Molecular Analysis of Cytolysin A (ClyA) in Pathogenic Escherichia coli Strains

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Cytolysin A (ClyA) of Escherichia coli is a pore-forming hemolytic protein encoded by the clyA (hlyE, sheA) gene that was first identified in E. coli K-12. In this study we examined various clinical E. coli isolates with regard to the presence and integrity of clyA. PCR and DNA sequence analyses demonstrated that 19 of 23 tested Shiga toxin-producing E. coli (STEC) strains, all 7 tested enteroinvasive E. coli (EIEC) strains, 6 of 8 enteroaggregative E. coli (EAEC) strains, and 4 of 7 tested enterotoxicogenic E. coli (ETEC) strains possess a complete clyA gene. The remaining STEC, EAEC, and ETEC strains and 9 of the 17 tested enteropathogenic E. coli (EPEC) strains were shown to harbor mutant clyA derivatives containing 1-bp frameshift mutations that cause premature termination of the coding sequence. The other eight EPEC strains and all tested uropathogenic and new-born meningitis-associated E. coli strains (n = 14 and 3, respectively) carried only nonfunctional clyA fragments due to the deletion of two sequences of 493 bp and 204 or 217 bp at the clyA locus. Expression of clyA from clinical E. coli isolates proved to be positively controlled by the transcriptional regulator SlyA. Several tested E. coli strains harboring a functional clyA gene produced basal amounts of ClyA when grown under standard laboratory conditions, but most of them showed a ClyA-dependent hemolytic phenotype only when SlyA was overexpressed. The presented data indicate that cytolysin A can play a role only for some of the pathogenic E. coli strains.

Many bacterial pathogens produce toxins that kill and lyse host cells by interacting with the plasma membrane and by disrupting the function of this membrane as a permeability barrier. The majority of these cytolytic toxins are pore-forming proteins, and several of them have been shown to represent important virulence factors of the corresponding bacteria.

In Escherichia coli several different pore-forming cytolsins have been identified. The one most extensively studied is α-hemolysin (HlyA), which is produced by many uropathogenic E. coli (UPEC) strains and which contributes to their virulence as shown in several animal models (14, 48). E. coli α-hemolysin is encoded by the hlyCAB operon and belongs to the family of RTX (repeats-in-toxin) toxins that are widespread among gram-negative pathogens (12, 26). Several UPEC strains have been shown to carry the hly gene cluster within unique chromosomal inserts called pathogenicity islands that are absent from the nonpathogenic E. coli laboratory strain K-12 (15).

A toxin related to α-hemolysin, enterohemorrhagic E. coli (EHEC) hemolysin (EHEC-HlyA), has been identified in EHEC strains of serotype O157:H7, which represent the major etiological agents of the hemolytic-uremic syndrome and of hemorrhagic colitis worldwide (4, 28, 38). The EHEC HlyA operon, EHEC-hlyCABD (shcCABD), is located on a large plasmid that is present in almost all clinical E. coli O157:H7 isolates (4, 38). Recent studies revealed that EHEC-hlyA is also present in most EHEC strains belonging to less prevalent serotypes, such as O157:H⁻, O26:H11/H⁻, and O103:H2 (7, 20, 40).

A novel pore-forming hemolysin not related to HlyA, cytoliysin A (ClyA), has recently been detected in E. coli K-12. ClyA is a 34-kDa protein that is encoded by a chromosomal gene denoted clyA (also referred to as hlyE and sheA) (3, 8, 13, 25, 30, 31). The ClyA protein is not produced at phenotypically detectable levels when E. coli K-12 is grown under standard conditions on blood agar. This is apparently due to repression of the transcription of clyA by the nucleoid protein H-NS (49). Nevertheless, the expression of clyA in E. coli K-12 can be activated to a level that suffices to evoke a hemolytic phenotype when certain transcriptional regulators, such as SlyA from E. coli or Salmonella enterica serovar Typhimurium (24, 25, 30), MprA (EmrR) from E. coli (8), HlyX from Actinobacillus pleuropneumoniae (13), or FnrP from Pasteurella haemolytica (43) are overproduced in this strain.

Lipid bilayer experiments and electron microscopic studies have shown that ClyA forms stable pores in target membranes by assembling into ring-shaped toxin oligomers (25, 47). Due to this pore-forming activity, ClyA lyases erythrocytes from several mammalian species. In addition, it has been reported that ClyA is cytotoxic towards cultured mammalian cells and that it induces macrophage apoptosis (22, 31), which suggests that this toxin might contribute to the virulence of pathogenic E. coli strains. Consistent with this, some EHEC strains of serotype O157:H7 have recently been shown to harbor a complete clyA gene whose predicted product is almost identical in amino acid sequence to ClyA from E. coli K-12 (ClyA,K-12) (9, 17, 36). Apart from that, however, the presence of clyA in the different
Functional clyAb gene, which in turn indicates that ClyA can play a role only for a subset of the pathogenic E. coli strains. The incidence of functional copies of clyA particularly showed a correlation with several E. coli pathogroups causing enteric diseases, while all E. coli strains isolated from extraintestinal infections merely harbored nonfunctional clyA fragments.

**MATERIALS AND METHODS**

Bacteria, plasmids, and culture conditions. The E. coli wild-type strains used in this study are listed in Table 1. E. coli DH5α (F- Δ(lacZΔM15) ΔlacZYA-argF) U169 deoR recA1 endA1 phoA hsdR17 (rK-mK) supE44 Δ(lac-proAB) thi-1 gyrA96.
**TABLE 2. Plasmids used in this work**

<table>
<thead>
<tr>
<th>Plasmid(s)</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tr>
<td>pUC18, pUC19</td>
<td>Cloning vectors; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>New England Biolabs</td>
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<td>pAL105</td>
<td>pBluescript II SK (+) carrying shlya&lt;sub&gt;K-12&lt;/sub&gt;; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pAL115</td>
<td>pUC18 carrying shlya&lt;sub&gt;K-12&lt;/sub&gt;; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pAL201</td>
<td>pUC18 carrying chyl&lt;sub&gt;K-12&lt;/sub&gt; under control of lacZ&lt;sub&gt;P&lt;/sub&gt;; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>25</td>
</tr>
<tr>
<td>pAL202</td>
<td>pUC19 carrying chyl&lt;sub&gt;K-12&lt;/sub&gt;; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>25</td>
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<td>pCLYA3232/96</td>
<td>pUC18 carrying chyl from STEC strain 3232/96; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pCLYA12860</td>
<td>pUC19 carrying chyl from STEC strain 12860; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pCLYA5477/94</td>
<td>pUC18 carrying chyl from EÆEC strain 5477/94; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pCLYA1253</td>
<td>pUC19 carrying chyl from ETEC strain G1253; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pCLYA284/97</td>
<td>pUC19 carrying chyl from ETEC strain 284/97; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pCLYA297/87</td>
<td>pUC18 carrying chyl from ETEC strain 297/87; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pCLYA212/87</td>
<td>pUC18 carrying chyl from EPEC strain 212/87; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pCVD442</td>
<td>Positive-selection suicide vector containing the pir-dependent R6K replicon and sacB of Bacillus subtilis; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>pCHIΔchyl&lt;sup&gt;A&lt;/sup&gt;</td>
<td>pCVD442 carrying chyl&lt;sub&gt;K-12&lt;/sub&gt;-flanking sequences but lacking chyl&lt;sub&gt;K-12&lt;/sub&gt;; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pANN202-812</td>
<td>pBR322 carrying the E. coli α-hemolysin gene cluster (chl&lt;sub&gt;AB&lt;/sub&gt;CD) from plasmid pHly152; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
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**Cloning of chyl from E. coli wild-type strains.** The chyl<sub>A</sub> genes of the E. coli strains 3232/96, 12860, 5477/94, G1253, 284/97, 297/87, and 212/87 were amplified by PCR, using several forward primers designed according to DNA sequences present 244 to 563 bp upstream from the start codon. The PCR products were cloned into pUC18 or pUC19. Recombinant plasmids carrying the chyl<sub>A</sub> genes of the above strains in opposite orientation relative to lacZ<sub>A</sub> were selected and named pCLYA3232/96, pCLYA12860, pCLYA5477/94, pCLAY1253, pCLYA284/97, pCLYA297/87, and pCLYA212/87. In all these plasmids the inserted chyl<sub>A</sub> gene is controlled only by its native 5′-flanking regulatory region. An additional isolated plasmid, pCVD442<sub>787</sub>, carries chyl<sub>A</sub> from E. coli 284/97 in the same orientation as lacZ<sub>A</sub>. In this case, the chyl<sub>A</sub> gene is consequently not only under control of its native promoter region but also under control of the lacZ<sub>A</sub> promoter (lacZ<sub>P</sub>).
erythrocytes in 0.9% NaCl, containing about 7.0 × 10^8 red blood cells per ml. Immunoblot analysis was performed with bacterial cell lysates which were prepared as described for E. coli strain 212/87 (AY576663); EPEC strain E2348/69 (AY576664); uropathogenic E. coli (UPEC) strain 536 (AY576665); UPEC strain RZ443 (AY576666); new-born meningitis-associated E. coli (NMEC) strain IHE3034 (AY576667).

Preparation of a polyclonal rabbit anti-ClyA antiserum. ClyA was overexpressed in E. coli DH5α from plasmid pAL201 and was isolated by osmotic shock from the periplasm of bacteria grown to the stationary phase. The periplasmic proteins were mixed with 1 volume of 2× sample buffer lacking β-mercaptoethanol and bromophenol blue (100 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS) and separated without previous boiling by SDS-PAGE. The predominant 34-kDa protein was used for rabbit immunization. The serum taken 11 days after the third injection was used as polyclonal anti-ClyA antiserum. This serum reacted strongly with ClyA as established by Western blot analysis, while the preimmune serum did not recognize ClyA (data not shown).

Determination of hemolytic activity. Quantitative hemolytic activity assays were performed with bacterial cell lysates which were prepared as described above by ultrasonic treatment of bacteria suspended in PBS. Different volumes (0.5 to 50 μl) of the lysates were mixed with 600 μl of a suspension of horse erythrocytes in 0.9% NaCl containing about 7.0 × 10^8 red blood cells per ml. After incubation at 37°C for 30 min, the erythrocytes were pelleted by centrifugation. The amount of hemoglobin released into the supernatant was measured spectrophotometrically at 543 nm.

Nucleotide sequence accession numbers. The clyA sequences of the following strains have been submitted to the EMBL/GenBank/DDBJ databases (accession numbers are given in parentheses): Shiga toxin-producing E. coli (STEC) strain 3232/96 (AY576656); EIEC strain 4608-58 (AY576657); EIEC strain 12860 (AY576658); enterohaemorrhagic E. coli (EHEC) strain 5477/94 (AY576659); enterotoxigenic E. coli (ETEC) strain G1253 (AY576660); ETEC strain 284/97 (AY576661); ETEC strain 297/87 (AY576662); enteropathogenic E. coli (EPEC) strain 212/87 (AY576663); EPEC strain E2348/69 (AY576664); uropathogenic E. coli (UPEC) strain 536 (AY576665); UPEC strain RS226 (AY576666); new-born meningitis-associated E. coli (NMEC) strain IHE3034 (AY576667).

RESULTS

PCR analysis of E. coli strains. A significant number of clinical E. coli isolates belonging to the most representative groups of pathogenic E. coli was analyzed by PCR with regard to the presence of the clyA gene, using primers designed according to clyA_1A_12 and flanking DNA sequences. The tested strains included 23 STEC, 7 EIEC, 8 EAEC, 7 ETEC, 17 EPEC, 14 UPEC, and 3 NMEC strains (Table 1). Two additional strains which were isolated from the stool of healthy individuals, E. coli 764 and E. coli RS226, have not been assigned to a specific pathogroup but belong to serotypes that are frequently encountered among UPEC and NMEC strains, respectively.

By using several primer combinations, DNA fragments could be amplified from all STEC, EIEC, and EAEC strains, from 5 of the 7 ETEC strains (117/86, 147/1, G1253, 164/82, and ST3135B/01), and from 9 of the 17 EPEC strains (111/87, 212/87, 402/87, 227/63, 315/60, 12810, 16-2, 6447/89, and 6587/85) that were indistinguishable in size, when analyzed by agarose gel electrophoresis, from the PCR products obtained under the same conditions from E. coli K-12 (Fig. 1). Slightly shorter DNA fragments were amplified from the ETEC strains 284/97 and 297/87, indicating the presence of small deletions in the clyA genes of these strains. DNA fragments about 0.7 kb shorter than expected were amplified from the remaining eight EPEC strains, from all UPEC and NMEC strains, and from E. coli 764 and E. coli RS226 when the PCR was conducted with primers binding more than 0.16 kb upstream and immediately downstream from clyA_12. In addition, no PCR products were obtained from these strains when primers binding to the 5′-terminal two-thirds of clyA_12 or to the DNA region preceding clyA_12 were used, suggesting that the latter strains have chromosomal deletions of about 0.7 kb affecting clyA and the 5′-flanking DNA sequence.

Characterization of the clyA sequences of E. coli wild-type strains. Sequencing of the PCR-amplified clyA-carrying or clyA-related DNA fragments from all 81 E. coli strains listed in Table 1 yielded the following results.

(i) Identification of an intact clyA gene in STEC, EIEC, EAEC, and ETEC strains. A complete clyA gene encoding, like clyA_12, a protein of 303 amino acid residues was found in 19 of the 23 tested STEC strains, in all 7 tested EIEC strains, in 6 of 8 EAEC strains, and in 4 of the 7 tested ETEC strains (Tables 1 and 3). The clyA genes of some of these strains (EAEC strains 17-2, 5477/94, and OPA065 and ETEC strain (Fig. 1. (A) Schematic presentation of the clyA gene from E. coli K-12. The position of the SlyA-controlled clyA promoter (p) (25) is indicated by an open box, and the binding sites of several primers used for PCR are shown by arrows (P1, 5′-GCCAGCAGATCAATCTGTG-3′; P2, 5′-CATAGTAGGATCCGTACC-3′; P3, 5′-CTTATG GATAGCAGAGTAAG-3′; P4, 5′-CAATGGACCGTGACGA CACC-3′). The position of deletion I and deletion II at the clyA locus of UPEC, NMEC, and several EPEC strains is indicated by solid bars. (B) Agarose gel electrophoresis of the PCR products obtained with the primer combinations P1-P2 (lanes 1 to 6) and P3-P4 (lanes 7 to 12) from E. coli K-12 (lanes 1 and 7), STEC (EHEC) strain 3232/96 (lanes 2 and 8), ETEC strain 284/97 (lanes 3 and 9), ETEC strain 297/87 (lanes 4 and 10), UPEC strain J96 (lanes 5 and 11), and UPEC strain 536 (lanes 6 and 12). Lane M, DNA size markers (SPP1 DNA cleaved with EcoR1).
117/86) were identical in sequence to clyA K-12, but in most cases several nucleotide substitutions (between 5 and 16) were detected. Some of these substitutions proved to be highly conserved in strains belonging to the same E. coli pathogroup or even in members of different pathogroups.

The amino acid sequences predicted for the clyA gene products of the above strains were either identical to that of ClyA K-12 (EAEC strains 17-2, 5477/94, and OPA065; ETEC strain 117/86 and G1253) at least the first 185 bp preceding clyA were the same as those in E. coli K-12. This DNA region carries the SlyA-controlled promoter of clyA (25). The remaining strains exhibited a few nucleotide substitutions in the clyA 5'-flanking sequence, but the −10 and −35 signals of the clyA promoter were generally not affected. Most of the latter strains, including all serogroup O157 STEC strains, exhibited a C→T and a G→T exchange in the spacer between the −10 and −35 signals and a T→C substitution 61 bp upstream from the clyA start codon.

(ii) Detection of frameshift mutations in clyA of several STEC, ETEC, and EPEC strains. Several E. coli wild-type strains were shown by DNA sequencing to harbor mutant clyA derivatives containing 1-bp frameshift mutations that cause premature truncation of the clyA open reading frame (ORF) (Tables 1 and 3, Fig. 2). In three serogroup O157 STEC strains (3817/96, 4299/96, and 4304/96) we found, for example, a clyA derivative exhibiting a unique 1-bp deletion in codon 248.

![Table 3. Characteristics of the clyA gene in E. coli wild-type strains analyzed in this study](image)

<table>
<thead>
<tr>
<th>E. coli pathotype</th>
<th>No. of strains tested</th>
<th>No. containing functional clyA</th>
<th>No. containing mutant clyA sequencesa</th>
<th>Strains with 1-bp frameshift mutations</th>
<th>Strains with large deletions (deletions I and II)</th>
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</thead>
<tbody>
<tr>
<td>STEC</td>
<td>23</td>
<td>19</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EIEC</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EAEC</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ETEC</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EPEC</td>
<td>17</td>
<td>0</td>
<td>9</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>UPEC</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>NMEC</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

a Results obtained by DNA sequencing.

![Figure 2. Alignment of the amino acid sequences predicted for the truncated clyA gene products of several clinical E. coli isolates with the amino acid sequence of ClyA from E. coli K-12. Amino acid substitutions in the ClyA derivatives of the clinical E. coli isolates are indicated by boldface type. The underlined C-terminal amino acid sequences of the truncated ClyA derivatives resulted from frameshift mutations in the corresponding clyA genes. The asterisk behind the last amino acid residue indicates the presence of a stop codon in the DNA sequence.](image)
Interestingly, this clyA derivative was otherwise identical in sequence to the intact clyA gene found in all other tested O157 STEC strains. In the clyA genes of the ETEC strains 284/97 and 297/87, which were already predicted from the PCR data to contain small deletions, we detected not only an in-frame deletion of the codons 179 to 182 but also an identical 1-bp insertion in codon 163. Furthermore, a unique 1-bp deletion was found in codon 15 of clyA from strain 297/87. All nine EPEC strains that did not exhibit noticeable clyA defects upon PCR analysis (111/87, 212/87, 402/87, 227/63, 315/60, 12810, 16-2, 6447/89, and 6587/85) were shown by DNA sequencing to harbor a mutant clyA gene exhibiting a specific 1-bp deletion in codon 165. The same deletion was also found in the EAEC strains DEF2 and DEF53, in ETEC strain 164/82, and in serogroup O128 STEC strain ST3494/03. In clyA of strain ST3494/03 we detected, in addition, a unique 1-bp deletion in codon 278. The clyA genes exhibiting the 1-bp deletion in codon 165 generally proved to be very similar or even identical in sequence. All of them encode an identical C-terminally truncated ClyA derivative with a predicted molecular mass of 19.03 kDa.

The promoter regions of the mutant clyA genes from the above strains were either identical in sequence to that of clyA<sub>KE12</sub> (ETEC strains 284/97, 297/87, and 164/82; all mentioned EPEC strains; EAEC strains DEF52 and DEF53; STEC strain ST3494/03) or corresponded to the clyA promoter regions of the O157 STEC strains harboring an intact clyA gene (STEC strains 3817/96, 4299/96, and 4304/96), suggesting that all these clyA derivatives may be expressed under appropriate conditions.

(iii) Detection of deletions at the clyA locus in UPEC, NMEC, and several EPEC strains. Sequencing of the strikingly short clyA-related PCR products obtained from all tested UPEC and NMEC strains as well as from E. coli 764, E. coli RS226, and eight EPEC strains (700-36/85, 22CH, 273-4, 12-1, 1104/80, 3715/67, E2348/69, and 1083-36/91) demonstrated that all these strains harbor only DNA sequences corresponding to an internal fragment and to the 3'-terminal region of clyA. The sequence data further indicated that these clyA-related sequences are left from two deletions at the clyA locus which we refer to as deletion I and deletion II (Fig. 1, Tables I and 3). Deletion I generally comprised the 204-bp fragment spanning the 160 bp preceding clyA and the first 333 bp of clyA. Deletion II was found in two versions: in the UPEC strains 536, RZ460, and RZ485 it comprised the 217-bp fragment spanning the nucleotides 377 to 593 of clyA, while in all other strains it comprised the 204-bp fragment from nucleotides 382 to 585 of clyA (codons 128 to 195).

The residual clyA sequences of the above-mentioned E. coli strains were at least 96% identical to the corresponding fragments of clyA<sub>KE12</sub>. Several nucleotide substitutions were found in all or in most of these strains, whereas others could be detected only in strains belonging to the same pathotype. The following groups of strains harbored identical residual clyA sequences: (i) the EPEC strains 700-36/85, 22CH, 273-4, 1104/80, 3715/67, and E2348/69, UPEC strain RZ533, and E. coli 764; (ii) the three UPEC strains exhibiting the larger version of deletion II (536, RZ460, and RZ485); (iii) all tested UPEC strains containing the smaller version of deletion II, except J96 and RZ533; (iv) all tested NMEC strains (IHE3034, IHE3036, and RS218) and E. coli RS226. The clyA sequences of UPEC strain J96 differed from those of the NMEC strains only at a single nucleotide position.

It is unlikely that these residual clyA sequences are expressed, because they lack a translational start codon and a fortuitous TAA stop codon is present 22 bp upstream from deletion I, in frame with the clyA coding sequence. Furthermore, the clyA promoter region is completely removed by deletion I.

Analysis of the stability of clyA in E. coli wild-type strains. In order to test the stability of the clyA sequence in E. coli wild-type strains, four randomly selected clyA<sup>+</sup> strains (STEC 3232/96, EIEC 4608/58, EAEC 5477/94, and ETEC G1253) were grown for 7 days in 2×YT broth with daily dilution of the cultures (1:100) into fresh medium. Subsequently, the clyA gene was amplified by PCR, using in each case the bacteria from 1 μl of the final culture as template. Sequencing of the PCR products yielded definite clyA sequences identical to those originally determined for the corresponding strains, indicating that the clyA genes of these strains are quite stable upon prolonged subculturing.

Expression of clyA from clinical E. coli isolates in E. coli K-12. The clyA genes of several clinical E. coli isolates were cloned into pUC18 and pUC19 as described in Materials and Methods. Four of the resulting plasmids, pCLYA3232/96, pCLYA12860, pCLYA5477/94, and pCLYAG1253, carrying the functional clyA genes from STEC 3232/96, EIEC 12860, EAEC 5477/94, and ETEC G1253 under control of their native promoter regions, caused a hemolytic phenotype when introduced into the E. coli K-12 strain DH5α. The hemolytic activity on blood agar resembled in each case that of DH5α carrying clyA<sub>KE12</sub> on plasmid pAL202 (Fig. 3). As shown in Fig. 4, these recombinant DH5α clones also produced amounts.
of the 34-kDa ClyA protein similar to amounts produced by E. coli DH5α/pAL202. Transformation of a slyA<sub>K-12</sub>-carrying plasmid (pAL108) into the DH5α clones harboring pAL202, pCLYA3232/96, pCLYA12860, pCLYA5477/94, and pCLYAG1253 resulted in each case in enhanced production of ClyA and in a significantly stronger hemolytic phenotype on blood agar, demonstrating that the clyA genes of the corresponding clinical E. coli isolates are positively controlled by SlyA, like clyA<sub>K-12</sub> (Fig. 3 and 4). It should be pointed out that the stronger hemolytic phenotype of the DH5α double transformants does not completely reflect the enhancement of clyA expression, because ClyA overproduced in E. coli accumulates in the periplasmic space and only small amounts of it are released from the bacteria (25 and data not shown).

By using the method of quantitative real-time reverse transcription-PCR we recently observed that transcription of clyA in exponentially growing E. coli DH5α/pAL202/pAL108 is 5- to 10-fold stronger than in E. coli DH5α carrying pAL202 only in combination with the vector pACYC184 (C. von Rhein and A. Ludwig, unpublished data). Similar results would be expected for corresponding experiments performed with isogenic E. coli DH5α clones carrying pCLYA3232/96, pCLYA12860, pCLYA5477/94, or pCLYAG1253 instead of pAL202.

The plasmids pCLYA212/87, pCLYA284/97, and pCLYA297/87, carrying the mutant clyA genes of EPEC 212/87 (clyA<sub>212/87</sub>), ETEC 284/97 (clyA<sub>284/97</sub>), and ETEC 297/87 (clyA<sub>297/87</sub>) under control of their own promoter sequences, did not cause a hemolytic phenotype when introduced into E. coli DH5α. Furthermore, transformation of pAL108 into the DH5α clones harboring these plasmids caused in each case only very weak hemolytic activity on blood agar due to the SlyA-mediated induction of the chromosomal clyA<sub>K-12</sub> gene (Fig. 3 and 4). Proteins corresponding in size to the predicted products of clyA<sub>212/87</sub> (19.03 kDa) and clyA<sub>284/97</sub> (19.28 kDa) were specifically detected by Western blot analysis in cell lysates of E. coli DH5α harboring pCLYA212/87 and pCLYA284/97, respectively. The corresponding DH5α double transformants carrying pAL108 as well produced markedly larger amounts of these ClyA derivatives, confirming that clyA<sub>212/87</sub> and clyA<sub>284/97</sub> are positively controlled by SlyA (Fig. 4). Nevertheless, in the absence as well as in the presence of pAL108 the cellular levels of ClyA<sub>212/87</sub> and ClyA<sub>284/97</sub> were significantly lower than those of complete, functional ClyA proteins expressed under identical conditions, which suggests that these truncated ClyA derivatives are more unstable. E. coli DH5α transformed with pCLYA284/97A, a pUC18 derivative carrying clyA<sub>284/97</sub> under control of the lacZ promoter, produced rather large amounts of ClyA<sub>284/97</sub> (Fig. 4) but was also nonhemolytic on blood agar. In addition, no significant hemolytic activity could be detected in cell lysates of this strain by a quantitative hemolytic activity assay. The product of clyA<sub>297/87</sub> (predicted molecular mass, 2.63 kDa) could be detected neither in lysates of E. coli DH5α/pCLYA297/87 nor in those of DH5α carrying both pCLYA297/87 and pAL108.

**Analysis of the expression of clyA in clinical E. coli isolates.**

Several E. coli strains possessing a functional clyA gene, such as STEC (EHEC) 3232/96, EIEC 12860, EIEC 4608-58, and ETEC G1253 showed a nonhemolytic phenotype when grown overnight on blood agar containing horse erythrocytes (the weak enterohemolytic phenotype of STEC strain 3232/96 caused by the production of EHEC-HlyA was visible only on sheep blood agar). Nevertheless, the colonies of EIEC strain 12860 developed a hemolytic phenotype on horse blood agar when the agar plate was stored for several days at 4°C after the initial overnight incubation at 37°C. A clyA knockout mutant of strain 12860 (E. coli 12860ΔclyA) remained nonhemolytic under the same conditions, demonstrating that this hemolytic phenotype is clyA dependent (Fig. 5A).
apparently not due to a stronger expression of similar to each other, indicating that all these strains expressed due to enhanced release of the toxin from the bacteria. pAL115 (1d), clyA 12860/H9004 E. coli pAL115 (1b), clyA 12860/H9004 E. coli (2b), clyA 12860/H9004 E. coli (2c), and E. coli 4608-58ΔclyA/pAL115 (2d). A single colony of each strain was picked onto a blood agar plate. The agar plate was incubated overnight at 37°C and then was stored for 2 weeks at 4°C prior to taking the photograph. (B) Analysis of the production of ClyA protein in different E. coli strains by immunoblotting using a polyclonal anti-ClyA antiserum. Lane 1, ClyA purified from the periplasmic protein fraction of E. coli DH5α/pAL201 employing the Model 491 Prep Cell (Bio-Rad); lane 2, E. coli DH5α; lane 3, E. coli 4608-58; lane 4, E. coli 12860; lane 5, E. coli 12860ΔclyA. All except lane 5 are whole-cell proteins of approximately 10^7 bacteria harvested from overnight cultures of the specified strains were analyzed. The immunoreactive bands were visualized with the ECL plus Western Blotting Detection System (Amersham Biosciences). The data presented are representative of three independent experiments.

To further study the expression of clyA in E. coli strains possessing a functional chromosomal clyA gene, we analyzed the cellular ClyA levels in stationary-phase cultures by immunoblotting with a polyclonal anti-ClyA antiserum. When a highly sensitive Western blotting detection system was employed, a protein of about 34 kDa corresponding to ClyA could be specifically detected in cell lysates of all tested E. coli strains harboring an intact clyA gene, such as DH5α, 4608-58, and 12860, but not in lysates of E. coli 12860ΔclyA (Fig. 5B). The amounts of ClyA found in the different clyA+ strains were very similar to each other, indicating that all these strains expressed clyA at similar low, basal levels. According to these data, the hemolytic phenotype of older colonies of EIEC strain 12860 is apparently not due to a stronger expression of clyA in this strain compared to that in the other strains but most likely is due to enhanced release of the toxin from the bacteria.

Introduction of a slyA K-12-carrying plasmid (pAL105 or pAL115) into the E. coli strains 3232/96, 12860, 4608-58, and G1253 by electroporation caused in each case a hemolytic phenotype, in line with the finding that the functional clyA genes of these strains are positively controlled by SlyA. Consistent with this, it was recently shown at the protein level that overexpression of SlyA in EIEC strain 12860 causes enhanced production of ClyA (41). As shown in Fig. 5A, E. coli 12860/pAL115 exhibited clearly stronger hemolytic activity on blood agar than E. coli 4608-58/pAL115, again suggesting that strain 12860 releases ClyA more readily than other clyA+. E. coli strains. clyA knockout mutants of E. coli 12860 and E. coli 4608-58 (12860ΔclyA and 4608-58ΔclyA) remained nonhemolytic after introduction of pAL115, confirming that the hemolytic activity of the SlyA-overproducing wild-type strains is dependent on clyA.

ETEC strain 297/87 and EPEC strain 212/87 (Amp+) were nonhemolytic on blood agar and retained this phenotype after introduction of slyA K-12-carrying plasmids (pAL105 and pAL108, respectively), consistent with the finding that the clyA genes of both strains encode only truncated, obviously nonhemolytic ClyA derivatives. ETEC strain 284/97 (Cm+), also harboring a defective clyA gene (see above), exhibited a strongly hemolytic phenotype that was not affected by introduction of pAL105. Southern blot analysis of genomic DNA from E. coli 284/97 using an E. coli α-hemolysin-specific DNA probe isolated from plasmid pANN202-812 revealed a single DNA fragment that hybridized with this probe. In addition, a protein possessing a molecular mass similar to that of HlyA (approximately 110 kDa) was specifically detected in culture supernatants of E. coli 284/97 by Western blot analysis using a polyclonal anti-HlyA antiserum (data not shown). These findings indicated that the hemolytic activity of E. coli 284/97 is most likely due to the production and secretion of α-hemolysin or of a closely related toxin.

DISCUSSION

Recent studies have shown that the ClyA protein of E. coli K-12 is a pore-forming toxin which lyses erythrocytes from various species and which exhibits cytotoxic and apoptotic activity towards cultured mammalian cells (22, 25, 30, 31, 47). Based on these findings the questions arose whether E. coli strains are generally able to produce ClyA and whether this toxin is involved in the virulence of strains causing intestinal or extraintestinal infections.

The data presented in this study demonstrate that only part of the pathogenic E. coli strains possess a functional clyA gene, while others harbor mutant clyA derivatives or even only clyA fragments. In particular, an intact clyA gene was found in all tested EIEC strains, in most of the tested STEC and EAEC strains, and also in several ETEC strains, but it was not detected in any of the tested EPEC, UPEC, and NMEC strains. Some STEC, EAEC, and ETEC strains and about half of the tested EPEC strains were shown to harbor clyA derivatives containing 1-bp frameshift mutations that cause premature truncation of the clyA ORF. In the remaining EPEC strains and in all tested UPEC and NMEC strains we found only nonfunctional clyA fragments that are apparently left from two deletions at the clyA locus. One of these deletions, denoted here as deletion I, generally comprised a 493-bp fragment spanning the 160-bp sequence preceding clyA and the 5′-terminal 111 clyA codons. The other deletion (deletion II) proved to be slightly heterogeneous in size, because in three of the tested UPEC strains it comprised a 217-bp fragment spanning the nucleotides 377 to 593 of clyA while in all other strains it comprised only the 204-bp fragment from nucleotides 382 to 585 of clyA (codons 128 to 195).

Interestingly, deletion I and deletion II were found only in
combination, but we do not know whether these deletions occurred simultaneously or separately. It is also unclear whether or to what extent deletions I and II occurred independently in different E. coli strains. In any case, vertical DNA transfer most likely played an important role in the spreading of these deletions, even if they occurred in several strains. It is remarkable in this context that we did not observe the appearance of these deletions (nor of any other clyA mutations) upon prolonged cultivation of several E. coli strains harboring a functional clyA gene, which suggests that the clyA sequence is quite stable and not particularly prone to mutations.

Regarding deletion I, it is interesting that in E. coli K-12 and other clyA+ E. coli strains two very similar sequence motifs of 22 and 23 bp (AAGCATGCCATAATGACATT and AAGCATCCGGCCAGAAAGACATT) are centered 160 bp upstream and 333 bp downstream, respectively, from the 5′ end of clyA (i.e., at the end points of the sequence that is removed by deletion I). This suggests that deletion I occurred by homologous recombination between these related sequences. The processes that resulted in deletion II are, however, less clear because the corresponding deleted clyA fragment is not flanked by obvious direct repeats. Nevertheless, it is noteworthy that the 48-bp (or 43-bp) fragment left between deletion I and deletion II includes an imperfect palindromic sequence.

Results from immunoblot analyses demonstrated that E. coli K-12 and several tested clinical clyA+ E. coli isolates produce ClyA at similar low, basal levels when grown in rich medium. Consistent with this, a basal-level expression of ClyA in E. coli K-12 has recently also been observed by Oscarcson et al. (32). Thus, clyA is not totally silent in E. coli strains under in vitro cultivation conditions. The amounts of ClyA produced in E. coli K-12 and several other clyA+ E. coli strains are, however, apparently below the threshold that has to be passed to cause detectable hemolysis on blood agar. Nevertheless, in the case of EIEC strain 12860 we observed that the colonies grown on blood agar develop a clyA-dependent hemolytic phenotype when the agar plate is stored for several days at 4°C. To our knowledge, E. coli 12860 is the first reported phenotypically hemolytic E. coli wild-type strain in which the clyA gene has been identified as the genetic determinant of the hemolytic activity.

Western blot analyses of recombinant E. coli DH5α clones revealed that the clyA genes from clinical E. coli isolates are positively controlled, like clyA\textsubscript{K-12}, by the transcriptional regulator SlyA. In line with this finding, several tested E. coli wild-type strains possessing a functional clyA gene showed a clyA-dependent hemolytic phenotype when SlyA was overexpressed. This in turn indicates that these strains are able to release substantial amounts of ClyA under environmental conditions that cause increased cellular levels of SlyA and/or other factors involved in the positive regulation of clyA. It is tempting to speculate that such conditions might exist during the infection of host organisms.

Given the data presented in this work, it appears to be quite possible that cytolsyn A contributes to the virulence of several STEC, EIEC, EAEC, and ETEC strains. In line with this, it was recently observed that EIEC strain 4608-58 exhibits significantly stronger cytotoxic activity towards J774 macrophage-like cells than a clyA knockout mutant of this strain (C. Hüttinger, W. Goebel, and A. Ludwig, unpublished data). The finding that all tested UPEC, NMEC, and EPEC strains are unable to produce functional cytolsyn A suggests, on the other hand, that this toxin is not an important virulence factor for the latter groups of strains.

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