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Enterohemolytic Phenotypes and Genotypes of Shiga Toxin-Producing *Escherichia coli* O111 Strains from Patients with Diarrhea and Hemolytic-Uremic Syndrome

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Thirty-six Shiga toxin-producing *Escherichia coli* (STEC) O111:H$^-$ strains, 18 of which were isolated from patients with hemolytic-uremic syndrome (HUS) and 18 from patients suffering from diarrhea, were investigated for their enterohemolytic phenotypes and genotypes. Twenty-two strains were EHEC hemolysin (EHEC Hly) positive by probe hybridization and by PCR with sequences complementary to the EHEC *hlyA* gene of *E. coli* O157:H7, but only 20 of these were hemolytic on blood agar plates. The remaining 14 strains were EHEC Hly negative according to DNA-based methods and did not express the enterohemolytic phenotype. The enterohemolytic phenotype was observed in 16 of 18 (88%) strains from patients with HUS but only in 4 of 18 (22.2%) of the STEC O111:H$^-$ strains from patients with diarrhea. All STEC O111:H$^-$ strains carried large plasmids, as shown by plasmid analysis, but only plasmids of EHEC Hly probe-positive strains hybridized with the CVD419 probe. A *Bam*HI fragment of approximately 12 kb was cloned from the large plasmid of the *E. coli* O111:H$^-$ strain 78/92 and shown to mediate hemolytic activity when transformed into the *E. coli* laboratory strain HB101. The EHEC O111 *hlyA* gene was sequenced completely and shown to have 99.4% sequence identity to the corresponding EHEC O157 *hlyA* gene of the *E. coli* O157:H7 strain EDL 933. Our results indicate that detection of EHEC Hly either by DNA-based methods or by investigation of the enterohemolytic phenotype on blood agar alone is insufficient for screening STEC O111 strains. However, the high incidence of EHEC Hly in isolates from patients with HUS and its rare occurrence in isolates from patients with diarrhea may indicate that STEC O111 strains have a distinct pathogenic potential for humans and that the presence of EHEC Hly increases the ability of an STEC O111 strain to cause extraintestinal complications in humans.

Shiga toxin-producing *Escherichia coli* (STEC), previously referred to as Shiga-like-toxin- or verotoxin-producing *E. coli*, causes a variety of symptoms, of which hemorrhagic colitis and systemic toxemic complications, such as hemolytic-uremic syndrome (HUS), are clinically the most important (10). In addition to Shiga toxins (Stx), most strains isolated from patients with HC and HUS produce Intimin (15) and are CVD419 probe positive (2, 14). The CVD419 probe is a 3.4-kb *Hin*dIII fragment derived from the large plasmid of the *E. coli* O157:H7 strain EDL 933 and has been shown to hybridize with 99% of the tested enterohemorrhagic *E. coli* (EHEC) O157:H7 strains (14, 19). We have recently shown that the large plasmid pO157 of strain EDL 933 harbors the EHEC *hly* operon encoding an RTX (repeats in toxin) toxin designated EHEC hemolysin (Hly) (19). The EHEC *hly* operon consists of the four EHEC genes *hlyC*, *hlyA*, *hlyB*, and *hlyD*, which are necessary for synthesis and export of this RTX toxin (21). Hybridization experiments have shown that the CVD419 probe covers most of the EHEC Hly structural gene *hlyA* as well as the 5′ region of EHEC *hlyB* gene (19). The CVD419 probe-positive STEC causing HC and HUS has been termed EHEC (14).

The serotype most important worldwide is *E. coli* O157:H7, although a large number of other serotypes have been implicated in both sporadic cases and outbreaks of HUS (10). Among the non-O157 STEC strains, the nonmotile STEC O111 strains are most frequently found in HUS patients from Europe. In Canada they were associated with HUS as early as 1983 (11, 12). Two outbreaks of HUS caused by STEC O111 in South Australia (9) and Italy (8) have recently been reported.

The magnitude of the public health problem posed by *E. coli* O111 and other non-O157:H7 STEC strains can be only roughly estimated since most laboratories do not screen stool samples for these pathogens. In contrast to serotype O157:H7, which possesses a range of biochemical characteristics greatly easing the efficiency of detection, no distinguishing biochemical marker has been found for non-O157 STEC up to now. Such strains are identified by DNA-based methods with *stx*- or *eaeA*-specific probes and/or the pCVD419 probes (2, 14, 22). Probe-positive colonies are subsequently subjected to slide agglutination with antiserum against the O antigen. In addition, it has been suggested that the enterohemolytic phenotype, detected on blood agar plates containing washed, defibrinated sheep erythrocytes, is highly efficient for detection of most STEC strains pathogenic to humans. In one study, all *E. coli* O111 strains expressed the enterohemolytic phenotype (2). The aim of this study was to characterize the enterohemolytic phenotypes and genotypes of STEC O111 strains. In addition, we describe the identification and nucleotide sequence of the EHEC *hlyA* gene of STEC O111:H$^-$ and compare it with the EHEC O157 *hlyA* gene described previously (19), and we show that these genes have very similar sequences.

MATERIALS AND METHODS

Bacterial strains and plasmids. Fifteen *E. coli* O111 strains from patients with HUS from Germany and Italy were recently described by Rüssmann et al. (17), and four *E. coli* O111 strains from patients with diarrhea were described by Bitzan et al. (6). Three strains from patients with HUS and 14 strains from patients suffering from diarrhea were isolated between 1990 and 1995 from unassociated patients in Germany by colony hybridization with probes complementary to *stx-1* and *stx-2*. Thirty-four strains harbored *stx-1*, and nine of them

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TABLE 1. Enterohemolytic phenotypes and genotypes of STEC O111:H$^-$ strains used in this study and their disease association

No. of strains	Entero-hemolytic phenotype	EHEC *hly* PCR result	Hybridization with:		No. of strains from patients with:	
			EHEC *hly* probe	CVD419 probe	HUS	Diarrhea
20	+	+	+	+	16	4
2	−	+	+	+	1	1
14	−	−	−	−	1	13

also had *stx-2*. Two strains carried the *stx-2* gene only. All strains were shown to possess *eaeA* sequences as shown by PCR with primers LP1 and LP3 (23). The 36 STEC O111 strains used in this study were all from sporadic cases of diarrhea and HUS. The laboratory *E. coli* strain HB101 was used as the host for cloning experiments. Plasmid pK18 was used as the cloning vector (16).

Recombinant DNA techniques. Restriction endonuclease digestion, ligation, transformation, and purification of DNA from agarose gels were performed according to standard methods (18). Restriction enzymes and T4 DNA ligase were from Gibco BRL (Eggenstein, Germany). Plasmid preparations were carried out with the Nucleobond AX kit (Macherey and Nagel, Düren, Germany).

Molecular detection methods. Southern blot hybridization was conducted with the digoxigenin labeling and detection kit (Boehringer GmbH, Mannheim, Germany) as described previously (20). Probes were labeled either by random hexamer labeling (CVD419) or by incorporation of digoxigenin-11-dUTP during PCR (hlyA1-hlyA4 probe). PCR for detection of EHEC *hlyA* sequences was performed with the GeneAmp PCR System 9600 (Perkin Elmer-Applied Biosystems, Weiterstadt, Germany) with primers hlyA1 and hlyA4 as described previously (19). The nucleotide sequence of the EHEC *hlyA* gene of the STEC O111:H$^-$ strain 78/92 was determined by *Taq* cycle sequencing with customized primers as described previously (19).

Detection of the enterohemolytic phenotype. STEC O111:H$^-$ strains were streaked onto agar plates containing defibrinated, washed sheep erythrocytes as described previously (19). After overnight growth at 37°C, the enterohemolytic phenotype was detected by the occurrence of small, turbid zones of hemolysis.

Statistical analysis. The correlation between EHEC Hly positivity and the occurrence of HUS was assessed by determining χ^2 with a 2-by-2 table.

Nucleotide sequence accession number. The nucleotide sequence of the EHEC *hlyA* gene of the STEC O111:H$^-$ strain 78/92 was entered in the EMBL database library and assigned the accession number X94129.

RESULTS

Investigation of enterohemolytic phenotypes and genotypes of STEC O111:H$^-$ strains from patients with HUS and diarrhea. Probe hybridization was achieved with an EHEC *hlyA*-specific probe generated by PCR with primers hlyA1 and hlyA4. Twenty-two strains were positive by probe hybridization as well as by PCR with primers hlyA1 and hlyA4 (Table 1). Seventeen of these strains were from patients with HUS, and five strains were from patients with diarrhea. The remaining 14 strains neither hybridized with the gene probe nor reacted in the PCR assay. Thirteen of these strains were from stools of patients with diarrhea, and only one was from a HUS patient. The CVD419 probe assay, based on hybridization results with plasmids purified from *E. coli* O111 strains, demonstrated complete concordance with the hlyA1-hlyA4 probe hybridization and PCR results (Table 1).

The enterohemolytic phenotyping of STEC O111 strains was done with blood agar plates containing washed sheep erythrocytes. Evaluation of the plates after 4 to 6 h and 18 to 24 h of incubation allowed optimal assessment of the hemolytic phenotype (either α-hemolysin or enterohemolysin) and gave definitive results. Twenty strains showed the typical enterohemolytic phenotype, with small, turbid zones of hemolysis occurring after 18 to 24 h of incubation. Of these, 16 strains were from patients with HUS and 4 were from patients with diarrhea. Fourteen strains showed neither the enterohemolytic phenotype nor the EHEC *hlyA* genotype by PCR and hybridization. Two strains were nonhemolytic, but DNA-based methods showed them to be carrying EHEC *hlyA* sequences (Table 1). In all, the enterohemolytic phenotype was observed in 16 of 18 (88%) strains from patients with HUS but only in 4 of 18 (22.2%) of the STEC O111 strains from patients with diarrhea.

Plasmid analyses of STEC O111:H$^-$ strains and sequencing of the EHEC O111 *hlyA* gene. Plasmid preparations of the *E. coli* O111 strains were digested with restriction endonuclease *Bam*HI, separated on 0.6% (wt/vol) agarose gels and hybridized with the internal EHEC *hlyA*-specific probe. Examples are shown in Fig. 1. With this technique, probe-positive *Bam*HI fragments of approximately 12 kb were detected in all strains expressing the enterohemolytic phenotype and in two of the nonhemolytic strains. It is of interest that, with the plasmid preparation of strain ED142, which is nonhemolytic on blood agar plates but EHEC Hly positive by PCR, two bands of approximately 12 kb hybridize with the probe (Fig. 1B). In order to determine the genetic relationship between the hemolysins of *E. coli* O111 and *E. coli* O157 strains, we cloned an approximately 12-kb *Bam*HI fragment of strain 78/92 by random ligation of *Bam*HI fragments in vector pK18. After transformation of the ligations in *E. coli* HB101, EHEC *hly*-positive transformants were identified by the production of small, turbid zones of hemolysis on blood agar plates containing 100 μg of kanamycin per ml. Single colonies, which showed the enterohemolytic phenotype, were isolated and shown to carry the EHEC *hlyA* gene by PCR with primers hlyA1 and hlyA4. The recombinant plasmid carrying the 12-kb *Bam*HI fragment of STEC O111 strain 78/92 was designated pSK78-1. The nucleotide sequence of the EHEC O111 *hlyA* gene was determined with the aid of customized primers. The two strands were sequenced at least twice, and sites with a sequence different from that in EHEC O157 *hlyA* were analyzed at least three times to exclude sequencing errors. An open reading frame of 2,997 bp was found with high levels of nucleotide (99.4%) and

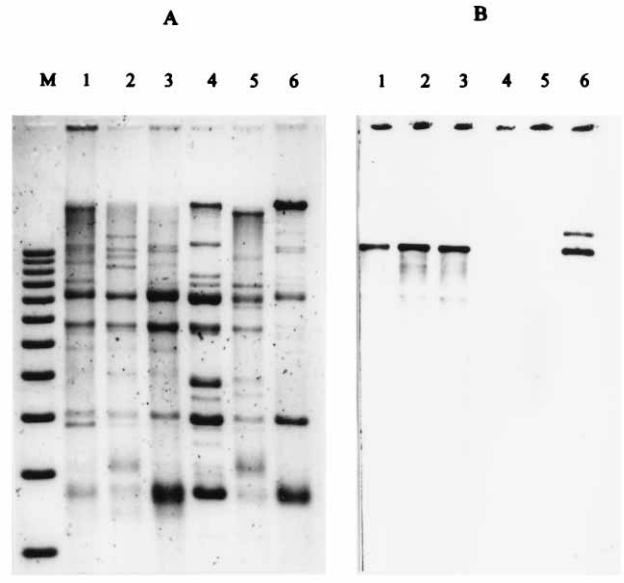

FIG. 1. Agarose gel electrophoresis (A) and Southern blot hybridization (B) of plasmid preparations of STEC O111:H$^-$ strains ED31 (lanes 1), ED82 (lanes 2), 78/92 (lanes 3), ED71 (lanes 4), ED72 (lanes 5), and ED142 (lanes 6). Whereas ED31, ED82, 78/92, and ED142 were isolated from patients with HUS, ED71 and ED72 were isolated from patients with diarrhea. Plasmids were digested with the restriction endonuclease *Bam*HI. Hybridization was conducted with the hlyA1-hlyA4 PCR-generated gene probe. The 1-kb ladder (Gibco BRL) was used as a molecular size marker (M).

amino acid (98.7%) sequence identity to the EHEC O157 *hlyA* gene. Substitution of 16 bases dispersed throughout the nucleotide sequence led to the exchange of 10 amino acid residues in the putative EHEC O111 HlyA protein. The respective RTX regions were identical in the two proteins. Furthermore, amino acid exchanges did not occur in the amino and carboxy termini of the putative EHEC O111 HlyA protein.

Statistical analysis. Since most STEC O111 isolates from patients with HUS were EHEC *hly* positive and most isolates from patients with diarrhea were EHEC *hly* negative, the null hypothesis was used to test the possibility of an association between the occurrence of HUS and EHEC Hly. According to a 2-by-2 table for assessment of the null hypothesis and the alternative hypothesis of association, χ^2 (Yates corrected) was 14.14. Since $\chi^2_{1;0.001}$ equals 6.635, the occurrence of EHEC Hly-producing STEC O111 strains in patients with HUS is highly significant at the 1% level.

DISCUSSION

The production of EHEC Hly (an enterohemolysin) is virtually an obligate characteristic of *E. coli* of serogroup O157 and is closely associated with STEC of other serogroups (3–5, 19). The study discussed here focused on the enterohemolytic phenotypes and genotypes of STEC O111 strains isolated from patients with diarrhea and HUS. EHEC Hly production by *E. coli* O111 strains has been reported previously by at least two authors. Campos et al. (7) investigated 152 *E. coli* O111 isolates, mostly from patients suffering from diarrhea in Brazil, by multilocus enzyme electrophoresis and characterization of their virulence properties. Strains could be subdivided into 15 electrophoretic types (ET). Nearly 70% of the isolates belonged to a particular electrophoretic type (ET12) and showed the characteristics of class I enteropathogenic *E. coli*. The production of EHEC Hly was restricted to ET8, ET9, ET16, and ET10. However, most enterohemolytic isolates (O111:H9, O111:H$^-$, and H not determined) belonged to ET9. Moreover, Stx-1 production was restricted to this ET. The virulence pattern observed in strains of this ET was common in *E. coli* of other serogroups, such as O55 and O26, and was suggested to reflect a novel mode of pathogenesis (7). Beutin et al. (5) investigated 64 verotoxin (VT)-producing *E. coli* strains from humans and animals with respect to their enterohemolytic phenotypes and found that 93.8% of the VT$^+$ strains produced hemolysins. Six of these strains were of serogroup O111, and these were all hemolytic. Moreover, all *E. coli* O111 strains produced VT1 (Stx-1), and two of these also produced VT2 (Stx-2).

In this study, using blood agar plates, we investigated the capacity of STEC O111 strains to produce the enterohemolytic phenotype, determined the enterohemolytic genotype, and, in the case of one strain, sequenced the EHEC *hlyA* gene. These investigations disclosed two key findings: (i) STEC O111:H$^-$ strains differ in their capacity to produce EHEC Hly, and (ii) this characteristic was far more frequent in strains from patients with HUS than in strains from patients with diarrhea (88 versus 22.2%). This observation is of interest since a clear association between virulence markers and disease may improve the classification of STEC-caused diseases.

The results of our study do not indicate that EHEC Hly may act as sole contributor to the development of HUS, but it appears that patients infected with enterohemolytic STEC O111 are at a higher risk for developing HUS than patients infected with EHEC Hly-negative strains. However, at present, the role of plasmid-encoded proteins in the pathogenesis of HUS is not known. The hemolysin characterized here, together with the EHEC O157 Hly, belongs also to the recently discovered RTX toxin family and is closely related to *E. coli* α-hemolysin. Both proteins are pore-forming toxins whose toxicity is attributable to their insertion into the cytoplasmic membrane of eucaryotic cells and the subsequent permeability changes. The in vivo action of RTX toxins ultimately causes physical damage to a variety of eucaryotic cells. In addition to the important cytotoxic activities, sublytic concentrations of RTX toxins are believed to modulate the immune response after infection with a given pathogen (24). At present it is not clear if EHEC Hly contributes to STEC pathogenicity and to extraintestinal complications in particular, if it is a virulence marker necessary for maintenance in cattle (1), or if it is required for iron acquisition during infection (13).

The results of plasmid analyses showed that in most EHEC Hly-positive strains the EHEC O111 *hlyA* gene is located on an approximately 12-kb *Bam*HI fragment, similar to the situation found in *E. coli* O157. Moreover, the nucleotide sequence of EHEC O111 *hlyA* is nearly identical to that of EHEC O157 *hlyA*. This finding supports the findings of Campos et al. (7), who suggested that the rate of acquisition and loss of virulence factors is rapid in comparison with the rate of mutations in so-called housekeeping genes in the evolution of *E. coli* O111 lineages. Moreover, because of the high sequence conservation of EHEC *hlyA* in *E. coli* O157 and *E. coli* O111 strains, one may hypothesize that *E. coli* O111 acquired the EHEC Hly genes late during development of distinct *E. coli* O111 clones. Despite considerable advances in the development of diagnostic methods in recent years, non-O157 STEC strains still pose a diagnostic challenge for microbiological laboratories.

The results of our study indicate that primary screening on blood agar plates alone is unsuitable for detection of STEC O111 strains because only 66% of the strains investigated here showed the enterohemolytic phenotype and because some strains apparently carry silent EHEC Hly genes. As a consequence, routine screening for STEC O111 and other non-O157 STEC strains in the laboratory should include determination of Stx by cytotoxicity assays with Vero cells or by PCR with primers complementary to *stx* genes. If positive results are obtained by the screening test, the enterohemolysin agar may assist in the detection of particular EHEC strains. However, if STEC colonies producing the enterohemolytic phenotype cannot be identified on this agar, we recommend the use of colony hybridization with digoxigenin-labeled *stx* probes.

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