions of identity with the *ail* gene from *Y. enterocolitica* (black background) and conserved changes (shaded background) are indicated.

predominately in the outer membrane of DHα(pET185). N-terminal amino acid sequencing data suggest that this protein undergoes posttranslational processing, resulting in the cleavage of the first 25 amino acids from the nascent Tia molecule. Additionally, the first 25 amino acid residues of Tia possess features conserved in many bacterial signal peptides, and the carboxy terminus of Tia contains a motif common to a number of outer membrane proteins. Together, these data support the localization of the mature Tia protein to the outer membrane.

The role, if any, for invasion of intestinal epithelial cells in the pathogenesis of diarrheal disease caused by ETEC remains to be established. Certainly, cultured epithelial cell monolayers possess a limited capacity to reproduce the complex environment presented to bacteria by the intestinal mucosa. However, Elsinghorst and Kopecko showed that both the *tia* and the *tib* loci can promote transcytosis of *E. coli* HB101 through polarized HCT8 monolayers (17) suggesting that the receptors for the *tia* gene product are normally present on the mucosal epithelial surface.

Several lines of evidence suggest that nontoxigenic ETEC strains may be diarrheagenic and that there may be mechanisms in addition to enterotoxin production by which ETEC may cause diarrhea. A spontaneous LT^- ST^- mutant of an ETEC strain caused mild diarrhea in human volunteers (35). These same strains consistently caused diarrhea when used in a variety of rabbit models (42, 44, 51). Although the mechanisms by which nonenterotoxigenic strains cause diarrhea are not yet defined, Schlager et al. (44) have shown that colonization with such strains may be associated with net fluid secretion and impaired villus function. At least some strains of ETEC may promote diarrhea by eliciting a mucosal inflammatory response. Diarrhea caused by ETEC is usually classified as a noninflammatory illness, yet fecal leukocytes can be detected in the stools of some patients with ETEC diarrhea (24), suggesting that some strains may promote migration of leukocytes into the gut lumen. The clinical presentation of ETEC diarrheal illness may be more heterogeneous than previously appreciated. Data presented by Arduino et al. suggest that in addition to fecal leukocytes, a proportion of patients with untreated ETEC infections may have occult or even gross blood in their stools (2). Preliminary studies in our laboratory have shown that H10407 is an efficient promoter of interleukin-8 production by HCT8 cells in vitro (20). The relationship of epithelial cell invasion and adherence to cytokine production is being evaluated. Alternatively, the *tia* and *tib* loci may typically trigger adherence to the human small-intestinal mucosa, but this tight adherence may be translated into invasion in certain cell lines, such as HCT8 cells.

While cholera toxin is efficiently exported in *V. cholerae*, the structurally similar LT remains in the periplasmic space of ETEC (25). In addition, Zafriri et al. showed that adherent bacteria are enhanced in their toxicity (54) and that anti-LT antibodies did not neutralize the toxicity of adherent bacteria (40). These observations suggest that the delivery of poorly secreted toxins, such as LT, to target epithelial cells may be enhanced by direct entry of ETEC into the cell.

Additionally, epithelial cell penetration could provide a protected niche for the organism or stimulate the production of unidentified toxins or virulence determinants. We are currently using H10407 strains bearing both *tia* and *tib* locus deletions to evaluate the relevance of epithelial cell invasion in the pathogenesis of ETEC.

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