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An *Edwardsiella tarda* Strain Containing a Mutation in a Gene with Homology to *shlB* and *hpmB* Is Defective for Entry into Epithelial Cells in Culture

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***Edwardsiella tarda* is an enteric pathogen that causes diarrhea, wound infections, and death due to septicemia. This species is capable of invading human epithelial cell lines, and we have now been able to follow the entry and replication of *E. tarda* within tissue culture host cells. *E. tarda* escapes from the endocytic vacuole within minutes of entry and then replicates within the cytoplasm. Unlike other well-studied bacteria that replicate and reside in the cytoplasm, we never observed this organism moving directly from cell to cell; instead the bacteria spread by lysing the plasma membrane after several rounds of replication. Efforts to study the interactions of *E. tarda* with tissue culture cells are complicated by the presence of a potent cytotoxin that the bacterium produces. Using transposon mutagenesis, we isolated a noncytotoxic strain of *E. tarda*. This mutant is also defective for hemolysin production. The dual phenotype of this strain is consistent with the hypothesis that cytotoxicity is due to the previously characterized *E. tarda* hemolysin activity. The nonhemolytic strain is also unable to enter HEp-2 cells. The disrupted gene has sequence similarity to members of a family of genes required for transport and activation of the hemolysin genes, *shlA* and *hpmA*. A cosmid bearing 40 kb of *E. tarda* DNA, including wild-type copies of the *E. tarda* homologs of the transporter-activator protein and the hemolysin itself, confers hemolytic, cytotoxic, and invasive abilities upon normally nonhemolytic, noncytotoxic, and noninvasive strains of *Escherichia coli*. Sequence data indicate that the genes required for hemolytic activity are linked to a transposable element, suggesting that they arose in the *E. tarda* genome by horizontal transfer.**

Edwardsiella tarda, a member of the family *Enterobacteriaceae*, causes illness in both humans and animals (11, 12, 16, 17, 33). *E. tarda* is ordinarily associated with gastroenteritis, but it can also cause wound infections and septicemia in patients with underlying immune disorders. The organism is common in tropical and subtropical environments and appears to be spread by contact with infected marine life, including ornamental fish and turtles (12, 31).

The virulence determinants of *E. tarda* are largely unknown. The clinical findings that *E. tarda* gastrointestinal infections can precede bacteremia and that the organism can cause bloody colitis (11) suggest that this pathogen can invade cells to spread systemically and cause tissue damage in vivo. Several research groups have shown that *E. tarda* enters both HEp-2 (14) and HeLa (19) cells; however, events subsequent to entry remain obscure.

Efforts to examine the behavior of *E. tarda* inside host cells over a period of hours have been hampered by the activity of a bacterial cell-associated cytotoxin that rapidly destroys the tissue culture monolayer. It was suggested by Janda and colleagues that the cytotoxicity is due to the activity of a cell-associated hemolysin (10, 14, 32).

In order to further examine the behavior of *E. tarda* within host cells, we used a two-pronged strategy, examining these interactions by microscopic and by molecular genetic approaches. We found that *E. tarda* replicates within the cytoplasm of host cells and eventually destroys the plasma, but not the nuclear membrane. The capacity to enter host cells appears to be related to the production of a cytotoxic hemolysin. Sequence analysis suggests that this activity is encoded by a gene that belongs to the *shlA* and *hpmA* gene family (1a).

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MATERIALS AND METHODS

Bacterial strains, tissue culture cells, and plasmids. *E. tarda* ET12 and SA8318 were obtained from J. Michael Janda, Microbial Diseases Laboratory, Berkeley, Calif. (13, 14). Streptomycin-resistant *E. tarda* ET12 was isolated by plating *E. tarda* ET12 on brain heart infusion (BHI) plates containing 200 μg of streptomycin sulfate/ml. Streptomycin-resistant mutants arose at a frequency of approximately 10^{-9}. These strains behaved similarly to the parent strain in terms of growth rates, cytotoxicity, HEp-2 cell invasion, and the ability to produce the sacs of bacterum-filled HEp-2 cells described in the text. HEp-2 tissue culture cells (24) were maintained in RPMI 1640 (Whittaker Bioproducts, Inc., Walkersville, Md.) and passaged every 2 to 3 days.

Media, reagents, and chemicals. Luria-Bertani (LB) broth and BHI broth were obtained from Difco (Detroit, Mich.) and prepared according to the manufacturer's instructions. Solid bacterial media were made by the addition of Bacto agar (1.5%). Antibiotics were added where appropriate to the following final concentrations: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; kanamycin, 50 μg/ml; and streptomycin sulfate, 200 μg/ml. Restriction endonucleases and DNA ligase were used according to the manufacturers' recommendations.

Vital dye exclusion assays. Trypan blue (0.1% final concentration) or eosin (1 mg/ml final concentration) was added to tissue culture cells in RPMI 1640. Cells with intact plasma membranes did not change appearance, while those with defective membranes allowed dye into the cytoplasm and appeared blue (trypan blue) or pink (eosin).

Hemolysin assay. Hemolysin activity was assayed on BHI agar plates containing 1% human erythrocytes, obtained by centrifuging whole blood that was gathered in heparinized collection tubes and washing it several times with phosphate-buffered saline (PBS). Bacterial strains to be tested were streaked onto these plates and incubated at 37°C overnight.

Electron microscopy. Electron microscopy was carried out as previously described (26). Bacterial cultures were grown to mid-logarithmic phase and diluted in PBS so that the appropriate number of bacteria would be contained in 100 μl (multiplicity of infection [MOI] of 1.0 for 0.5- to 4.5-h time points; MOI of 1.3 for 5-min time point). The bacteria were centrifuged onto HEp-2 cells that had been seeded the previous day at 10^5 cells/well on glass coverslips. The tissue culture medium was replaced with medium containing gentamicin at 30 min postinfection and again with medium lacking antibiotics at 90 min postinfection.

Gentamicin protection and survival assays. The assay was done as described previously (18) and below. Bacterial cultures were grown to mid-logarithmic phase and diluted in PBS so that the appropriate number of bacteria would be contained in 100 μl. Centrifugation in a clinical centrifuge at 470 × *g* for 5 min was carried out to increase the rate at which bacteria made contact with tissue culture cells. For survival assays, after the initial incubation in 100 μg of gentamicin/ml, the medium was replaced with RPMI 1640 that contained 4 μg of gentamicin/ml. (This concentration is also cytotoxic to *E. tarda*.)

Isolation of genomic and plasmid DNA. Genomic DNA from *E. tarda* was obtained by adding 1/12 volume of lysozyme (10 mg/ml) in 40 mM Tris–20 mM EDTA, pH 8.0, to a bacterial culture that had been grown overnight at 37°C and incubating for 30 min at 37°C. One-tenth volume of 10% sodium dodecyl sulfate was added, and the mixture was incubated at 65°C for 5 min and extracted three times with Tris-EDTA (TE)-saturated phenol, taking the interface each time. This step is critical to obtaining DNA from the *E. tarda* strains we have worked with. The mixture was then extracted three more times with TE-saturated phenol, taking only the aqueous layer, followed by extraction once with choloroform. NaCl (5 M) was added to a final concentration of 150 mM. Two volumes of ethanol was added, and the DNA was spooled onto a capillary tube which was sealed at the end. After resuspension in TE, the DNA was incubated at 65°C for 20 min and stored at 4°C.

Plasmid DNA was obtained from *E. tarda* by modifying the alkaline lysis protocol as follows. After the neutralization step (addition of sodium or potassium acetate), TE-saturated phenol was added and the samples were vortexed and then centrifuged in a microcentrifuge for 15 min. After this step, the standard protocol was followed (removal of the aqueous layer, isopropanol precipitation, etc.).

Plasmid DNA was obtained from *Escherichia coli* by the standard alkaline lysis method, by the manufacturer's suggested method included in Qiagen (Chatsworth, Calif.) kits, and by cesium chloride gradient purification.

Sequencing and PCR. Sequencing was carried out on double-stranded DNA with a 373A DNA sequencer (Applied Biosystems) with the ABI PRISM dye terminator cycle sequencing kit. Primers used were Tn*5*L2 (CCTCCAGA TCCTGGAAAACGGGA), for sequencing from the end of the Tn*5* derivative carried on pBDJ103, and HLY2RC (GTACCCGAGCCGTGATATACCC), which lies on the opposite strand from Tn5L2, within *ehlB*. PCR was carried out with the primers HLY2RC and HLY1 (CGCTAACCAAACAACAGCATGC).

In vitro transposon mutagenesis was carried out with Tn*7* (1) and transformed into *E. coli*. Cosmid-bearing bacteria that contained the transposon were selected by plating on kanamycin. Resulting transformants were then screened for hemolysin activity on blood plates containing kanamycin. The sequence adjacent to the site of transposon insertion was obtained with the primers NLC94 (AAAGTCCAGTATGCTTTTTCACAGCATAAC) and NLC272 (ATTTTCG TATTAGCTTACGACGCTACACCC).

Transposon mutagenesis. A plasmid (pBDJ103), carrying an ampicillin resistance gene, a Tn*5* derivative with a tetracycline resistance gene, and a streptomycin-sensitive allele of the ribosomal protein S12 gene, was obtained from Brad Jones (University of Iowa) (15). Although the transformation frequency of *E. tarda* is extremely low (more than 3 logs less than that of *E. coli*), we were able to obtain a Str^r ET12 colony bearing pBDJ103 by electrotransformation, using a Bio-Rad electroporator and plating on BHI containing ampicillin. A library of *E. tarda* transposon mutants was generated as described previously (15) with the following modifications. The Str^r ET12 strain carrying pBDJ103 was grown overnight in BHI containing ampicillin, plated for single colonies on BHI-ampicillin plates, and grown at 37°C. Replicas of the plates were made on BHI containing tetracycline, and single colonies were allowed to grow overnight; this step selects for the presence of the transposon and provides a period of time in which transposition can occur and the plasmid can be lost from the bacterial cell. Replicas of the plates were then made on BHI containing tetracycline and streptomycin sulfate; this step selects against the presence of the plasmid (streptomycin sulfate) but for the transposon (tetracycline). The absence of the plasmid in the mutant strains was confirmed by their lack of growth on BHI containing ampicillin.

Southern blot analysis was carried out by the Boehringer Mannheim digoxigenin chemiluminescence method according to the manufacturer's directions. Genomic DNA from ET12, Str^r Hly$^-$ ET12, and two hemolytic transposon mutant strains was digested with *Hin*dIII; in addition, DNA from pBDJ103 was included as a control. Probes were as follows: (i) the *Hin*dIII/*Xho*I fragment from pBDJ103, containing most of the tetracycline resistance gene; (ii) the *Bam*HI fragment from pBDJ103, containing only backbone plasmid; (iii) the *Bam*HI/*Eco*RI fragment from pBDJ103, containing the *lacZ* gene; and (iv) the *Hin*dIII fragment from pES37. Hybridization patterns indicated that there was only one transposon insertion in Str^r Hly$^-$ ET12.

Cloning of DNA containing transposon insertion. Genomic DNA from Str^r Hly$^-$ ET12 was digested with *Hin*dIII, which cuts once within the transposon, outside the end of the tetracycline resistance gene. pACYC177 was digested with *Hin*dIII and gel purified with the Geneclean system. Ligations were carried out overnight at 16°C and ligation mixtures were transformed by electroporation into *E. coli* DH12S and plated on LB agar plates containing tetracycline. Several hundred transformants were obtained by this method. Twelve of 12 transformants showed identical DNA fragment patterns, which we obtained by using several restriction enzymes.

Cloning of wild-type *ehl* locus. Genomic DNA from *E. tarda* ET12 was partially digested with *Sau*3A. pHC79 (Gibco BRL) was digested with *Bam*HI and gel purified with the Geneclean system. Ligation mixtures were incubated overnight at 16°C and packaged according to the manufacturer's instructions, using Gigapack II XL packaging extracts (Stratagene), which selectively package only cosmids with large (i.e., 40 to 45 kb) inserts. *E. coli* XL1 blue was infected with this mixture and plated on BHI agar plates containing 1% blood and ampicillin. In order to determine whether the hemolytic phenotype segregated with the cosmid, cosmid DNA was extracted from the hemolytic colony and retransformed into a different strain of *E. coli*, DH12S, and the electroporation mixture was plated on BHI-blood-ampicillin plates. All transformants were hemolytic.

RESULTS

***E. tarda* escapes from the host cell vacuole and replicates in the cytoplasm before lysing the plasma membrane.** Although it had previously been shown that *E. tarda* is capable of invading epithelial tissue cells in culture, characterization of bacterium-host cell interactions was complicated by the presence of a bacterium-produced cytotoxin that kills host cells and destroys the monolayer shortly after contact. In order to circumvent this problem, we infected at an approximately 10-fold-lower MOI than that previously used. HEp-2 cells were inoculated with bacteria at a MOI of approximately 1, and electron micrographs were obtained at several time points over a period of 4.5 h. Under these conditions, the monolayer remained intact, as measured by vital dye exclusion assays and visual examination.

Within 5 min of centrifuging the bacteria onto the tissue culture cells, individual organisms could be seen in tight association with the host cell plasma membrane (Fig. 1A). In some cases, the membrane was not well defined or was absent at the point of contact (Fig. 1B). After 30 min, bacteria were observed within membrane-bound vacuoles in the cytoplasm (Fig. 1C to E). Out of 12 intracellular bacteria seen at this time point or earlier, we never observed a bacterium completely encased by membranous material, which suggests that endocytosis is accompanied by the initial stages of membrane lysis (Fig. 1D and E).

After 1.5 h, small numbers of bacteria (usually one or two) could be seen in the cytoplasm of the host cell. At 2.5 and 3.5 h postinfection, multiple bacteria were present in the cytoplasm (Fig. 1F and G). We believe that the increased numbers of bacteria represent intracellular replication, because we prevented the possibility of continued infection by killing extracellular bacteria with gentamicin 30 min postinfection. After a 60-min incubation period with the drug, we placed the cells in antibiotic-free medium. This was critical in order to allow unencumbered bacterial growth: 2.5 to 3.5 h postinfection, the host cell membrane became compromised and allowed gentamicin into the cytoplasm. Four and one-half hours after initial contact with bacteria, the host cell cytoplasm had almost completely lost its electron-dense character (Fig. 1H), the plasma membrane was destroyed, and bacteria could sometimes be seen escaping from the cell. The breach in the integrity of the plasma membranes of infected cells identified visually at 4.5 h postinfection was confirmed by vital dye exclusion assays. In contrast to the plasma membrane, the nuclear membrane appeared intact (Fig. 1H).

We also investigated the replication of *E. tarda* inside HEp-2 cells by conducting survival assays. After allowing invasion to occur and killing extracellular bacteria with gentamicin, we lysed the tissue culture cells periodically and counted the intracellular bacteria. Between 1.3 and 2.3 h postinfection, the number of output bacteria increased, even in the presence of gentamicin (Fig. 2). After 2.3 to 3.5 h (depending on the exact experimental design), the number of recoverable CFU decreased dramatically if the tissue culture medium contained

gentamicin (Fig. 2), whereas the numbers continued to rise (until the end of the experiment, usually 7 or 8 h postinfection) if the medium contained no antibiotic (data not shown). The decreased numbers of bacteria in the presence of gentamicin several hours postinfection presumably reflect entry of the antibiotic into the HEp-2 cells due to the impaired integrity of the plasma membranes. The general features of the intracellular behavior of *E. tarda* indicated by visual examination were thus corroborated by the results of survival assays.

It was also possible to identify individual infected cells in the tissue culture monolayer by phase microscopy: HEp-2 cells containing large numbers of *E. tarda* organisms 4.5 h postinfection were beginning to detach from the tissue culture well, appeared round, and were filled with motile bacteria (data not shown). Although the host cells at this point were permeable to gentamicin, they were able to contain the writhing *E. tarda* organisms, whose movements were clearly confined by the plasma membrane.

An *E. tarda* mutant strain defective for hemolysin production is not cytotoxic to HEp-2 cells. Although it was possible to observe interactions between *E. tarda* and host cells over several hours, it was necessary to use a fairly low MOI in order to minimize the cytotoxicity resulting from exposing the tissue culture cell monolayer to large numbers of microorganisms. We reasoned that a strain defective in the well-established hemolytic activity elaborated by *E. tarda* (10) might allow us to study bacterium-host interactions without the complications due to bacterium killing of host cells.

We constructed a collection of 400 *E. tarda* strains bearing random chromosomal insertions of a Tn*5* derivative (see Materials and Methods). These mutants were tested for hemolytic activity on BHI broth plates containing human blood. Of the 400 strains, two were nonhemolytic. One of these displayed marked autoagglutination, settling to the bottom of a culture tube after standing for several minutes; this strain was not characterized further. The other strain (Str^r Hly^- ET12) appeared identical to the parent (in terms of growth characteristics and the physical appearance of the culture) except for its inability to lyse erythrocytes (Fig. 3).

We compared the cytotoxicities of the parental strain and the nonhemolytic mutant by assessing their abilities to remove cell monolayers from plastic wells. Approximately 75-fold more Str^r Hly^- ET12 than Str^r ET12 bacteria were required to destroy tissue culture cells, as assessed by Giemsa staining the monolayer. In order to determine whether the toxic agent was secreted into the medium, we obtained bacterium-free culture supernatant in two ways: (i) by centrifugation, to pellet the bacteria, and subsequent decanting and (ii) by filtering the culture through 0.2-μm-pore-size filters. These bacterium-free supernatants were tested with HEp-2 cells in the vital dye exclusion assay, and we found that the cytotoxic effects were associated uniquely with the bacteria, not the supernatant, a property shared with the previously characterized cell-associated *E. tarda* hemolysin (10).

The hemolysin mutant is defective for entry into HEp-2 cells. The hemolysin mutant was tested for entry into HEp-2 cells in a standard gentamicin protection assay (18). The number of CFU obtained by this technique is proportional to the number of intracellular organisms at the time of gentamicin addition. As shown in Fig. 4, Str^r Hly^- ET12 enters HEp-2 cells two to three orders of magnitude less efficiently than its parental strain, Str^r ET12, although they adhere at similar levels (data not shown). This defect in HEp-2 cell entry appears to be specific for the nonhemolytic mutant strain, as other transposon mutants invade at levels similar to that of Str^r ET12 (data not shown).

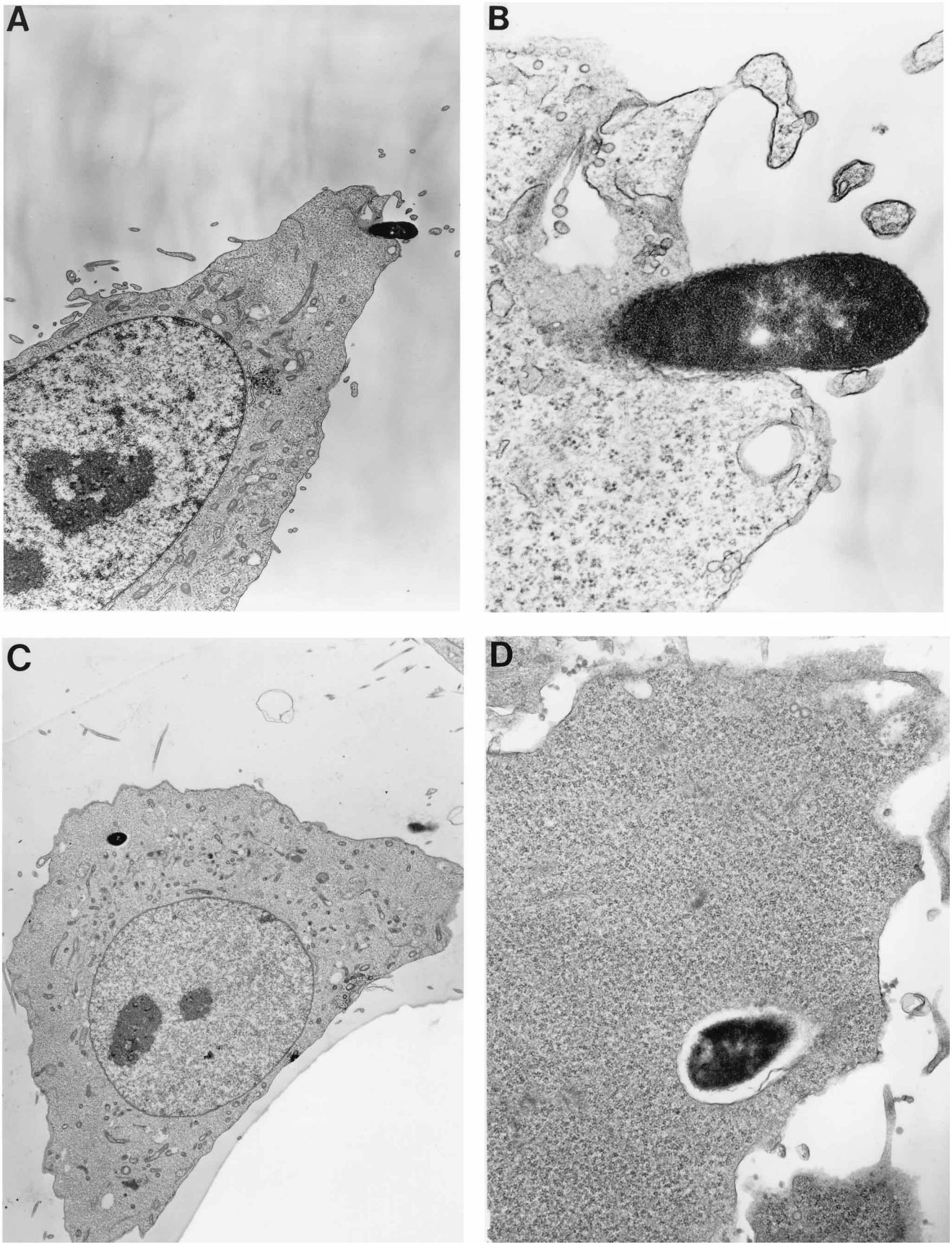
The transposon insertion carried by Str^r Hly^- ET12 interrupts a gene that is similar to *shlB* and *hpmB*. In order to locate the site of the transposon gene disruption, we first confirmed that there was only a single insertion in Str^r Hly^- ET12, using Southern blot analysis. A chromosomal fragment of DNA containing a portion of the transposon and flanking DNA was isolated by ligating random 8- to 12-kb pieces of *Hin*dIII-cut chromosomal Str^r Hly^- ET12 DNA to pACYC177. The ligation mixture was transformed into *E. coli* DH12S and plated on LB agar containing tetracycline in order to select for the presence of the tetracycline resistance gene from the transposon. In this way, a plasmid containing a portion of the transposon along with a piece of flanking chromosomal DNA was isolated (pES37).

Sequence information from the DNA adjacent to the transposon insertion site was obtained by using a primer that lies close to the end of Tn*5*. Subsequently, other primers, designed to hybridize within the disrupted gene, were used to confirm the sequence. Examination of the 225-nucleotide DNA sequence revealed an open reading frame of 75 amino acids, with significant homology to the *shlB* gene (BLASTP probability score, 8.5e-13) of *Serratia marcescens* and the *hpmB* gene (BLASTP probability score, 4.9e-11) of *Proteus mirabilis* (Fig. 5) (27, 30). Each of these genes encodes a protein that is required for outer membrane transport and activation of the hemolysins encoded by *shlA* and *hpmA* (1a). The *E. tarda* locus was named *ehl* (*E. tarda* hemolysin), by analogy with the nomenclature of the first member of this gene family, the *S. marcescens* hemolysin operon (3, 27).

A cosmid containing wild-type *ehl* confers hemolysin activity upon normally nonhemolytic *E. coli*. In order to determine whether the region closely linked to *ehlB* includes genes sufficient to produce an active hemolysin molecule, we sought the wild-type gene. Genomic DNA from the wild-type *E. tarda* ET12 was digested with *Sau*3A and ligated into the *Bam*HI site of pHC79. The ligation mixtures were packaged and used to infect *E. coli*; the resultant mixture was plated on BHI containing ampicillin and human blood. In this way, we obtained a cosmid (pES185) that contains approximately 40 kb of insert DNA and confers hemolytic activity on two different nonhemolytic strains of *E. coli* (Fig. 6 and data not shown). *E. coli* bearing pES185 also produces cytotoxic effects similar to those of *E. tarda*; in contrast to HEp-2 cells incubated with *E. coli* carrying pHC79, those incubated with the hemolysin-producing cosmid round up and detach from plastic culture dish wells.

In order to confirm that this cosmid contains the *ehlB* gene, we sequenced a portion of the cosmid, using primers designed to hybridize within the *ehlB* region. Specificity of primer binding was shown by carrying out PCR. As expected, after agarose gel electrophoresis, we observed PCR amplification products

FIG. 1. Electron microscopy of interactions between *E. tarda* and HEp-2 cells. (A) *E. tarda* adheres to HEp-2 cells 5 min after having been centrifuged onto the monolayer. Magnification, ×4,500. (B) The plasma membrane of the host cell appears fuzzy and dissolved at the point of contact. Magnification, ×30,000. (C) After 30 min, HEp-2 cells containing single bacteria within membrane-bound vacuoles are seen. Magnification, ×3,000. (D and E) High magnification of bacteria within vacuoles at 30 min postinfection (the same bacterium is shown in panels C and D). Magnification, ×15,000. (F and G) HEp-2 cells containing *E. tarda* at 2.5 and 3.5 h postinfection. Magnification, ×4,500 (F) and ×3,000 (G). (H) The cytoplasm of an infected cell has lost its electron-dense character at 4.5 h postinfection, and there is a breach in the integrity of the plasma membrane; the nuclear membrane remains intact. Magnification, ×2,000.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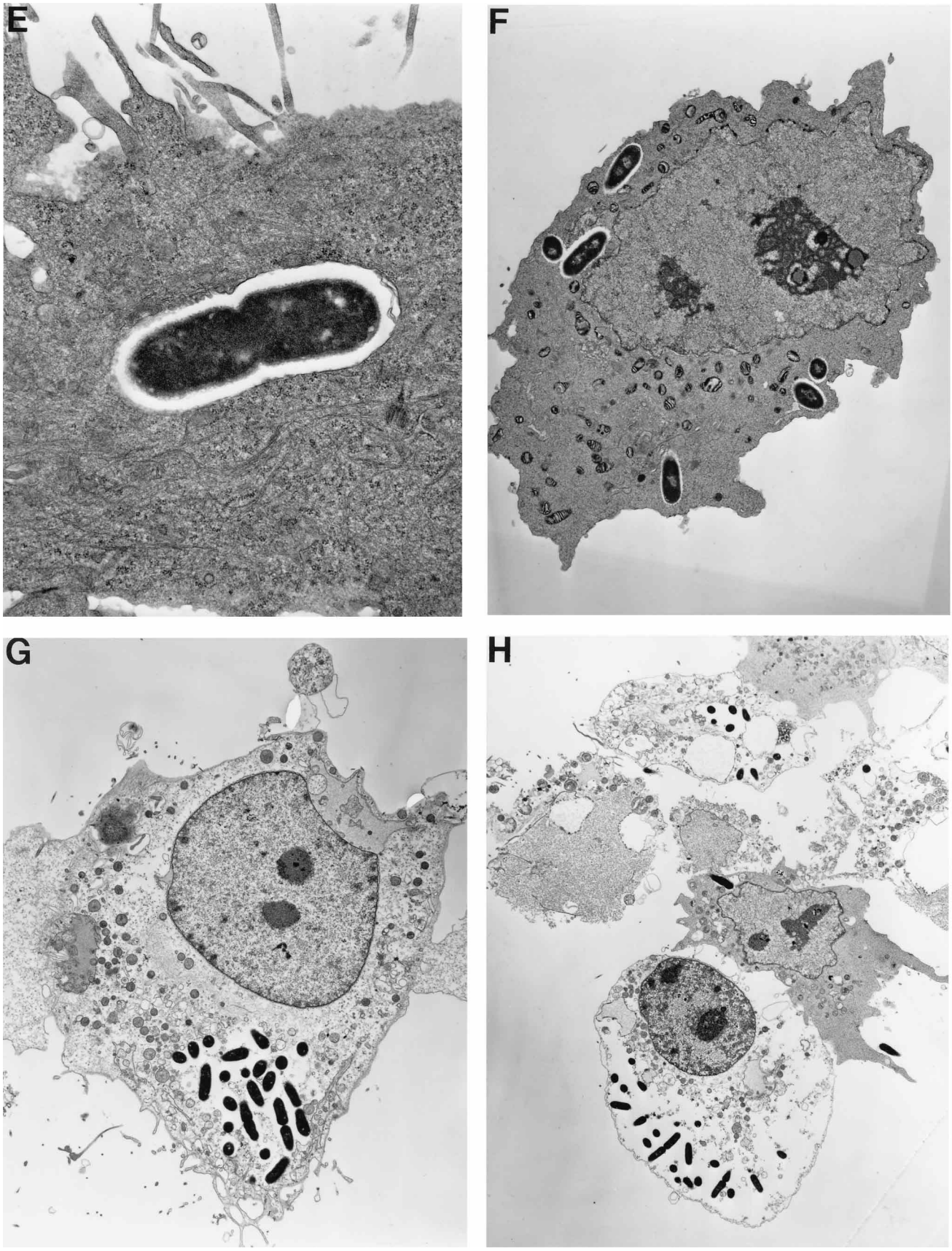

FIG. 1—*Continued.*

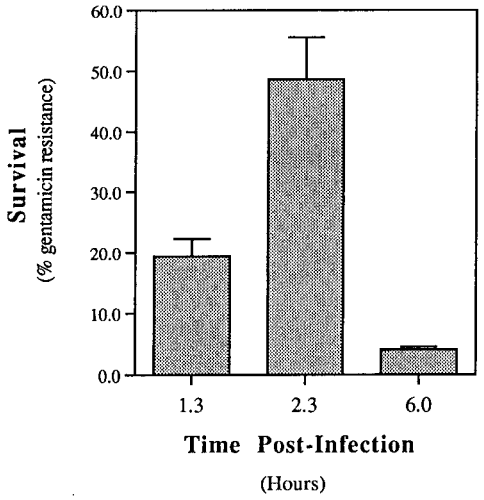

FIG. 2. *E. tarda* replicates within HEp-2 cells in the presence of gentamicin at early time points. Invasion efficiency is represented as the percentage of input bacteria that survive gentamicin treatment (1.3-h time point). When the HEp-2 cells are cultured continuously in gentamicin, the number of recoverable bacteria increases between 1.3 and 2.3 h postinfection and then decreases between 2.3 and 6.0 h postinfection. Each sample was assayed in triplicate, and the experiment was repeated three times. The graph shows results from a representative experiment. Error bars represent standard deviations.

of the same size (241 nucleotides) from both pES185 and pES37; production of these products was dependent on the presence of both primers and was not observed with two different sister cosmids, containing different pieces of *E. tarda* DNA, as templates. These results confirm that *ehlB* is carried on pES185.

A cosmid containing wild-type *ehl* confers invasive ability upon normally noninvasive *E. coli*. The noninvasive phenotype of the Str^r Hly$^-$ ET12 mutant raises the possibility that the hemolysin itself functions as an invasin. Although it will not be possible to resolve this issue until we have an isolated piece of DNA that contains solely the (presumptive) *ehl* operon, we sought to address the issue in a different way.

Random transposon insertions in the cosmid pES185 were

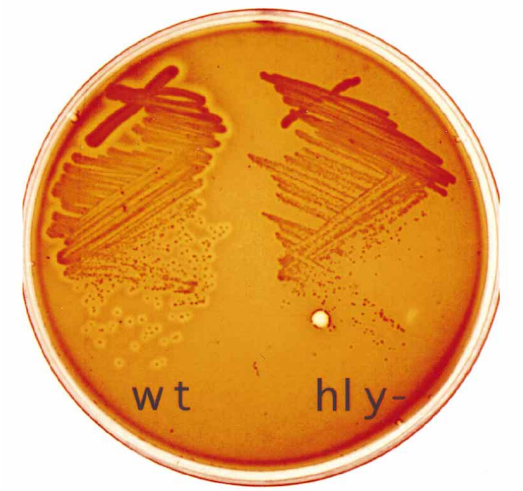

FIG. 3. *E. tarda* Str^r Hly$^-$ ET12 is nonhemolytic. ET12 (wild type [wt]) and Str^r Hly$^-$ ET12 (nonhemolytic mutant [hly−]) were streaked on BHI blood agar plates.

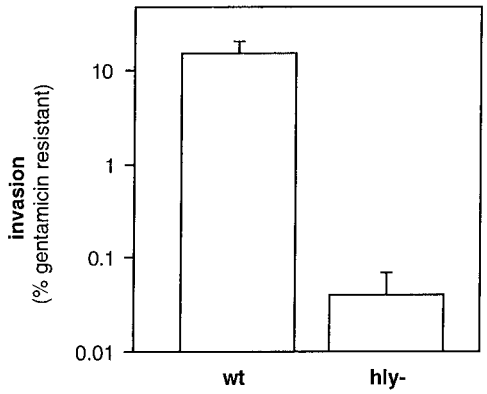

FIG. 4. The nonhemolytic mutant strain, Str^r Hly$^-$ ET12 (hly−), is defective for entry into HEp-2 cells. Invasion efficiency is represented as the percentage of input bacteria that survive gentamicin treatment. Wild type (wt; Str^r ET12) is the hemolytic parental strain. Invasion for each strain was assayed in triplicate, and the experiment was repeated three times. The graph shows results from a representative experiment. Error bars represent standard deviations.

constructed in vitro. Following transformation into *E. coli* with selection for the transposon, we screened for hemolytic and nonhemolytic isolates. We chose six *E. coli* strains: three retained hemolytic activity and three had no hemolytic activity. These strains represented cosmids with transposon insertions at different sites (confirmed by DNA sequence analysis). Sequence analysis revealed that the transposon in several of the nonhemolytic strains is inserted into an open reading frame with significant homology to the genes *shlA* (BLASTP probability score, 4.9e-15), *hpmA* (BLASTP probability score, 4.5e-17), and *hhdA* (a third member of this family found in *Haemophilus ducreyi*; BLASTP probability score, 6.4e-6) (25), suggesting the presence of a gene (*ehlA*) on the cosmid that encodes the hemolysin itself (Fig. 7).

We tested the six strains in the gentamicin protection assay. As shown in Fig. 8, the hemolytic strains invaded HEp-2 cells more than two orders of magnitude more efficiently than the nonhemolytic strains did; invasion by the nonhemolytic strains was below the detection level of the assay. The levels of invasion by the hemolytic strains were approximately 10-fold lower than that exhibited by wild-type *E. tarda*, suggesting that additional factors in *E. tarda* are required for efficient invasion. (Invasion levels were 20- to 50-fold higher for *E. coli* containing the hemolysin-bearing cosmid than for the Str^r Hly$^-$ ET12 mutant strain.)

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shlB   ESRRALQDSTREVNQLIEQRRYQQLKQQ
       |||||||+|||++| |+||+   |+| +|
ehlB   ESRRALQNSTRQLNSLLEQQTLQRLTKQ
       |||||||+| |++| |+||    |+| ++
hpmB   ESRRALQDSQREINQLIEQNRYQQLQEK

shlB   CLPIAGVYLQGVTLLSPADL
       ||||+|||| |+|||| ||
ehlB   CLPISGVYLTGITLLSLHDL
       |||| |||+ |||||+  ||
hpmB   CLPIKGVYIQGITLLTEKDL
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FIG. 5. The transposon insertion in Str^r Hly$^-$ ET12 interrupts a gene that is similar to *shlB* and *hpmB*. For ShlB, the amino acid sequence blocks are residues 34 to 61 (upper alignment) and residues 78 to 97 (lower alignment). For HpmB, the amino acid sequence blocks are residues 32 to 59 (upper alignment) and 76 to 95 (lower alignment). Lines indicate identical residues. Pluses indicate conserved amino acid changes.

FIG. 6. A cosmid carrying the *ehl* locus confers a hemolytic phenotype on a normally nonhemolytic *E. coli* strain. *E. coli* XL1 blue, bearing the cosmid with the *ehl* locus (pES185), and a sister cosmid (pES184), with a similarly sized fragment of *E. tarda* DNA, were streaked on BHI blood agar plates.

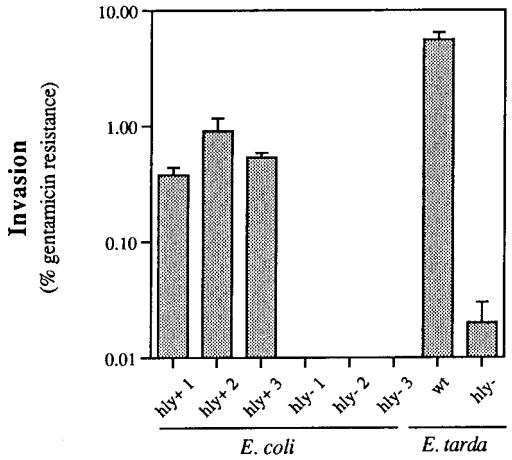

FIG. 8. Hemolytic *E. coli* invade HEp-2 cells. *E. coli* bearing hemolysin-producing (hly+) and hemolysin-defective (hly−) cosmids were tested in the gentamicin protection assay. Wild-type (wt; Str^r ET12) and nonhemolytic (Str^r Hly^- ET12) *E. tarda* strains are shown for comparison. The invasion for each strain was assayed in triplicate, and the experiment was repeated three times. The graph shows results from a representative experiment. Error bars represent standard deviations.

DISCUSSION

We have undertaken the study of *E. tarda* and its interactions with tissue culture cells and have begun to characterize an apparently novel strategy by which this organism interacts with host cells in culture. After entry into an epithelial cell line, HEp-2, the bacteria escape from the host cell vacuole and replicate within the cytoplasm (Fig. 1). After several hours of infection, the integrity of the plasma membrane is seriously impaired, while the nuclear membrane appears to remain intact (Fig. 1). Eventually the bacteria escape into the extracellular medium.

In contrast to other bacteria that replicate in the cytoplasm, such as *Listeria monocytogenes* and *Shigella flexneri* (29), *E. tarda* does not appear to harness actin or engage in direct cell-to-cell spread. In the electron micrographs, we never observed the bacterium-containing protrusions characteristic of intercellular movement. Furthermore, it was quite common to see an infected HEp-2 cell adjacent to one or more uninfected cells; there was no evidence of clusters of infected cells, as would be expected at late time points if the bacteria could move directly from one cell to another. Finally, *E. tarda* does not form plaques (data not shown).

Previous attempts to characterize the behavior of *E. tarda* inside host cells were confounded by the activity of a potent cytolysin that destroyed the tissue culture cell monolayer with which the bacteria were interacting. In the electron microscopy study described above, we avoided this problem by infecting at an MOI of 1 instead of 10 and incubating for 30 min or less before killing the extracellular bacteria. We were curious, however, whether we could construct a strain that was not cytotoxic by interfering with the production of the well-characterized *E. tarda* hemolysin (10). We generated a bank of transposon-mutagenized strains and found a mutant defective in β-hemolysin production, as assayed on blood agar plates. As predicted, this strain has decreased lethal effects on HEp-2 cells. The site of the transposon insertion suggests that the phenotype is a direct result of the genetic disruption. The sequence immediately adjacent to the transposon is highly homologous to the sequence of genes known to be required for hemolysin activity and secretion; further sequence analysis revealed that the cosmid also contains a sequence homologous to the hemolysin gene itself (27, 30). Moreover, a cosmid containing a wild-type copy of the disrupted gene (as well as additional sequences) confers hemolytic and cytotoxic activities on nonhemolytic, noncytotoxic strains of *E. coli.*

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shlA   ATGAQVVDIVAPNGNGLSHNQYQDFNVNQPGAVLNNSREAGLSQ
ehlA   ANGAQVVNIVKPTDQGLSHNQYQDFNVNRPGAVFNNALSAGQSQ
hpmA   NGGTQVINIVTPNNEGISHNQYQDFNVGKPGAVFNNALEAGQSQ
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FIG. 7. Transposon insertions that destroy hemolytic activity in several of the cosmids (pES185) interrupt a gene that is similar to *shlA* and *hpmA*. For ShlA, the amino acid sequence blocks are residues 48 to 91. For HpmA, the amino acid sequence blocks are residues 47 to 90. Lines indicate identical residues. Pluses indicate conserved amino acid changes.

The two other members of this gene family, *shlB* and *hpmB*, encode integral outer membrane proteins that are responsible for activating and secreting the *shlA* and *hpmA* gene products, respectively (4). These secreted hemolysins are distinct from the well-known family of RTX proteins that includes the HlyA hemolysin of *E. coli* (1a). Distinguishing features of the ShlA-HpmA family are classic Sec-dependent transport across the inner bacterial membrane, ShlB- or HpmB-dependent activation and transfer through the outer membrane, and a conserved operon structure in which the B gene lies upstream of the A gene (2, 27, 30). Like the *E. tarda* hemolysin, the homologous *P. mirabilis* and *S. marcescens* proteins can be expressed in functional form in *E. coli* (3, 30) and there is evidence that they are produced in response to iron starvation (2, 30).

Although it is possible that the transposon insertion interferes with the production of a hemolysin gene downstream of the presumptive *ehl* operon or that there is another (unmarked) genetic lesion in this strain, the most parsimonious hypothesis with which to explain these results is that the putative A-B operon encodes the cell-associated hemolysin of *E. tarda* that has been described by other groups. Production of this hemolysin increases in response to iron starvation (10), and the protein is cell associated except under this growth condition. In contrast, Chen and colleagues recently reported

a hemolysin-encoding gene in *E. tarda*, whose sequence is unrelated to that of the gene we have identified in this work and whose product is secreted (7). These investigators suggest that the reason for the apparently discrepant results regarding hemolysin characteristics is due to strain differences (6).

In addition to the hemolytic defect and decrease in cytotoxicity, the nonhemolytic mutant is defective for entry into HEp-2 cells. Although we have not determined whether the invasion defect of this strain is a direct effect of the insertion, our results are consistent with a critical role for this putative hemolysin activator-transporter protein in entry, presumably because transcription of *ehlA* is coupled to *ehlB* transcription and/or by virtue of the *ehlB* gene product's ability to transport and activate the hemolysin. The cosmid bearing the wild-type *ehlA* and *ehlB* genes confers substantial invasive ability upon normally noninvasive *E. coli*. Furthermore, random mutations in the cosmid that eliminate hemolytic activity also eliminate invasive functions; conversely, random mutations that retain cosmid-encoded hemolytic functions maintain invasive functions. Interestingly, a naturally occurring nonhemolytic *E. tarda* strain (SA8318) was previously shown to invade HEp-2 cells less efficiently than a nonisogenic wild-type strain (14). At least one hemolysin, the *ipaB* gene product of *S. flexneri*, is required for invasion of epithelial cells (9). IpaB also mediates escape from the vacuole, and our results are consistent with a similar role for the *ehl* locus or another gene on the cosmid. Using electron microscopy, we have observed *E. coli* bearing pES185 in the cytoplasm of HEp-2 cells, partially surrounded by pieces of membranous material; furthermore, data from survival assays (similar to that shown in Fig. 2) are also consistent with a scenario in which *E. coli* bearing the cosmid gain entry to the cytoplasm, where the bacteria replicate (data not shown).

These results do not prove that the hemolysin itself functions in invasion, although they are suggestive. It is possible that the correlation between disruption of the hemolytic phenotype and invasive ability is due to polar effects on downstream genes. This raises the possibility that a number of presumptive virulence functions, encoded by multiple linked genes, are present on pES185. Indeed, one of the transposon insertions that eliminated hemolysin activity lies in an open reading frame with substantial homology to a transposable element, suggesting that the hemolysin of *E. tarda* is associated with a mobile genetic element. Numerous pathogenic bacteria contain pieces of apparently foreign DNA that encode multiple virulence functions; notably, hemolysins are common components of these pathogenicity islands (20). Perhaps all or part of the 40-kb piece of DNA carried on the cosmid represents another pathogenicity island. Genetic proof of a direct role for *ehl* in invasion awaits functional complementation with the cloned hemolysin gene alone in Str^r Hly^- ET12. Although it is sometimes possible to transform *E. tarda* (as we did to introduce the transposon we used for mutagenesis), transformation efficiencies are very low, at best, and we have been unable to obtain an *E. tarda* strain containing pES185. Finally, although the cosmid confers hemolytic, cytotoxic, and invasive abilities upon normally nonhemolytic, noncytotoxic, and noninvasive *E. coli*, it is formally possible that the cosmid DNA functions differently in *E. tarda* than in *E. coli*. The activities we observe in *E. coli* might only coincidentally provide the same functions as the ones disrupted in the *E. tarda* strain containing the transposon insertion at the same locus.

Like the *E. tarda* hemolysin, HpmA is cytotoxic to tissue culture cells; the protein, however, does not appear to be required for host cell entry (8, 23). In contrast to the *E. tarda* hemolysin mutant, a *P. mirabilis* strain defective for hemolytic activity is protected from gentamicin killing at 10- to 100-fold higher levels than the wild-type parent, as expected if the hemolysin plays no role in invasion but kills tissue culture cells during the course of the assay. The two organisms differ in entry mechanisms in other ways as well: *E. tarda* appears to use the host cytoskeleton in this process, as invasion is inhibited by cytochalasin D (14), whereas *P. mirabilis* entry is unaffected by this agent (8). For both *E. tarda* and *P. mirabilis*, it appears that the hemolysin is the predominant, if not the only, cytotoxic factor. Although there is some evidence that HpmA contributes to virulence, this has not yet been established (21, 22, 28). Similarly, the role of the *shl* locus in *S. marcescens* virulence remains obscure (5).

E. tarda is a relatively rare pathogen that causes illness predominantly in tropical and subtropical regions of the world. Illness due to this species can be serious and even lethal, particularly in people with underlying liver disorders or other immunocompromising conditions. The behavior of *E. tarda* in tissue culture cells might reflect its intracellular replication in a host organism, with subsequent spread. Possible functions for the hemolysin include a role in invasion, escape from the host cell vacuole, and/or a contribution to invasive disease or systemic spread. It is also possible that the cytotoxic property of this molecule is used to kill immune cells or other host cells. While our results are consistent with these scenarios, the function of the *ehl* locus remains speculative at the moment. Future studies should further elucidate the molecular mechanisms of virulence of this interesting bacterial pathogen.

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ADDENDUM IN PROOF

After this paper was accepted, we became aware of a publication that reported the cloning and sequencing of an *E. tarda* hemolysin gene with homology to the *hpmA*/*shlA* genes (I. Hirono, N. Tange, and T. Aoki, Mol. Microbiol., **24:**851–856, 1997).

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