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Molecular Analysis of the Plasmid-Encoded Hemolysin of *Escherichia coli* O157:H7 Strain EDL 933

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In this study, we determined the nucleotide sequence of the 5.4-kb *Sal*I restriction fragment of the recombinant plasmid pEO40-1, cloned from the large plasmid of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 strain EDL 933. This revealed two open reading frames which shared approximately 60% homology to the *hlyC* and *hlyA* genes of the *E. coli* α-hemolysin (α-*hly*) operon. We termed these genes EHEC-*hlyA* and EHEC-*hlyC* to distinguish them from the α-*hly* genes. Preliminary sequence analysis indicated that another open reading frame homolog to the *hlyB* gene is located close to the 3′ end of EHEC-*hlyA*. The predicted molecular masses of the EHEC-*hlyA* and EHEC-*hlyC* gene products were 107 and 19.9 kDa, respectively. The EHEC hemolysin protein (EHEC-Hly) was not secreted into the culture supernatant by the strain EDL 933. However, hemolytic activity was found in the broth culture supernatant after transforming EDL 933 with the recombinant plasmid pRSC6 carrying the *hlyB* and *hlyD* genes from the *E. coli* α-hemolysin operon. The EHEC hemolysin was precipitated and used as an antigen for immunoblot analysis. This demonstrated that 19 of 20 reconvalescent-phase serum samples from patients with hemolytic uremic syndrome reacted specifically with the antigen; conversely, only 1 of 20 control serum samples demonstrated reactivity. To investigate the prevalence of EHEC hemolysin genes in diarrheagenic *E. coli*, a PCR was developed to specifically detect EHEC-*hlyA*. All Shiga-like toxin-producing O157 strains and 12 of 25 Shiga-like toxin-producing non-O157 strains were PCR positive; strains of other categories of diarrheagenic *E. coli* were PCR negative. All PCR-positive strains hybridized with the CVD 419 probe. We found the CVD 419 probe to be identical to the 3.4-kb *Hin*dIII fragment of plasmid pEO40 carrying most of the EHEC-*hlyA* gene and a part of the putative EHEC-*hlyB* gene. In this study, the newly discovered EHEC hemolysin was shown to be responsible for the enterohemolytic phenotype and demonstrated to be related but not identical to α-hemolysin. The EHEC hemolysin appears to have clinical importance because it occurs in all O157 strains tested and is reactive to sera of patients with hemolytic uremic syndrome.

Escherichia coli strains which cause extraintestinal diseases often have the ability to lyse erythrocytes in different mammalian species (hemolysis) (7). Several types of hemolysins have been described for *E. coli* from different pathogroups (1), the best characterized being α-hemolysin (1, 7, 44). The production of active extracellular α-hemolysin requires the products of the four linked genes *hlyC*, *hlyA*, *hlyB*, and *hlyD* (45). α-Hemolysin is synthesized as an inactive polypeptide and converted in its active form by the addition of a fatty acid group catalyzed by the HlyC protein (18). The secretion of α-hemolysin is signal peptide independent and mediated by a specific membrane translocator system encoded by *hlyB* and *hlyD* (44). The outer membrane protein TolC, encoded by a gene located outside the *hlyCABD* gene cluster, is also required for secretion (43).

Another type of hemolysin, termed enterohemolysin, was shown to be closely associated with *E. coli* serotypes O157, O26, and O111 (2, 3). Recently, we have shown that the enterohemolysin of O157:H7 strains is encoded on their large virulence plasmid. Hybridization studies revealed that the hemolysin is related to *E. coli* α-hemolysin (36).

E. coli O157:H7 strains are the predominant serotype causing hemorrhagic colitis, sometimes followed by extraintestinal complications. Much attention has been directed to the function of Shiga-like toxins and the intimin (9, 25, 28, 46) in these bacteria, but little is known about other factors of pathogenicity.

Hemolysins (cytolysins) were described to be important virulence factors of bacteria causing extraintestinal diseases (7) and are active on different cells, such as lymphocytes, granulocytes, erythrocytes, and renal tubular cells (6, 8, 16, 38). By analysis of the DNA sequences of two of the genes encoding the enterohemorrhagic *E. coli* (EHEC) hemolysin determinant, we can attribute this new hemolysin to the RTX (repeats in toxin) family of pore-forming cytolysins. Furthermore, we demonstrate that the enterohemolytic phenotype found with the EHEC hemolysin is related to a defective hemolysin secretion system. A possible clinical significance of EHEC hemolysin is pointed out by the specific immune response of O157-associated hemolytic uremic syndrome (HUS) patient sera to the EHEC hemolysin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used for characterization of EHEC hemolysin were described elsewhere (36). The clinical isolates used for PCR analysis, originating from patients with diarrheal diseases, were from our strain collection. For routine purposes, *E. coli* strains were grown in Luria broth. For purification of the EHEC hemolysin, *E. coli* strains WAF100/pSF4000 and C600/pEO40 + pRSC6 were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.). For maintenance of recombinant plasmids, 30 μg of chloramphenicol per ml, (pRSC6), 50 μg of kanamycin per ml (pEO40-1, pEO40-2, and pEO40-3), and 50 μg of ampicillin per ml (pEO40) were added to the culture media. For detection of enterohe-

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molysis, blood agar plates with defibrinated and washed sheep erythrocytes were used as described previously (36). The plasmid pRSC6 (*hlyB*$^+$ *hlyD*$^+$) was kindly provided by Ivo Gentschev, Würzburg, Germany. Plasmids pUC18 (42) and pK18 (29) were used as cloning vectors.

General recombinant DNA methods. Plasmid DNA was purified with the Qiagen plasmid Midi kit (Qiagen, Düsseldorf, Germany). Restriction endonuclease digestion, ligation, agarose gel electrophoresis, and transformation of plasmids were performed according to standard methods (32). Purification of DNA fragments from agarose gels was carried out with the Prep a Gene kit (Bio-Rad, Munich, Germany). Hybridization experiments were conducted with the digoxigenin labeling and detection kit (Boehringer GmbH, Mannheim, Germany) as previously described (36).

PCR. PCR for detection of EHEC-*hlyA* sequences was performed with the GeneAmp PCR System 9600 (Perkin-Elmer–Applied Biosystems GmbH, Weiterstadt, Germany). Primers hlyA1 (5′-GGT GCA GCA GAA AAA GTT GTA G-3′) and hlyA4 (5′-TCT CGC CTG ATA GTG TTT GGT A-3′) were designed to amplify a 1,551-bp fragment from the 5′ region of the EHEC-*hlyA* gene. Briefly, 10^4 bacterial cells derived from a single colony were suspended in saline (0.85% NaCl). PCR was performed by addition of 5 μl of this bacterial cell suspension to a reaction mixture containing 30 pmol of each primer per ml, 5 μl of 10-fold-concentrated polymerase synthesis buffer, 200 μM (each) deoxynucleoside triphosphate, and 2.0 U of *Taq* polymerase (Amersham Laboratories, Buckinghamshire, United Kingdom). The samples were filled up with water to a final volume of 50 μl. After an initial denaturation of 5 min at 94°C, the samples were subjected to 30 cycles of amplification, each of which consisted of 30 s at 94°C, 90 s at 57°C, and 90 s at 72°C. Five microliters of the PCR products was mixed with gel loading buffer and electrophoresed on 0.7% agarose gels.

Nucleotide sequencing and sequence analysis. The nucleotide sequence was determined by an automated fluorescence procedure based on the Sanger dideoxy chain termination method (33). The *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Inc.) was used. Stock solutions of double-stranded plasmids pEO40 and pEO40-1 were used as template DNA. For starting the sequencing reactions, 9.5 μl of dye terminator premix was mixed with 1.5 μg of plasmid DNA, 10 pmol of primer, and distilled water to reach a final volume of 20 μl. The samples were subjected to the following PCR procedure. The DNA was denatured for 15 s at 96°C, and then the primers were annealed at the appropriate temperature (50 to 64°C, depending on the oligonucleotide sequences) for 15 s and then extended for 4 min at 60°C. After 25 cycles, the samples were phenol-chloroform extracted twice and ethanol precipitated and the pellet was dissolved in 4 μl of formamide–50 mM EDTA (pH 8.0 [5:1]). After being heated for 2 min to 90°C, the samples were ready for loading on the gel.

Nucleotide sequence data were obtained by stepwise sequencing. Initially, universal and reverse primers for pUC18 (Boehringer GmbH) were used for sequencing various subclones. As new sequence data became available, customized synthetic primers (22-mers) were utilized (Roth, Karlsruhe, Germany). The sequencing reaction products were analyzed with the Applied Biosystems model 373A DNA sequencer (Applied Biosystems, Inc.). Both strands of DNA were completely sequenced. Nucleotide sequence analyses and the searches for homologous DNA sequences in the EMBL and GenBank database libraries were performed with the program package HUSAR (Heidelberg Unix Sequence Analysis Resources; German Cancer Research Center, Heidelberg, Germany), as well as with the DNASIS program (Hitachi Software, San Bruno, Calif.).

Preparation of EHEC hemolysin and α-hemolysin. The preparation of secreted *E. coli* hemolysins was done according to a procedure originally described by Bhakdi et al. (5), with minor modifications. A 600-μl sample of an overnight broth culture (10^9 bacteria per ml) was added to 200 ml of prewarmed Todd-Hewitt broth (Difco Laboratories) containing 5 mM $CaCl_2$ and the appropriate antibiotics for maintenance of recombinant plasmids. After 90 min of vigorous agitation in a rotary shaker at 37°C, the culture was further grown at 30°C for another 3 h. The bacteria were then pelleted by centrifugation (Sorvall centrifuge RC-5B, rotor type GSA; 8,000 rpm for 15 min at 4°C). The supernatant was made 3% (vol/vol) in glycerol, and 40 g of solid polyethylene glycol 4000 (Merck Laboratories, Darmstadt, Germany) was then added. The solution was stirred for approximately 5 min until the polyethylene glycol 4000 had dissolved, after which incubation was continued at 4°C for another 60 min. The polyethylene glycol 4000 precipitate was collected by centrifugation (Sorvall centrifuge RC-5B, rotor type GSA; 12,000 rpm for 30 min at 4°C), the supernatant was discarded, and the pellet was dissolved in 500 μl of saline (0.85% NaCl). These preparations were used freshly or were immediately stored at −20°C for up to 1 week.

Immunoblot analysis. The EHEC and α-hemolysin preparations were mixed to equal parts with twofold concentrated sodium dodecyl sulfate (SDS) sample buffer containing 125 mM Tris-HCl (pH 6.8), 4% (vol/vol) SDS, 20% (vol/vol) glycerol, 10% (vol/vol) β-mercaptoethanol, and 0.002% (wt/vol) bromphenol blue and boiled for 5 min. After electrophoresis on a 12% SDS–polyacrylamide gel, the proteins were electroblotted on nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany) by using a Bio-Rad Mini-Protean II Dual Slab Cell. Filters were blocked in a solution made of 0.1% (wt/vol) sodium azide–0.05% (vol/vol) Tween 20 in phosphate-buffered saline (PBS-Tween) and were allowed to react overnight with patient serum diluted 1:100 in PBS-Tween. Filters were washed three times for 30 min in PBS-Tween and then incubated with alkaline phosphatase-coupled goat anti-human immunoglobulin G (IgG; Dianova, Hamburg, Germany) for 2 h. Antibody binding was detected by developing the blots with 0.01% (wt/vol) Nitro Blue Tetrazolium (Sigma) and 0.0005% (wt/vol) 5-bromo-4-chloro-3-indolylphosphate (Sigma) dissolved in 2% (vol/vol) diethanolamine (pH 9.8 [Merck]).

Twenty serum samples were collected in 1993 from HUS patients not geographically related from all parts of Germany. The ages of the patients ranged between 4 and 15 years, and none of them had received transfusions with plasma or Igs. The sera were positive in both IgG and IgM O157 lipopolysaccharide (LPS) immunoblots. Twenty serum samples were from age-matched control patients suffering from diseases other than O157-associated HUS.

Nucleotide sequence accession number. The nucleotide sequences for the EHEC-*hlyA* and EHEC-*hlyC* genes have been entered into the EMBL database library under the titles "*E. coli* O157:H7 (EDL 933) plasmid-encoded *hlyA* gene," accession no. X79839, and "*E. coli* O157:H7 (EDL 933) plasmid-encoded *hlyC* gene," accession no. X80891.

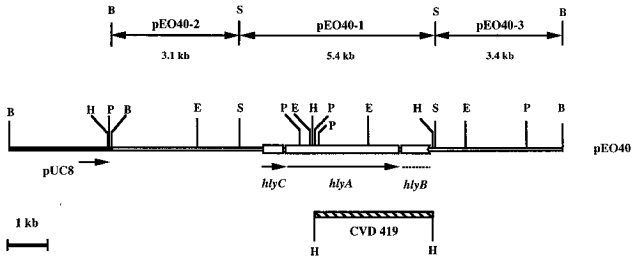

FIG. 1. Map of recombinant plasmid pEO40. The restriction sites for *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), and *Sal*I (S) are indicated. The open reading frames of EHEC-*hlyC* and EHEC-*hlyA* are depicted as boxes. The broken box shows the beginning of an open reading frame homologous to *hlyB*. The position of the CVD 419 probe is indicated by the hatched bar. Arrows indicate the direction of transcription. Above the restriction and gene map of pEO40, the size and location of subclones pEO40-1, pEO40-2, and pEO40-3, including the respective restriction sites, are shown.

RESULTS

DNA sequence analysis of the EHEC-Hly plasmid pEO40-1. Three subclones of plasmid pEO40 were analyzed for hemolytic activity. When transformed in *E. coli* C600, clones pEO40-2 and pEO40-3 were not able to mediate hemolysis on blood agar plates. However, the recombinant plasmid pEO40-1 mediated a weak enterohemolytic phenotype on blood agar plates similar to that previously described (36). Consequently, we suggested that the structural gene for the EHEC hemolysin is located on the 5.4-kb *Sal*I restriction fragment of pEO40-1 (Fig. 1). Several subclones prepared from pEO40-1 were initially sequenced with the pUC universal and reverse primers. For further sequencing of pEO40-1, internal primers were used. To minimize sequencing errors generated by *Taq* cycle sequencing reactions, each base of both strands was determined an average of four times. Analysis of the DNA sequence revealed two open reading frames. The longer of these consisted of 2,997 bp (Fig. 2B) and could encode a protein of 999 amino acids. Significant homologies were found when we compared this sequence with sequences of the EMBL database library for *E. coli* α-hemolysin and other members of the RTX family of pore-forming cytolysins (44). In a 2,830-bp overlap, 62.1% of this nucleotide sequence was identical to the sequence of the chromosomal *hlyA* gene of *E. coli* J96 (10); 61.8% of the nucleotide sequence in a 2,830-bp overlap was identical to that of the plasmid-encoded (pHly 152) *hlyA* gene (15); 61.8% was identical in a 2,687-bp overlap to that of the *Actinobacillus pleuropneumoniae apxICA* gene (19); 58.9% was identical in a 2,643-bp overlap to that of the *Actinobacillus actinomycetemcomitans lktA* gene (23); and 54.5% was identical to that of the *Pasteurella haemolytica* leukotoxin gene in a 2,994-bp overlap (direct submission to EMBL; accession no. Z26247). The overall homology between this open reading

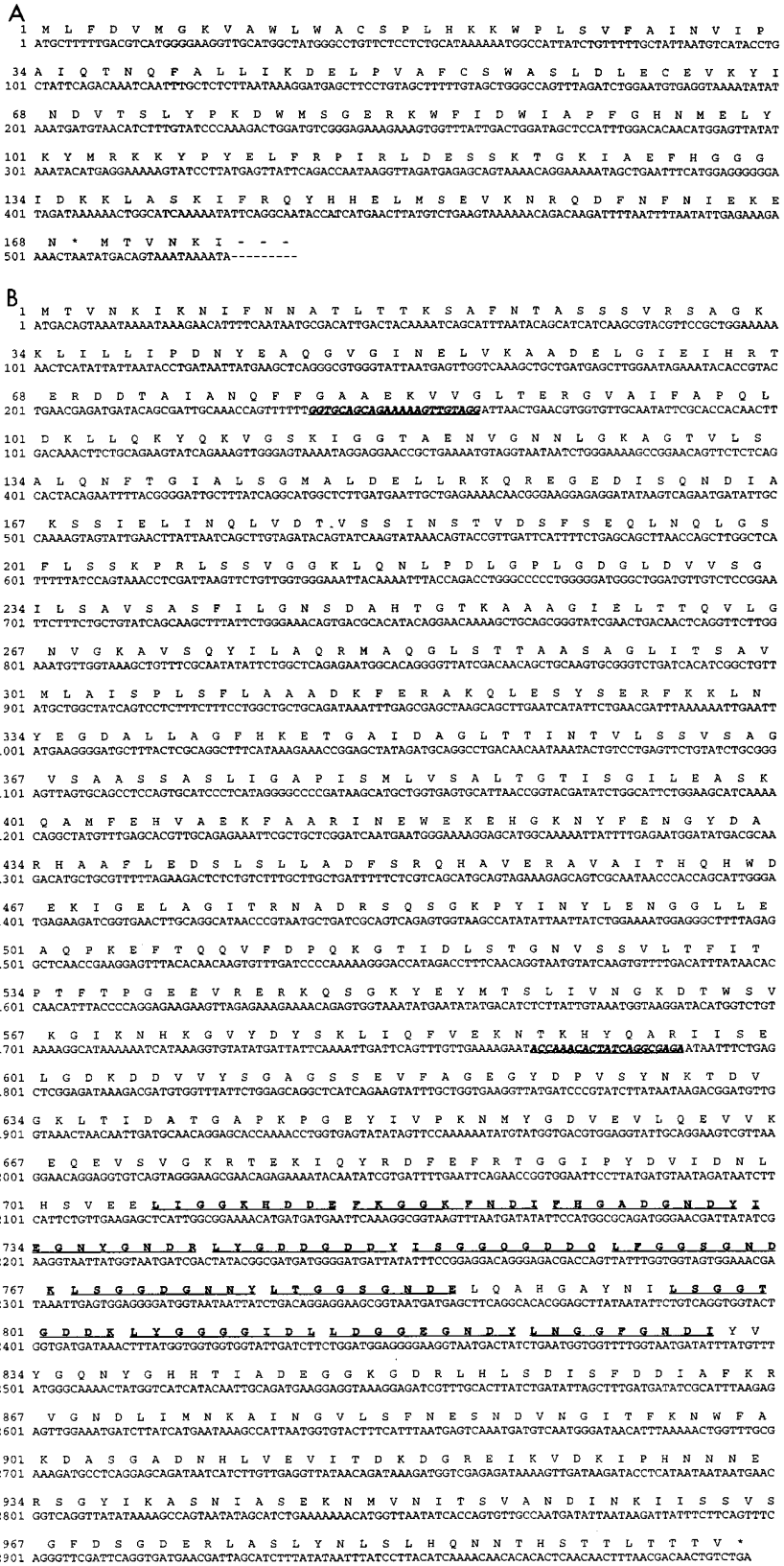

FIG. 2. Nucleotide sequence of the EHEC-*hlyC* (A) and EHEC-*hlyA* (B) genes. The DNA translation is depicted with the one-letter code positioned above the nucleotide sequence at every second nucleotide of the codon. (A) Nucleotide and putative amino acid sequence of the open reading frame encoding the *hlyC* gene. Spaced by one nucleotide, the start codon and the first nucleotides of *hlyA* are depicted in a 3′ direction behind the *hlyC* stop codon starting at position 509 in the nucleotide sequence. (B) Nucleotide and amino acid sequence of EHEC-*hlyA*. The locations of the PCR primers hlyA1 and hlyA4 are indicated by italic underlined letters, whereas the RTX repeats are indicated by boldface underlined letters.

frame and the chromosomally encoded α-*hlyA* gene was 61.4%.

We termed the gene "EHEC-*hlyA*" to distinguish it from the structural gene of the *E. coli* α-hemolysin operon. The position of EHEC-*hlyA* in plasmid pEO40 is indicated in Fig. 1. The second open reading frame, 507 bp in size, was located directly upstream of the EHEC-*hlyA* gene and was separated from this gene by only one nucleotide (Fig. 2A); it could encode a protein of 168 amino acids (Fig. 2A). This open reading frame was 67.5% homologous to the *E. coli hlyC* gene and was termed EHEC-*hlyC* (Fig. 1). Preliminary sequence analysis of the region downstream of the EHEC-*hlyA* gene revealed a nucleotide sequence (approximately 1,050 bp) with 64% homology to the *hlyB* gene located in the *E. coli* α-hemolysin operon (Fig. 1). Therefore, the hemolysin genes of EDL 933 appear to be organized in an operonlike structure similar to the *E. coli* α-hemolysin or *A. actinomycetemcomitans* leukotoxin genes (23, 45).

On the basis of the predicted amino acid content, the hemolysin of EDL 933 is an acidic protein with a molecular mass of 107.05 kDa and an isoelectric point of 4.86. We did not find an abundance of any particular amino acid or class of amino acids except cystein, which is absent in EHEC hemolysin. The *hlyC* gene encodes a protein with a predicted molecular mass of 19.9 kDa which has an isoelectric point of 8.39. The amino acid sequence homology of EHEC-HlyC and α-HlyC was 66%; that of EHEC-HlyA and α-HlyA was 61.2%.

Because of the relationship of EHEC-Hly to members of the RTX family, we looked for conserved sequence features present in these proteins. A common structural characteristic of the RTX proteins is the presence of tandem arrays of a nine-amino-acid repeat with the consensus sequence L/I/F-X-G-G-X-G-N/D-D-X (11, 44). α-Hemolysin of *E. coli* contains 13 tandemly repeated sequences, whereas the hemolysins of *P. haemolytica* and *A. pleuropneumoniae* each only contain 11 repeats (267, 463, and 441). Like α-HlyA, the EHEC-HlyA protein shows 13 tandem repeats at the C terminus, spanning amino acid residues 706 to 832 (Fig. 2B). Whereas repeats 1 to 9 are continuous, there is a larger gap of nine amino acids between repeats 9 and 10. A similar situation is found in the α-hemolysin of *E. coli* (12, 27).

A second primary structural feature of the RTX proteins is the presence of a stretch of ca. 200 hydrophobic amino acids in the N-terminal region. By comparing EHEC-Hly with α-hemolysin, the presence of such a hydrophobic stretch in EHEC-Hly is demonstrated. The distributions of hydrophobicity are similar in both α-hemolysin and the predicted amino acid sequence of EHEC hemolysin (35).

Secretion of the EHEC-*hlyA* gene product. The hemolytic phenotypes of the strains used in this study are shown in Fig. 3. A blood agar plate with defibrinated, washed sheep erythrocytes was inoculated with the different *E. coli* strains and incubated for 24 h at 37°C. The strains EDL 933, C600/pSK3, and C600/pEO40-1 showed an enterohemolytic phenotype, with small turbid zones of hemolysis which were only detectable after 24 h of incubation. In contrast, the plasmid-cured strain EDL 933-Cu and laboratory strains C600 and C600/pRSC6 were nonhemolytic. EDL 933/pRSC6 and C600/pEO40-1/pRSC6 showed the typical α-hemolytic phenotype, with large clear zones of hemolysis occurring after 4 to 8 h of incubation.

After growth of the EHEC hemolysin-positive strain EDL 933 in broth, no hemolytic activity could be detected in the culture supernatant. The hemolytic activity of this strain remained fully cell associated (35). It was suggested that the EHEC hemolysin produced by EDL 933 is deficient in the transport system necessary for hemolysin excretion. EDL 933 and the EHEC-Hly-positive strains C600/pEO40 and C600/pEO40-1 were transformed with the recombinant plasmid pRSC6 carrying the α-*hlyB* and α-*hlyD* genes, which are needed for secretion of *E. coli* α-hemolysin. The pRSC6 transformants showed big, clear zones of hemolysis on blood agar plates, resembling α-hemolysin (Fig. 3).

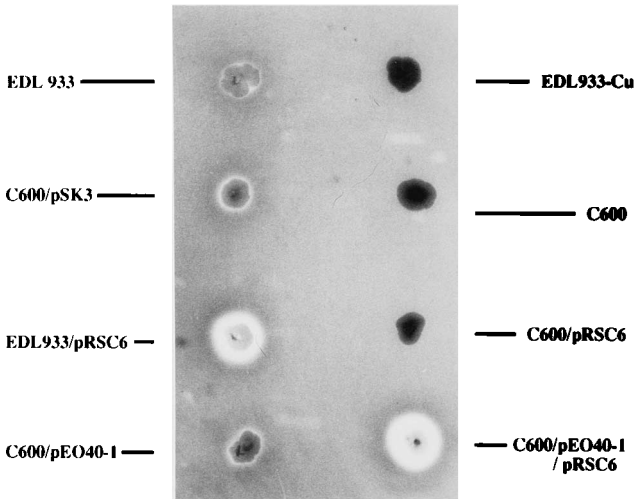

FIG. 3. Blood agar plate with defibrinated, washed sheep erythrocytes inoculated with *E. coli* strains used in this study and incubated for 24 h at 37°C.

By growing the pRSC6 transformants in Todd-Hewitt broth, we were able to detect active EHEC hemolysin in the culture supernatant from mid-log-phase cultures of EHEC-Hly-positive strains EDL 933/pRSC6, C600/pEO40/pRSC6, and C600/pEO40-1/pRSC6. We precipitated the EHEC hemolysin with polyethylene glycol 4000 and subsequently incubated the precipitate with washed sheep erythrocytes for 1 h at 37°C to detect hemolysis. After this procedure, we prepared the EHEC hemolysin and α-hemolysin from the culture supernatants of *E. coli* C600/pEO40/pRSC6 and *E. coli* WAF100/pSF4000, respectively, and used these preparations for SDS-polyacrylamide gel electrophoresis and immunoblot experiments. A protein with a size of approximately 107 kDA was detected in EHEC-Hly- and α-Hly-positive strains (Fig. 4) but not in the Hly-negative control strain, C600/pK18/pRSC6 (35). Even though the α-hemolysin has a higher molecular mass than the EHEC-hemolysin, the mobility is slightly higher, presumably depending on posttranslational modification of the EHEC hemolysin.

Immune response to the EHEC-*hlyA*-associated protein in serum samples of HUS patients. The immune responses of HUS patients infected with EHEC O157 strains to the EHEC-*hlyA*-associated 107-kDa protein were investigated. Twenty serum samples of HUS patients with positive IgM and IgG O157 LPS immunoblots and 20 serum samples of age-matched patients suffering from diseases other than O157-associated HUS were incubated with EHEC hemolysin as described above. In a second approach, the same serum samples were incubated with α-hemolysin. Nineteen of the 20 serum samples from patients with O157-associated HUS reacted with the EHEC hemolysin, whereas only one of the 20 control serum samples reacted with EHEC hemolysin. In contrast, all serum samples demonstrated reactivity with the α-hemolysin. These results indicate that patients suffering from O157-associated HUS show a specific immune response to the EHEC hemolysin produced by these strains.

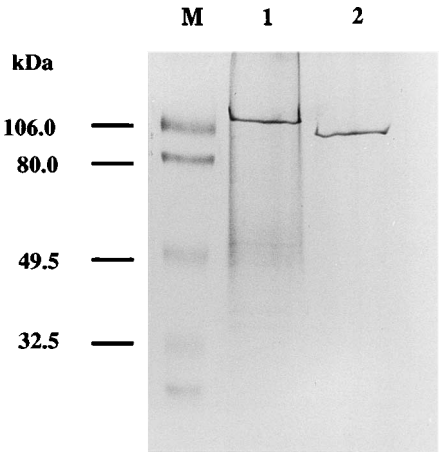

FIG. 4. SDS-polyacrylamide gel electrophoresis of EHEC hemolysin (lane 1) and α-hemolysin (lane 2). Both proteins were purified from broth culture supernatants of *E. coli* C600/pEO40 + pRSC6 and *E. coli* WAF100/pSF4000 as described in Materials and Methods. M indicates the molecular mass marker.

PCR for detection of EHEC-*hlyA* sequences. To assess the association of the plasmid-encoded EHEC hemolysin with Shiga-like toxin-producing strains of *E. coli*, we designed a PCR with primers hlyA1 and hlyA4 (Fig. 2B) to amplify an internal fragment of EHEC-*hlyA* with a size of 1,551 bp. As negative controls, we included the α-hemolysin reference strain WAF100/pSF4000, the enterohemolysin-producing strains JM83/pEO39 (4) and JM83/pEO21 (39), as well as enteropathogenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, and enterotoxigenic *E. coli* strains. All strains were PCR negative (i.e., α-hemolysin-, enterohemolysin-producing strains; enteropathogenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, and enterotoxigenic *E. coli*), except for EDL 933, which was clearly PCR positive. This demonstrated the specificity of the primers for EHEC-*hlyA*. Table 1 shows the distribution of the *hlyA* sequences throughout a set of 53 clinical isolates of various serotypes. All O157:H7 strains were positive in the PCR assays for Shiga-like toxin genes (*slt*) and EHEC-*hlyA*. The *slt*-negative O157:H45 strains were all *hlyA* negative. Of the O111:H$^-$/H2 strains, only the toxigenic strains carried the *hlyA* gene, and the O26 strain was *hlyA* positive.

Localization of CVD 419 sequences in plasmid pEO40. The CVD 419 probe described by Levine et al. (24) is a 3.4-kb *Hin*dIII fragment derived from the 90-kb plasmid of *E. coli* O157:H7 strain EDL 933. This probe hybridized with 99% of *E. coli* O157:H7 and 77 to 81% of non-O157 Shiga-like toxin-positive strains isolated from diseased humans (24). A preliminary sequence analysis of the CVD 419 probe (35) suggested a homology between the EHEC hemolysin determinant and this probe. To further analyze the relationship between CVD 419 and EHEC-Hly, we hybridized the *Hin*dIII-restricted insert of pEO40, as well as the *Sal*I insert of pEO40-1 and the *Sal*I-*Bam*HI insert of pEO40-3, with the 3.4-kb *Hin*dIII fragment representing the CVD 419 probe. A positive hybridization result was found with the 5.4-kb *Sal*I insert of pEO40-1 and the 3.4-kb *Hin*dIII fragment of pEO40 but not with pEO40-3 (35). This clearly indicated that the CVD 419 probe is identical to the internal 3.4-kb *Hin*dIII fragment of pEO40 carrying most of EHEC-*hlyA* gene, as well as the 5′ region of the putative EHEC-*hlyB* gene.

TABLE 1. Linkage of Shiga-like toxin and EHEC hemolysin production in toxigenic and nontoxigenic *E. coli* strains of patients with diarrheal diseases

Serotype (no. of strains)	No. of strains				
	slt$^+$	EHEC-*hlyA*$^+$	*slt*$^+$/*hlyA*$^+$	CVD419 probe positive	Hemolytic
O157:H7 (22)	22	22	22	22	22
O157:H45 (6)	0	0	0	0	0
O111:H$^-$/:H2 (14)	7	7	7	7	7
O117:H16 (1)	1	0	0	0	0
O26:H11 (1)	1	1	1	1	1
O119:H5 (2)	2	0	0	0	0
O98:H8 (5)	5	3	3	3	3
O91:H$^-$ (1)	1	1	1	1	1
O96:H$^-$ (1)	1	0	0	0	0

DISCUSSION

In this study, we described the nucleotide sequence analysis of two putative genes encoded on the large plasmid of strain EDL 933. This plasmid is also present in the vast majority of *E. coli* strains of serogroup O157 which are associated with hemorrhagic colitis and HUS (24). The predicted amino acid sequences from these two putative genes share significant similarity to those of the HlyA and HlyC proteins encoded by the *hlyCABD* operon present in the chromosome of some uropathogenic *E. coli* strains. The *hlyA*-like DNA sequence encodes an activity (EHEC hemolysin) previously referred to as enterohemolysin (2, 3).

On the basis of the nucleotide and predicted amino acid sequences, high levels of similarity between the primary structures of the EHEC-Hly and RTX proteins were revealed. RTX proteins demonstrate different target cell specificities. The *E. coli* α-hemolysin, as well as the *Bordetella pertussis* adenylate cyclase/hemolysin, is active against a wide range of nucleated cells from different animal species (6, 30, 31, 34, 37). Conversely, the cytolytic activity of the leukotoxins of *P. haemolytica* and *A. actinomycetemcomitans* is limited to neutrophils and monocytes from ruminant species, several primates, and humans (38). The hemolysin produced by *A. pleuropneumoniae* is erythrolytic against a variety of animal erythrocytes and cytotoxic to rabbit and porcine neutrophils (13, 31). It is still unclear which part of the protein is responsible for target cell specificity. Forestier and Welch proposed that the amino terminal one-fifth of the RTX proteins is important for target cell specificity (12). Furthermore, the different pIs of that region are believed to have a role in specificity for target cells (23). EHEC-HlyA demonstrates a low homology of only 40% in the first 50 N-terminal amino acid residues to α-HlyA, suggesting that the target cell specificity of EHEC hemolysin is different from that of α-hemolysin.

The genes of the EHEC hemolysin determinant appear to be organized in an operonlike structure similar to those of other RTX proteins. These proteins are found in various species and exert different functions. Past studies have primarily focused on the *E. coli* α-hemolysin. Some functions of *E. coli* α-hemolysin have been shown to depend upon its primary structure (10, 22, 26, 27). Several features which were described for α-hemolysin were also found in the EHEC hemolysin gene. These include the RTX structures and a hydrophobic stretch of approximately 200 amino acids in the N-terminal region.

The enterohemolytic phenotype shown by EHEC hemolysin was associated with defective hemolysin secretion. Moreover, by transforming the strain EDL 933 with the *hlyB* and *hlyD* genes derived from the α-hemolysin operon, this defect could

be complemented, resulting in an alpha-hemolytic phenotype. The *hlyB* and *hlyD* genes of different bacterial species are functionally interchangeable in a limited sense (44), meaning that the *hlyB*- and *hlyD*-encoded transport system works most efficiently when the transported HlyA protein is encoded by the same operon. Despite this, it is possible to efficiently secrete the *P. haemolytica* leukotoxin with *E. coli* HlyB and HlyD in an *E. coli* background (44).

The cell-free hemolytic activity coupled with secretion of the 107-kDa protein in EDL 933 carrying the *hlyB* and *hlyD* genes from the α-*hly* operon indicates that hemolysin transport is defective in EHEC strains. Extensive studies regarding the regulation and secretion of EHEC hemolysin need to be performed.

It has been reported that the hemolytic properties of RTX proteins are not its most important characteristics. RTX proteins appear to affect normal cell functions in different ways. Sublytic concentrations of α-hemolysin were reported to cause the release of leukotrienes from human granulocytes or prevent human neutrophils from binding chemotactic factors (34). When treated with picogram amounts of α-hemolysin, rat renal tubular cells demonstrated a 14-fold increase in superoxide production and a 2-fold decrease in ATP level (21). Bhakdi and coworkers found that sublytic amounts of α-hemolysin caused release of interleukin 1β from cultured monocytes (6, 14) and granulocytopenia and a drop in platelet levels in monkeys (41).

Because the mechanisms of pathogenesis leading to the complex symptoms of HUS are poorly understood, it would be of interest to determine which cells other than erythrocytes are susceptible to EHEC hemolysin. Endothelial cells have been implicated as the primary target of Shiga or Shiga-like toxins in the development of HUS. Shiga- and Shiga-like toxins show a tropism to glycolipid globotriaosylceramide-expressing endothelial cells, which are present in the colon, the central nervous system, the pancreas, renal glomerular microvasculature, and renal tissue cells. It has been shown that interleukin 1β increases the cytotoxicity of Shiga toxin toward human vascular epithelial cells by increasing globotriaosylceramide synthesis. Thus, infection by O157 strains may enhance interleukin 1β production because of the presence of LPS and possibly by the action of the EHEC hemolysin (20), similar to what was described for α-hemolysin (20, 40). If this is the case, than one could suggest that Shiga-like toxins and EHEC hemolysin act synergistically to disturb important cell functions.

Although a serological response against EHEC hemolysin was frequently seen in humans with O157-associated HUS, it was seldom observed among controls. These findings support our observation that there is a close association between the presence of EHEC hemolysin and Shiga-like toxin in *E. coli* O157. In contrast, patient and control sera were found to possess antibodies against α-hemolysin. The presence of anti-α-hemolysin antibodies might be due to the common exposure to bacteria producing RTX-like antigens. Exposure may also occur through intestinal colonization with α-hemolysin-producing *E. coli*. α-Hemolysin has been shown to be an effective immunogen when produced by bacteria in the human host (17).

The incidence of EHEC hemolysin in Shiga-like toxin-producing *E. coli* strains is high, implicating a linkage between these two proteins in the pathogenesis of hemorrhagic colitis and HUS. The specific association of EHEC hemolysin and Shiga-like toxin production in pathogenic *E. coli* points to the possible role of EHEC hemolysin in bacterial virulence. Future work is necessary to characterize the cytolytic activity and target cell specificity of EHEC hemolysin and its possible role in pathogenesis. This work requires further studies of EHEC hemolysin secretion in vitro and in vivo.

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