Characterization of the α-haemolysin determinant from the human enteropathogenic Escherichia coli O26 plasmid pEO5

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Abstract

The 157-kb conjugative plasmid pEO5 encoding α-haemolysin in strains of human enteropathogenic Escherichia coli (EPEC) O26 was investigated for its relationship with EHEC-haemolysin-encoding plasmids of enterohaemorrhagic E. coli (EHEC) O26 and O157 strains. Plasmid pEO5 was found to be compatible with EHEC-virulence plasmids and did not hybridize in Southern blots with plasmid pO157 from the EHEC O157:H7 strain EDL933, indicating that both plasmids were unrelated. A 9227-bp stretch of pEO5 DNA encompassing the entire α-hlyCABD operon was sequenced and compared for similarity to plasmid and chromosomally inherited α-hly determinants. The α-hly determinant of pEO5 (7252 bp) and its upstream region was most similar to corresponding sequences of the murine E. coli α-hly plasmid pHly152, in particular, the structural α-hlyCABD genes (99.2% identity) and the regulatory hlyR regions (98.8% identity). pEO5 and α-hly plasmids of EPEC O26 strains from humans and cattle were very similar for the regions encompassing the structural α-hlyCABD genes. The major difference found between the hly regions of pHly152 and pEO5 is caused by the insertion of an IS2 element upstream of the hlyC gene in pHly152. The presence of transposon-like structures at both ends of the α-hly sequence indicates that this pEO5 virulence factor was probably acquired by horizontal gene transfer.

Introduction

We have previously reported that human enteropathogenic Escherichia coli (EPEC) O26 strains harbour large plasmids encoding α-haemolysin (α-hly) (Beutin et al., 1986; Leomil et al., 2005). These strains were assigned to a clonal lineage of rhamnose–dulcitol fermenting (RDF), nonmotile EPEC O26 strains genetically and phenotypically different from rhamnose–dulcitol nonfermenting (non-RDF) enterohaemorrhagic E. coli (EHEC) O26[H11] (Leomil et al., 2005). A prototype α-hly plasmid of an EPEC O26 strain called pEO5 was shown to be transferable by conjugation into E. coli K-12 (Beutin et al., 1986). Possession of α-hly plasmids was found to be a typical trait of the RDF clone of EPEC O26 strains, but is not associated with non-RDF, EHEC O26 strains (Leomil et al., 2005). Non-RDF EHEC O26[H11] strains were assigned to a different clonal lineage and are characterized by the production of Shiga toxins (Stx) and EHEC-haemolysin (E-hly). E-hly is encoded by elih genes located on a virulence plasmid in EHEC O26[H11] strains (Leomil et al., 2005; Jenkins et al., 2008). Escherichia coli α- and E-hly can be discerned by their phenotypes on washed sheep blood agar plates (Beutin et al., 1996) and by PCR according to nucleotide variation (Schmidt et al., 1995). Both types of haemolysins are active on erythrocytes from numerous mammalian species including humans (Beutin et al., 1988; Welch, 1991). Binding of α-hly and activation of target cells was found to be independent of a cellular receptor that indicates how the toxin may lyse a variety of nucleated cells from different animal species (Welch, 1991; Valeva et al., 2005). E- and α-hly were shown to stimulate inflammation and cell damage, thus contributing to bacterial pathogenicity (Welch, 1991; Taneike et al., 2002; Aldick et al., 2007; Troeger et al., 2007).

E- and α-hly are encoded by polycistronic operons consisting of four genes arranged in the order of hlyCABD (Schmidt et al., 1996; Stanley et al., 1998). Both haemolysins are members of the calcium-dependent, pore-forming
cytolysins of the repeats in the toxin (RTX) family (Welch, 1991; Menestrina et al., 1994) and share 61.4% identity in their structural hlyA gene. The product of the hlyC gene is involved in activation of the haemolytic toxin that is the product of the hlyA gene. The products encoded by hlyB and hlyD, together with TolC, are involved in secretion of the haemolysin through the bacterial inner and outer membrane (Holland et al., 2005).

E-hly determinants have been found on large nonconjugative plasmids harboured by strains of EHEC (O26, O103, O111, O145 and O157) that cause haemorrhagic colitis and haemolytic uremic syndrome in humans (Schmidt et al., 1994; Brunder et al., 1999). α-hly-encoding determinants were found located on pathogenicity islands (PAI) in the chromosome of extraintestinal pathogenic E. coli strains (Dobrindt et al., 2000). Plasmids carrying α-hly genes are frequent in Shiga toxin-producing E. coli (STEC) causing oedema disease in pigs and in enterotoxigenic E. coli (ETEC) as diarrhoeal agents in domestic animals (Jorgensen & Poulsen, 1976; Prada et al., 1991). In contrast to EHEC-virulence plasmids, which were explored for their nucleotide sequence and virulence genes (Burland et al., 1998; Brunder et al., 1999, 2006), little is known regarding the EPEC O26 α-hly plasmids and their relationship with E-hly-encoding plasmids of E. coli.

In this work, we have investigated plasmid pEO5 and pEO5-related plasmids from EPEC O26 strains for similarity and relation to an E-hly-encoding plasmid. We have cloned the α-hly determinant of pEO5 and have determined its nucleotide sequence to compare it with already described plasmid and chromosomally inherited α-hly determinants of E. coli.

**Materials and methods**

**Escherichia coli strains**

The strains used in this work are listed in Table 1. Strain C4115, the source of the plasmid pEO5, the E. coli strain 374 carrying the α-hly plasmid pHly152 as well as other EPEC O26 strains were described previously (Beutin et al., 1986, 1988; Hess et al., 1986; Leomil et al., 2005). The properties of strains WAF100 as hosts of the α-hly recombinant plasmid pSF4000 and TPE1313 harbouring the EHEC virulence plasmid pO157::Tn801 (confering resistance to ampicillin) are listed elsewhere (Welch et al., 1983; Schmidt et al., 1994, 1995). Mating of pEO5-type plasmid carrying O26 wild type (C4115, CB9866, CB1027, and CB1030) and K-12 strains (TPE422 and TPE477) with E. coli recipients and isolation of haemolytic transconjugants were performed as described previously (Beutin et al., 1986; Prada et al., 1991). TPE477 carries a Tn5-tagged derivative of pEO5, conferring resistance to kanamycin that was used for selection of transconjugants.

**Phenotypic and genetic characterization of haemolysins**

Phenotypes corresponding to E. coli α-hly and E-hly were analysed on washed sheep blood agar (enterohaemolysin

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### Table 1. Properties of Escherichia coli strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Haemolysin phenotype</th>
<th>hly plasmid (kb)</th>
<th>Origin, references</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4115</td>
<td>O26:NM</td>
<td>α-hly</td>
<td>pEO5 (157)</td>
<td>Human, Germany (Beutin et al., 1986)</td>
</tr>
<tr>
<td>JC3272</td>
<td>Or:H48</td>
<td>—</td>
<td>—</td>
<td>E. coli K-12 (Beutin et al., 1986)</td>
</tr>
<tr>
<td>TPE422</td>
<td>Or:H48</td>
<td>α-hly</td>
<td>pEO5 (157)</td>
<td>JC3272 (pEO5) (Beutin et al., 1986)</td>
</tr>
<tr>
<td>TPE477</td>
<td>Or:H48</td>
<td>α-hly</td>
<td>pEO5::Tn5</td>
<td>JC3272 (pEO5::Tn5) (Beutin et al., 1986)</td>
</tr>
<tr>
<td>TPE2321</td>
<td>Or:H48</td>
<td>—</td>
<td>—</td>
<td>JC3272 (pACYC184) (Rose, 1988)</td>
</tr>
<tr>
<td>TPE2319</td>
<td>Or:H48</td>
<td>α-hly</td>
<td>pEO2319 (23)</td>
<td>This work</td>
</tr>
<tr>
<td>TPE2320</td>
<td>Or:H48</td>
<td>α-hly</td>
<td>pEO2320 (23)</td>
<td>This work</td>
</tr>
<tr>
<td>CB9866</td>
<td>O26:NM</td>
<td>α-hly</td>
<td>pEO5 like</td>
<td>Cattle, Brazil (Leomil et al., 2005)</td>
</tr>
<tr>
<td>CB1027</td>
<td>O26:NM</td>
<td>α-hly</td>
<td>pEO5 like</td>
<td>Human, Brazil (Leomil et al., 2005)</td>
</tr>
<tr>
<td>CB1030</td>
<td>O26:NM</td>
<td>α-hly</td>
<td>pEO5 like</td>
<td>Human, Brazil (Leomil et al., 2005)</td>
</tr>
<tr>
<td>IP187</td>
<td>O26:NM</td>
<td>α-hly</td>
<td>pEO5 like</td>
<td>Human, France (this work)</td>
</tr>
<tr>
<td>1040/83</td>
<td>O26:H11</td>
<td>E-hly</td>
<td>pO26 (90)</td>
<td>Human, Germany (this work)</td>
</tr>
<tr>
<td>TPE497</td>
<td>O26:H11</td>
<td>α-hly</td>
<td>pEO5::Tn5 &amp; pO26</td>
<td>TPE477 × 1040/83 (this work)</td>
</tr>
<tr>
<td>TPE1313</td>
<td>Or:H48</td>
<td>E-hly</td>
<td>pO157::Tn801 (92)</td>
<td>Schmidt et al. (1994)</td>
</tr>
<tr>
<td>TPE2611</td>
<td>Or:H48</td>
<td>α-hly</td>
<td>pO157::Tn801 &amp; pEO5::Tn5</td>
<td>TPE477 × TPE1313 (this work)</td>
</tr>
<tr>
<td>698/83</td>
<td>O26:H32</td>
<td>—</td>
<td>—</td>
<td>Human, Germany (this work)</td>
</tr>
<tr>
<td>374</td>
<td>Or:H48</td>
<td>α-hly</td>
<td>pHLY152 (48)</td>
<td>Mouse (Hess et al., 1986)</td>
</tr>
<tr>
<td>WAF100</td>
<td>Or:H48</td>
<td>α-hly</td>
<td>pSF4000 (12.5)</td>
<td>α-hlyCABD genes of 396 cloned on pACYC184 (Welch et al., 1983)</td>
</tr>
</tbody>
</table>

O: H serotype, Or, rough lipopolysaccharide, NM, nonmotile; α-hly, α-haemolytic and positive for α-hly genes; E-hly, enterohaemolysin and positive for ehr genes; —, negative for haemolysins.
Isolation of total and plasmid DNA

Total DNA of bacteria was isolated as described (Beutin et al., 2008). Purified plasmid DNA was isolated using the Large-Construct kit following the instructions of the manufacturer (Qiagen, Hilden, Germany).

Preparation of gene probes and DNA hybridization

Southern blot hybridization of plasmid DNA and labelling of gene probes with digoxigenin-11-dUTP were performed as described previously (Schmidt et al., 1994). Digoxigenin-labelled molecular markers (Roche, Mannheim, Germany) were used for size determination of hybridizing DNA fragments. The Aval-A fragment of pSF4000, which covers >90% of the complete x-hly determinant cloned on pSF4000 (Welch et al., 1983; Prada et al., 1992), was used as the gene probe for identification of pEO5 recombinant plasmids pEO2319 and pEO2320. For investigation of genetic similarity between pEO5 and pO157, the plasmids were separately digested with restriction enzymes ApaLI, EcoRI and HindIII and hybridized on Southern blots with a mixture of digoxigenin-labelled EcoRI restriction fragments of the respective plasmid. Detection of the EHEC-plasmid pO157 in Southern-blotted pulsed field gel electrophoresis (PFGE) gels was performed with a 1551-bp elxA-specific gene probe generated with primers HlyA1 and HlyA4 as described previously (Schmidt et al., 1995). A 666-bp PCR product of the x-hlyA gene generated with primers 10f+10r (Table 2) was used as the internal DNA probe for detection of x-hly-specific sequences. DNA hybridization and posthybridization washes were performed according to the protocol provided with the Dig DNA labelling and detection kit (Roche). Hybridization was performed overnight at 42 °C in digoxigenin-Easy Hyb buffer (0.1 M maleic acid and 0.15 M NaCl, pH 7.5) (Roche). After hybridization, filters were washed twice for 5 min in 2 × SSC (10 × SSC 0.15 M NaCl and 1.5 M NaCl, pH 7.0)+0.1% sodium dodecyl sulphate (SDS) at room temperature, followed by two high-stringency washes for 15 min at 68 °C in 0.5 × SSC and 0.1% SDS. PCR reactions were performed as described previously (Beutin et al., 2008).

Determination of plasmid size by PFGE

The size of large x- and EHEC-hly plasmids was determined by PFGE with S1 nuclease-treated total DNA of bacteria embedded in agarose plugs as described previously (Barton et al., 1995). The plugs were incubated with S1 nuclease (Amersham, Buckinghamshire, UK) following the conditions recommended by the supplier. A slice of agarose plug was sealed into a well of a 1% horizontal agarose gel. A λ ladder PFGE marker (New England Biolabs, Frankfurt/Main, Germany) and a low-range PFGE marker (Biolabs) were used as molecular weight standards. PFGE was performed with the clamped homogeneous electronic field (CHEF-DR II system, BioRad, Munich, Germany) with pulse times increasing from 2 to 30 s for 18.5 h at 210 V in 0.5 × TBE buffer at 10 °C. After the electrophoresis, gels were stained with ethidium bromide for visualization of single bands and photographed under UV light. Southern hybridization of PFGE gels was performed with x-hly- and EHEC-hly-specific gene probes as described above.

Molecular cloning of the x-hly determinant of pEO5

The E. coli K-12 strain TPE422 (Table 1) that carries pEO5 was used as a source for plasmid preparation. For cloning of pEO5-associated x-hly genes, pEO5 DNA was digested with Sall, which does not cut inside the x-hlyCABD operon (Felmlee et al., 1985; Hess et al., 1986). Sall-digested pEO5 DNA was ligated with Sall-digested vector plasmid pACYC184 and transformed into the nonhaemolytic E. coli K-12 strain C600 (Schmidt et al., 1994). Selection for transformants expressing x-hly was performed on sheep blood agar plates supplemented with 20 μg mL⁻¹ chloramphenicol for selecting pACYC184 recombinants. Haemolysin-expressing transconjugants were selected after an overnight incubation on enterohaemolysin agar plates inoculated with transformed C600. Plasmid DNA of x-hly recombinant strains TPE2319 and TPE2320 was isolated and the DNA insert size was determined by restriction endonuclease digestion of pEO2319 and pEO2320, followed

### Table 2. PCR primers and conditions for detection of x-hlyA and hlyC upstream sequences in pEO5 and related plasmids

<table>
<thead>
<tr>
<th>Target genes (position in pEO5 sequence FM180012)</th>
<th>Primer</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Tm (°C)</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>x-hlyA (1915–1936)</td>
<td>10f</td>
<td>GCTGCAATAAATTGCACTCAGCC</td>
<td>53.1</td>
<td>666</td>
</tr>
<tr>
<td></td>
<td>10r</td>
<td>CTGCACCGATATTATCAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hlyC – upstream hlyC (953–1630)</td>
<td>1f</td>
<td>GTAGTCAAAAGACAAACTCGTG</td>
<td>50.6</td>
<td>678</td>
</tr>
<tr>
<td></td>
<td>1r</td>
<td>ATCCCCGAAAGGAGCAATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hlyR – upstream hlyC (597–1267)</td>
<td>32f</td>
<td>GCCTTGCCGTACAATTTCC</td>
<td>56.5</td>
<td>671</td>
</tr>
<tr>
<td></td>
<td>32r</td>
<td>TCGTTTTATGTCATAACTCGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
by gel electrophoresis of restriction fragments. Fragments containing \(\alpha\)-hly-specific sequences were detected by hybridization of Southern-blotted digested pEO2319 and pEO2320 with the \(\alpha\)-hly-specific gene probe.

**Nucleotide sequencing of pEO5-encoded \(\alpha\)-hly genes and bordering sequences**

Nucleotide sequence analysis of the \(\alpha\)-hly determinants cloned on plasmids pEO2319 and pEO2320 was performed by PCR. Sequencing primers located in the vicinity of the SalI restriction site of pACYC184 (GenBank X06403) were developed with the aid of ACCelryS GENE v.2.5 software (Accelrys Ltd, Cambridge, UK). Analysis of the 23-kb insert from pEO5 and from recombinant plasmids pEO2319 and pEO2320 was performed by single-strand sequencing and primer walking. A 9227-bp fragment encompassing the \(\alpha\)-hlyCABD operon and adjacent regions was subjected to double-stranded sequencing with greater than or equal to sixfold coverage. PCR products were purified and used for sequencing applying the dye terminator chemistry (PE Applied Biosystems, Darmstadt, Germany) and separated on an automated DNA sequencer (ABI PRISM® 3100 Genetic Analyser, Applied Biosystems, Foster City, CA). The sequences were analysed using the LASERGENE software (DNASTAR, Madison, WI) and ACCelryS GENE v.2.5 software.

**Nucleotide sequence accession numbers**

The nucleotide sequence of the pEO5 \(\alpha\)-hly determinant and adjacent sequences has been entered in the EMBL nucleotide sequence database (accession number FM180012).

**Results**

**Presence and properties of pEO5-related plasmids in E. coli O26 strains**

A conjugative plasmid called pEO5 that encodes \(\alpha\)-hly was previously isolated from the EPEC strain C4115 and similar large plasmids over 150 kb conferring the \(\alpha\)-hly phenotype were detected in EPEC O26 from human patients (Beutin et al., 1986). The presence of \(\alpha\)-hly plasmids was associated with a distinct clonal, RDF-type of EPEC O26 isolated from humans and cattle (Leomil et al., 2005).

We have chosen five temporally and geographically varying RDF O26 strains from humans and cattle (C4115, CB1027, CB1030, CB9866 and IP187) to investigate the relationship between \(\alpha\)-hly genes and EPEC O26 plasmids. Two strains carrying E-hly plasmids (1040/83 and TPE1313) served as controls. The properties of the strains are listed in Table 1. We have analysed \(\alpha\)-hly and E-hly plasmids that were separated by PFGE. The PFGE gels were Southern blotted and hybridized with an \(\alpha\)-hly and an EHEC-hly gene probe (Fig. 1). The RDO O26 strains carried \(\alpha\)-hly plasmids of similar size as pEO5 ( > 150 kb) (Fig. 1). Smaller size differences between the \(\alpha\)-hly plasmids were found between wild-type E. coli O26 strains and strain constructs carrying \(\alpha\)-hly and E-hly plasmids (TPE497). Representative \(\alpha\)-haemolytic E. coli O26 strains (C4115, CB9866, CB1027 and CB1030) were shown to transfer their \(\alpha\)-hly plasmids to nonhaemolytic E. coli such as JC3272 (E. coli K-12) and 698/83 (E. coli O26:H32), which were subsequently converted to \(\alpha\)-hly production (Table 1).

**Relationship between pEO5 and plasmids encoding E-hly**

Non-RDF EHEC O26:[H11] strains are negative for \(\alpha\)-Hly and pEO5-related plasmids, but carry plasmids encoding E-hly (ehx), which is characterized by an enterohaemolytic phenotype (Leomil et al., 2005). Plasmids encoding E-hly are frequently found in EHEC O26-, O103-, O111-, O145- and O157-type strains and were shown to be genetically related (Brunder et al., 1999). We were interested in determining the relationship of the \(\alpha\)-hly plasmid pEO5 with E-hly-encoding plasmids. For this reason, we have transferred the Tn5-tagged pEO5 from strain TPE477 into the enterohaemolytic non-RDF strain 1040/83, which harbours two
large plasmids c. 70 and 90 kb in size; the transconjugant strain was called TPE497 (Table 1). The 90-kb plasmid was identified as the EHEC-virulence plasmid (pO26) as it hybridized with the EHEC-hly-specific gene probe (Fig. 1a and c, lane 5). EHEC-virulence plasmid-encoded genes elx, katP and esp were detected in 1040/83 and in the transconjugant strain TPE497 by PCR (Leomil et al., 2005; this work). Strain TPE497 was shown to carry a third plasmid of >150 kb size that was identified as AE05 by Southern hybridization with the \( \alpha \)-hly-specific gene probes (Fig. 1a and b, lane 5). The presence of \( \alpha \)-hly and EHEC-hly genes in TPE497 was confirmed by specific PCR as indicated in Material and methods. The finding that the E. coli O26 E-hly plasmid pO26 and the \( \alpha \)-hly plasmid AE05 were compatible with each other indicates that they are not closely related.

We were interested in investigating the genetic relationship between the E-hly and the \( \alpha \)-hly-encoding plasmids by DNA hybridization. Because the 90-kb E-hly plasmid pO26 could not be separated from the 70-kb plasmid in 1040/83, we chose the EHEC virulence plasmid plasmid pO157 (92,077 bp, GenBank AF074613) of the EHEC O157:H7 strain EDL933 (Burland et al., 1998) for this investigation. The E. coli K-12 strain TPE1313 harbouring a Tn801-tagged derivative of pO157 was used as a recipient in conjugation experiments and as a source of pO157 DNA (Table 1). pEO5::Tn5 was introduced from TPE477 into TPE1313 by conjugation. TPE1313 transconjugants carrying pO157::Tn801 and pEO5::Tn5 were selected by screening for Km\(^{R}\) and Amp\(^{R}\) colonies. Plasmids pO157 and pEO5 were found to coexist stably in the E. coli K-12 transconjugant strain TPE2611 (Table 1, Fig. 1). To investigate the overall genetic relationship between pEO5 and pO157, plasmid DNA of pEO5 and pO157 was isolated, digested with different restriction enzymes and used for Southern Blot hybridization experiments with digoxigenin-labelled pEO5 and pO157 as gene probes. Only weak hybridization signals were obtained by cross-hybridization of pEO5 and pO157, indicating that these plasmids do not share large regions of genetic similarity (Fig. 2).

**Cloning and nucleotide sequencing of the \( \alpha \)-hly determinant present on pEO5**

We were interested in characterizing the \( \alpha \)-hly determinant of pEO5 as it is so far the only virulence determinant found to be associated with this EPEC O26 plasmid. Cloning of pEO5-associated \( \alpha \)-hly genes on pACYC184 was performed as described in Materials and methods. Two colonies with an \( \alpha \)-haemolytic phenotype were obtained from about 48,000 screened chloramphenicol-resistant transformants. These pEO5-recombinant strains, called TPE2319 and TPE2320, were screened for DNA hybridization with the Sall-A fragment of plasmid psF4000, which encompasses the entire \( \alpha \)-hly-operon cloned from strain J96 (Welch et al., 1983) (Table 1). Both TPE2319 and TPE2320 were found to carry an \( \alpha \)-23-kb insert on pACYC184, which hybridized with the Sall-A gene probe. The vector pACYC184 and the \( \alpha \)-hly plasmid psF4000 served as negative and positive controls (data not shown).

Published nucleotide sequence data indicate that the pHly152-inherited \( \alpha \)-hlyCABD determinant is 96.0–96.4%
identical to chromosomally inherited α-hly determinants located on PAI I (GenBank AJ488511) and PAI II (GenBank AJ494981), respectively (Hess et al., 1986; Vogel et al., 1988; Nagy et al., 2006). Because there was only one plasmid (pHly152)-derived α-hlyCABD sequence available, which was from a murine E. coli strain (GenBank M14107), we have determined the sequence of a 9227-bp fragment of pEO5 encompassing the entire α-hly-determinant and adjacent sequences (GenBank FM180012) (Fig. 3).

The pEO5-derived sequence is flanked at both ends with transposon-associated sequences. On its left side, a 113-bp stretch with 98% similarity to a putative transposase gene (GenBank AF453441) is found. A 702-bp regulatory region with 98.8% similarity to the hlyR regulatory region of pHly152 (GenBank X07565) (Vogel et al., 1988) is located between positions 165 and 867 (FM180012). The hlyR region carries regulatory sequences (A, B and ops) (Vogel et al., 1988; Nagy et al., 2006) for enhancement of α-hly gene expression. A stretch of 465 bp upstream of hlyC in pEO5 (positions 868–1333) shows 99% homology to the corresponding region of plasmid pHly152 (GenBank M14107). This region, designated 'pHly152' (Fig. 3), does not contain orfs (Hess et al., 1986; this work). The entire α-hlyCABD determinant of pEO5 has a size of 7252 bp and shares 99.2% identity to that of pHly152. The hlyC (1334–1846) and hlyD (7149–8585) genes of pEO5 were found to be 99% similar to the corresponding genes of plasmid pHly152 and pEO5 hlyA (1858–4932), and hlyB genes (5007–7130) share 98% similarity to their pHly152 homologues. A stretch of 155 bp in pEO5 located immediately downstream of the hlyD gene (position 8586–8748) is 97% similar to the corresponding sequence of pHly152. In the pEO5 sequence, a 255-bp DNA segment is present (position 8749–9003) that has 91% similarity to the IS911 Shigella dysenteriae insertion sequence (GenBank X17613). The last 223 bp (position 9004–9227) of the pEO5 sequence are 97% similar to an IS transposase of the E. coli plasmid pCOO (GenBank CR942285).

**Similarity between pEO5-like α-hly plasmids in the region encompassing the hlyCABD determinant**

In order to investigate pEO5-related plasmids in EPEC O26 strains for similarity in the hlyC upstream region, we have developed a PCR (primers 1f and 1r, Table 2) amplifying a 678-bp DNA stretch encompassing hlyC upstream sequences and hlyC. The E. coli O26 strains carrying pEO5-like plasmids as well as pHly152 from strain 374 yielded PCR products that were of the same size and similar for their HinfI restriction profiles, corresponding to 23, 180, 202 and 273 bp in the pEO5 sequence (FM180012). A second PCR encompassing the region between the hlyR and the hlyC upstream region in pEO5 (primers 32f and 32r, Table 2) yielded 671 bp products with the pEO5-type plasmids carrying O26 strains, and the HinfI patterns obtained from digested PCR products corresponded to 46, 49, 90, 202 and 284 bp (FM180012). In contrast, a PCR product of about 2000 bp was obtained with pHly152. This finding can be explained by the presence of an IS2 element that is inserted between hlyR and hlyC in pHly152 and is encompassed by primers 32f and 32r (Hess et al., 1986; Knapp et al., 1985;
Vogel et al., 1988; this work). We could confirm this by nucleotide sequencing of the pHly152 PCR product generated with primers 321f/32r, showing that the IS2 element is directly inserted between the hlyR and the ‘phly152’ region located upstream of hlyC (GenBank M14107) (Fig. 3).

Discussion

We have previously described two clonal lineages of human pathogenic E. coli O26 strains that can be distinguished from each other by their phenotypes, their virulence attributes, by multilocus sequence analysis (MLST) and by their XbaI PFGE profiles (Leomil et al., 2005). The O26 RDF lineage is characterized by atypical EPEC strains that are positive for intimin (eae) and for α-hly, but are negative for EAF-plasmids and for virulence markers (stx, katP, espP, EHEC-hly and iutA) that are frequently associated with EHEC O26:[H11] strains.

We were interested in comparing the α-hly plasmid pEO5 of a human EPEC O26 strain with virulence plasmids of EHEC O26 and O157 strains that encode a related RTX-toxin called E-hly. While α-hly plasmids of different origins were found to be conjugative and could transfer the haemolytic phenotype to nonhaemolytic E. coli (Grunig & Lebek, 1988; Prada et al., 1991; this work), EHEC-virulence plasmids were shown to carry defective transfer genes (Burland et al., 1998) and are thus limited for their spread in the E. coli species. By DNA hybridization, pEO5 was not found to be similar to pO157, a prototype plasmid encoding E-hly. The pO157 plasmid-encoded E-hly operon (AF074613) and the α-hly operon of pEO5 (FM180012) share < 60% DNA homology, and α-hly-encoding plasmids, pHly152 and pEO5, differ from pO157 for the hlyCABD operon-encompassing sequences. These findings indicate that E-hly and α-hly have evolved separately in EHEC and EPEC strains.

Most of the earlier studies dealing with plasmid-encoded α-hly in E. coli were performed using the murine E. coli plasmid pHly152, which was sequenced for its α-hly determinant and adjacent regions (Vogel et al., 1988). Here, we present the first nucleotide sequence analysis of a plasmid-encoded α-hly determinant from a human pathogenic EPEC strain. The nucleotide sequence analysis of the hlyCABD determinant of pEO5 revealed 99.2% identity to the α-hly genes present on pHly152, in contrast to their different origin and size. Regulatory sequences (A, B and ops) in the hlyR and in the hlyC upstream (‘pHly152’) region that were reported to enhance synthesis and secretion of α-hly in pHly152 (Knapp et al., 1985; Vogel et al., 1988) were also detected in pEO5. In addition, an operon polarity suppressor sequence (ops) that is essential for RfaH-dependent transcriptional antitermination of the α-hly operon (Nieto et al., 1996; Leeds & Welch, 1997) is located in the hlyR region of pEO5 648 bp upstream of the hlyC start codon. An ops element is also found in the hlyR sequence of pHly152 (GenBank X07565) and ops elements were found located 334 and 667 bp upstream of hlyC in chromosomally encoded α-hly determinants (Nagy et al., 2006). However, the latter are negative for the plasmid-specific regulatory regions hlyR and ‘phly152’ and do not share genetic similarity in the hlyC upstream regions to those of α-hly plasmids pHly152 and pEO5 (Knapp et al., 1985; Nagy et al., 2006; this work).

Haemolytic toxins that are very similar to E. coli α-hly were identified in other bacterial species such as Proteus, Morganella and Mannheimia (former Pasteurella) haemolytica (Koronakis et al., 1987; Strathdee & Lo, 1987). On the basis of codon preferences and base composition studies, it was speculated that the α-hly genes were acquired by E. coli from a donor belonging to one of these species (Koronakis et al., 1987; Strathdee & Lo, 1987, 1989). For plasmid-encoded α-hly determinants, it was assumed that these were acquired through horizontal gene transfer as part of transposable elements (Knapp et al., 1985). Transposon-like structures were found at both ends of the pEO5 sequence (FM180012), indicating that the pEO5 α-hly-CABD determinant and adjacent sequences could have been introduced by transposition in E. coli.

Epidemiological studies have shown that α-hly-encoding plasmids are frequently associated with ETEC and STEC strains causing enteric disease in animals (Prada et al., 1991; Bertschinger & Gyles, 1994; Beutin, 1999). It was suggested that the production of α-hly contributes to the virulence of the bacteria and favours their proliferation in the intestine (Smith & Linggood, 1971; Hampson et al., 1988; Wu et al., 2007). Similar to E-hly, α-hly may also play a role in intestinal infections of humans with intestinal pathogenic E. coli. Both E-hly and α-hly have been shown to cause cellular damage and trigger inflammation in the mammalian host (Welch, 1991; Taneike et al., 2002; Aldick et al., 2007; Troeger et al., 2007). Further studies are needed to investigate the contribution of these cytolyins in enteric infections of humans.

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