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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 enterotoxigenic *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 (2).

In *Escherichia coli* several different pore-forming cytolysins 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 *hlyCABD* 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 hemolysin operon, EHEC-*hlyCABD* (*ehxCABD*), 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, cytolysin 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 lyses 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 ($ClyA_{K\text{-}12}$) (9, 17, 36). Apart from that, however, the presence of *clyA* in the different

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TABLE 1. *E. coli* wild-type strains used in this study

Strain	Serogroup or serotype	Clinical origin[a]	*clyA*[b]	Source of supply[c]	Reference
STEC strains[d]					
0136/96	O157:H7	HUS	+	I	
0296/96	O157:H$^-$	HUS	+	I	
1104/96	O157:H$^-$	HUS	+	I	
1885/96	O157:H$^-$	HUS	+	I	
1938/96	O157:H$^-$	D	+	I	
1994/96	O157:H$^-$	AC	+	I	
2313/96	O157:H$^-$	AC	+	I	
2471/96	O157:H$^-$	HUS	+	I	
2711/96	O157:H$^-$	AC	+	I	
3010/96	O157:H7	D	+	I	5
3172/96	O157:H$^-$	HUS	+	I	
3232/96	O157:H7	HUS	+	I	
3817/96	O157:H$^-$	HUS	(+)	I	5
4011/96	O157:H7	D	+	I	
4049/96	O157:H7	HUS	+	I	
4299/96	O157:H$^-$	D	(+)	I	
4304/96	O157:H$^-$	D	(+)	I	
5869/96	O157:H$^-$	HUS	+	I	
16110/96	O157:H$^-$	D	+	I	
86-24	O157:H7	HC	+	II	11
ST73/01	O157:H7	HUS	+	III	
ST2415/01	O26:H11	HUS	+	III	
ST3494/03	O128	HUS	(+)	III	
EIEC strains					
12860	O124	D	+	I	39
78-5	O124	D	+	I	
W7062	O124:H$^-$	D	+	II	
76-5	O143	D	+	I	
107-11	O143	D	+	I	
4608-58	O143	D	+	IV	16
309-36/85	O145	D	+	I	
EAEC strains					
17-2	O3:H2	D	+	I	45
D4140-86	O44	D	+	I	39
DEF40	O78	D	+	I	
5477/94	O86	D	+	I	
4185/95	O86	D	+	I	
DEF53	O111	D	(+)	I	39
OPA065	O119	D	+	I	
DEF52	O126	D	(+)	I	
ETEC strains					
117/86	O6:H$^-$	D	+	I	
297/87	O25:H42	D	(+)	I	
147/1	O128:H$^-$	D	+	I	
G1253	O147:H19:K88	D	+	I	
164/82	O148:H28	D	(+)	I	
284/97	O149:H$^-$	PD	(+)	I	
ST3135B/01	O167:H6	D	+	III	
EPEC strains					
700-36/85	O55	ID	Del.	I	
22CH	O55	ID	Del.	I	
273-4	O55	ID	Del.	I	
111/87	O111	ID	(+)	I	
212/87	O111	ID	(+)	I	
402/87	O111	ID	(+)	I	
227/63	O114	ID	(+)	I	
315/60	O114	ID	(+)	I	
12810	O114	ID	(+)	I	
12-1	O119	ID	Del.	I	
16-2	O119	ID	(+)	I	
1104/80	O127	ID	Del.	I	
3715/67	O127	ID	Del.	I	
E2348/69	O127:K63:H6	ID	Del.	II	23
6447/89	O128	ID	(+)	I	
6587/85	O128	ID	(+)	I	
1083-36/91	O157:H45	ID	Del.	I	5
UPEC strains					
J96	O4:K6	UTI	Del.	II	18
AD110	O6:K2:H1	UTI	Del.	II	44
RZ460	O6:K2:H$^-$	UTI	Del.	II	50
RZ485	O6:K2:H1	UTI	Del.	II	50
RZ486	O6:K2:H1	UTI	Del.	II	50
RZ439	O6:K5:H1	UTI	Del.	II	51
RZ440	O6:K5:H1	UTI	Del.	II	51
RZ442	O6:K5:H1	UTI	Del.	II	51
RZ443	O6:K5:H$^-$	UTI	Del.	II	51
RZ495	O6:K5:H$^-$	UTI	Del.	II	51
RZ498	O6:K5:H1	UTI	Del.	II	51
RZ513	O6:K5:H$^-$	UTI	Del.	II	51
RZ533	O6:K5:H$^-$	UTI	Del.	II	51
536	O6:K15:H31	UTI	Del.	II	6
NMEC strains					
IHE3034	O18ac:K1:H7	NBM	Del.	II	1
IHE3036	O18ac:K1:H7	NBM	Del.	II	1
RS218	O18ac:K1:H7	NBM	Del.	II	1
Additional *E. coli* strains					
RS226	O18ac:K1:H7	AC	Del.	II	1
764	O18:K5:H$^-$	AC	Del.	II	34

[a] HUS, hemolytic-uremic syndrome; D, diarrhea; AC, asymptomatic carrier; HC, hemorrhagic colitis; PD, pig diarrhea; ID, infant diarrhea; UTI, urinary tract infection; NBM, new-born meningitis.

[b] Results obtained by DNA sequencing (this study). +, functional *clyA* gene; (+), *clyA* present but containing a 1-bp frameshift mutation (two 1-bp frameshift mutations were detected in the *clyA* sequences of STEC ST3494/03 and ETEC 297/87); Del., extensive deletions (deletion I and deletion II) at the *clyA* locus.

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[d] STEC strains causing the hemolytic-uremic syndrome or hemorrhagic colitis are also referred to as EHEC.

pathogroups of *E. coli* has not yet been systematically studied. Interestingly, functional *clyA* homologues have recently been identified in *S. enterica* serovar Typhi and serovar Paratyphi A, demonstrating that $ClyA_{K\text{-}12}$ represents the prototype of a novel family of bacterial cytolysins (33, 35, 47).

In this study, we analyzed various *E. coli* wild-type strains belonging to different pathogroups with regard to the presence and sequence characteristics of *clyA*. In addition, we investigated the expression of *clyA* from several of these strains and studied the influence of SlyA on *clyA* regulation. The data presented show that only some of the tested strains harbor a functional *clyA* 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$^-$ ϕ80 d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 phoA hsdR17* (r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA96*

TABLE 2. Plasmids used in this work

Plasmid(s)	Relevant characteristics[a]	Source or reference
pUC18, pUC19	Cloning vectors; Amp^r	New England Biolabs
pAL105	pBluescript II SK(+) carrying $slyA_{K\text{-}12}$; Amp^r	24
pAL108	pACYC184 carrying $slyA_{K\text{-}12}$; Cm^r	25
pAL115	pUC18 carrying $slyA_{K\text{-}12}$; Amp^r	This work
pAL201	pUC18 carrying $clyA_{K\text{-}12}$ under control of *lacZp*; Amp^r	25
pAL202	pUC19 carrying $clyA_{K\text{-}12}$; Amp^r	25
pCLYA3232/96	pUC18 carrying *clyA* from STEC strain 3232/96; Amp^r	This work
pCLYA12860	pUC18 carrying *clyA* from EIEC strain 12860; Amp^r	This work
pCLYA5477/94	pUC18 carrying *clyA* from EAEC strain 5477/94; Amp^r	This work
pCLYAG1253	pUC19 carrying *clyA* from ETEC strain G1253; Amp^r	This work
pCLYA284/97	pUC19 carrying *clyA* from ETEC strain 284/97; Amp^r	This work
pCLYA284/97A	pUC18 carrying *clyA* from ETEC strain 284/97 under control of *lacZp*; Amp^r	This work
pCLYA297/87	pUC18 carrying *clyA* from ETEC strain 297/87; Amp^r	This work
pCLYA212/87	pUC18 carrying *clyA* from EPEC strain 212/87; Amp^r	This work
pCVD442	Positive-selection suicide vector containing the *pir*-dependent R6K replicon and *sacB* of *Bacillus subtilis*; Amp^r	10
pCHΔ*clyA*	pCVD442 carrying $clyA_{K\text{-}12}$-flanking sequences but lacking $clyA_{K\text{-}12}$; Amp^r	This work
pANN202-812	pBR322 carrying the *E. coli* α-hemolysin gene cluster (*hlyCABD*) from plasmid pHly152; Amp^r	46

[a] The $slyA_{K\text{-}12}$ gene in pAL105, pAL108, and pAL115 and the *clyA* genes in pAL202, pCLYA3232/96, pCLYA12860, pCLYA5477/94, pCLYAG1253, pCLYA284/97, pCLYA297/87, and pCLYA212/87 are under control of their native promoter regions.

relA1] was employed as cloning host and for the propagation of all plasmids except pCVD442 and pCVD442 derivatives, which were propagated in *E. coli* SY327λpir and *E. coli* SM10λpir (29). The plasmids used in this work are listed in Table 2. All *E. coli* strains were grown aerobically at 37°C in 2× yeast extract-tryptone (2×YT) broth (yeast extract, 10 g/liter; tryptone, 16 g/liter; NaCl, 10 g/liter) or on YT broth solidified with 1.5% (wt/vol) agar. For the preparation of blood agar plates, the YT agar was supplemented with 4% defibrinated horse blood (Oxoid). When appropriate, antibiotic selection was carried out using ampicillin (Amp), chloramphenicol (Cm), and streptomycin (Str) at final concentrations of 100, 30, and 30 μg/ml, respectively.

DNA manipulations. DNA manipulations were performed with standard protocols (37). PCR was conducted either with *Taq* DNA polymerase (Eppendorf), Deep Vent DNA polymerase (New England Biolabs), or Phusion High Fidelity DNA polymerase (Finnzymes). Only PCR products synthesized by one of the latter two DNA polymerases were cloned and sequenced. Nucleotide sequences of DNA fragments were determined by automated cycle sequencing with fluorescence dye terminator technology, using either the ABI PRISM 377 DNA Sequencer or the ABI PRISM 3700 DNA Analyzer (Applied Biosystems). Southern blot hybridizations were performed with the ECL direct nucleic acid labeling and detection system (Amersham Biosciences) following the recommendations of the manufacturer.

Cloning of *clyA* from *E. coli* wild-type strains. The *clyA* 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 *clyA* of *E. coli* K-12 ($clyA_{K\text{-}12}$) and using several reverse primers corresponding to DNA sequences present 13 to 55 bp downstream from $clyA_{K\text{-}12}$. The PCR products were cloned into pUC18 or pUC19. Recombinant plasmids carrying the *clyA* genes of the above strains in opposite orientation relative to *lacZ*α of the vector were selected and named pCLYA3232/96, pCLYA12860, pCLYA5477/94, pCLYAG1253, pCLYA284/97, pCLYA297/87, and pCLYA212/87. In all these plasmids the inserted *clyA* gene is controlled only by its native 5′-flanking regulatory region. An additionally isolated plasmid, pCLYA284/97A, carries *clyA* from *E. coli* 284/97 in the same orientation as *lacZ*α. In this case, the *clyA* gene is consequently not only under control of its native promoter region but also under control of the *lacZ* promoter (*lacZp*).

Construction of plasmid pAL115. The *slyA* gene of *E. coli* K-12 ($slyA_{K\text{-}12}$) was amplified by PCR from strain CC118 (27) using the forward primer 5′-GAAG CAGGCGGTCGACGACAAGCC-3′, which was designed to introduce a SalI restriction site (underlined) 316 bp upstream from *slyA*, and the reverse primer 5′-GTTTCTCCGCGCTGGATCCGTTTGCGTGTG-3′, which introduced a BamHI site 43 bp downstream from the *slyA* stop codon. The 0.83-kb PCR product was cleaved with SalI and BamHI, and the generated 0.8-kb SalI-BamHI fragment was subsequently cloned into pUC18, resulting in pAL115. The authenticity of the insert of pAL115 was established by sequencing.

Construction of *clyA* knockout mutants of *E. coli* strains. To delete *clyA* in the enteroinvasive *E. coli* (EIEC) strains 12860 and 4608-58, a 0.9-kb SalI-BamHI fragment comprising the DNA sequence present 1.04 to 0.14 kb upstream from *clyA* was isolated by PCR with the forward primer 5′-TAGCTCTTCCAGCGTCGACATCACCCG-3′ and the reverse primer 5′-TATCAAACAGGATCCAATGTCATTATGGCG-3′. Furthermore, a 1.03-kb BglII-SacI fragment representing the DNA sequence immediately downstream from *clyA* was amplified by PCR with the primers 5′-GTACCTGAAAGATCTTAAGCGATTATTCTC-3′ and 5′-GCGTTTGAGAGCTCTTGTCCGCTTTCC-3′. The restriction sites at the ends of these DNA fragments were introduced by the PCR primers (see underlined sequences; only the SalI site was naturally present). The two DNA fragments were fused with each other by ligation of the BamHI and BglII sites and then were inserted between the SalI and SacI sites of the suicide vector pCVD442, resulting in plasmid pCHΔ*clyA*. pCHΔ*clyA* was transferred by conjugation from *E. coli* SM10λ*pir* into in vitro-selected Str^r derivatives of *E. coli* 12860 and *E. coli* 4608-58. Transconjugant clones were selected on Amp/Str agar plates and were analyzed by Southern blot hybridization of EcoRV-digested genomic DNA using the insert of pCHΔ*clyA* as probe. In both cases a clone was identified in which pCHΔ*clyA* was inserted into the chromosomal DNA sequence downstream from *clyA*. These clones were then grown in the absence of antibiotics to allow for excision of the suicide plasmid by a second event of homologous recombination. Descendants that had lost the plasmid (including the plasmid-encoded *sacB* gene) were selected on YT agar plates supplemented with 1% (wt/vol) sucrose, and those descendants in which the plasmid was excised by recombination between the SalI-BamHI fragment of pCHΔ*clyA* and the corresponding chromosomal DNA fragment were identified by Southern blot analysis, which was performed as described above. In the latter clones, the excision of the suicide plasmid concomitantly caused the deletion of the chromosomal *clyA* gene. This was confirmed for two of these clones, *E. coli* 12860Δ*clyA* and *E. coli* 4608-58Δ*clyA*, by sequencing of the *clyA* locus. In both *clyA* knockout mutants we detected the expected deletion of a 1.06-kb fragment spanning the entire *clyA* gene and the 149-bp sequence preceding *clyA*, which carries the *clyA* promoter region.

Isolation and analysis of proteins. The periplasmic proteins of *E. coli* strains were isolated by osmotic shock as described previously (25), precipitated by addition of ice-cold trichloroacetic acid (final concentration, 10%), pelleted by centrifugation at 12,000 × *g*, washed with acetone, dried under vacuum, and dissolved in sample buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate [SDS], 0.05% bromophenol blue). Extracellular proteins of *E. coli* strains were precipitated from cell-free culture supernatants by addition of 10% trichloroacetic acid and then were processed as described above for the periplasmic proteins. To analyze the proteins of whole cells, bacteria grown in 2×YT broth were harvested by centrifugation, washed with phosphate-buffered saline, pH 7.4, (PBS) (37), resuspended in PBS, and broken by ultrasonic treatment (10 times for 15 s each) at 4°C. Subsequently, the cell lysate was mixed with an equal volume of 2× sample buffer. Protein samples were neutralized, if required, by addition of saturated Tris solution and were boiled at 99°C prior to loading onto SDS-polyacrylamide gels. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (21). Western immunoblotting was conducted according to Towbin et al. (42) with the modification that the proteins were blotted onto polyvinylidene difluoride membrane using a Tris-glycine transfer buffer (25 mM Tris, 192 mM glycine,

10% methanol [pH 8.3]). Immunoblot analysis was performed either with a polyclonal antiserum raised in a rabbit against purified $ClyA_{K-12}$ (see below) or with a polyclonal rabbit anti-HlyA antiserum (19). Horseradish peroxidase-conjugated anti-rabbit secondary antibodies were used to detect the immunoreactive protein bands. These bands were finally visualized by using either the ECL plus Western Blotting Detection System (Amersham Biosciences) (in this case, the first and secondary antibodies were used at final dilutions of 1:10,000 and 1:50,000, respectively) or by using 0.02% 4-chloro-1-naphthol/0.01% H_2O_2 in Tris-buffered saline (TBS) (10 mM Tris-HCl [pH 7.5], 150 mM NaCl) for chromogenic detection (in this case, both antibodies were used at final dilutions of 1:1,000 or 1:2,000).

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 ClyA protein band was excised from the gel, and ClyA was eluted by diffusion at 4°C into PBS. About 0.5 mg of the purified ClyA 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); enteroaggregative *E. coli* (EAEC) 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 RZ443 (AY576666); newborn 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 $clyA_{K-12}$ 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 $clyA_{K-12}$. In addition, no PCR products were obtained from these strains when primers binding to the 5′-terminal two-thirds of $clyA_{K-12}$ or to the DNA region preceding $clyA_{K-12}$ were used, suggesting that the latter strains have chromosomal deletions of about 0.7 kb affecting *clyA* and the 5′-flanking DNA sequence.

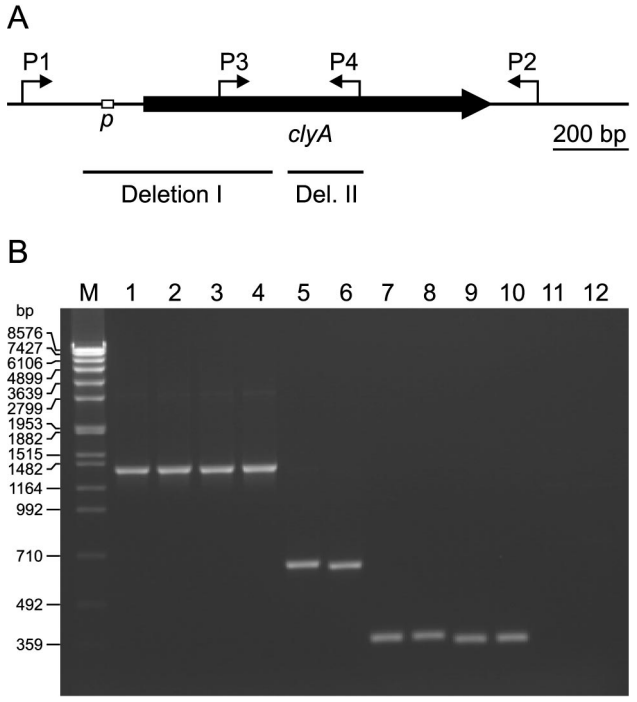

FIG. 1. (A) Schematic presentation of the *clyA* gene from *E. coli* K-12. The position of the SlyA-controlled *clyA* promoter (*p*) (25) is indicated by an open box, and the binding sites of several primers used for PCR are shown by arrows (P1, 5′-GCCAGCAGATCAATACTG-3′; P2, 5′-CATAATGAGAGTTACCCGATACC-3′; P3, 5′-CTTATG GATAGCCAGGATAAG-3′; P4, 5′-CAAATGGACCGTCGACGA 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 EcoRI).

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 $clyA_{K-12}$, 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

TABLE 3. Characteristics of the *clyA* gene in *E. coli* wild-type strains analyzed in this study

E. coli pathotype	No. of strains tested	No. containing functional *clyA*[a]	No. containing mutant *clyA* sequences[a]	
			Strains with 1-bp frameshift mutations	Strains with large deletions (deletions I and II)
STEC	23	19	4	0
EIEC	7	7	0	0
EAEC	8	6	2	0
ETEC	7	4	3	0
EPEC	17	0	9	8
UPEC	14	0	0	14
NMEC	3	0	0	3

[a] Results obtained by DNA sequencing.

117/86) were identical in sequence to $clyA_{K\text{-}12}$, 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 $ClyA_{K\text{-}12}$ (EAEC strains 17-2, 5477/94, and OPA065; ETEC strain 117/86; serogroup O26 STEC strain ST2415/01) or contained between one and three amino acid exchanges, which corresponds to a sequence identity of >99%. In particular, compared to $ClyA_{K\text{-}12}$ the following amino acid substitutions were found: a single N220→S exchange in the putative ClyA proteins of several EIEC (78-5, 107-11), EAEC (D4140-86, 4185/95), and ETEC strains (147/1, G1253, ST3135B/01); K75→N/A199→T in ClyA of the EIEC strains 76-5, 4608-58, and 309-36/85; L99→F/V185→I in ClyA of the EIEC strains 12860 and W7062 and of EAEC strain DEF40; D64→N/N220→S/K279→N in the ClyA proteins of all 18 serogroup O157 STEC strains harboring an intact *clyA* gene.

The DNA sequences flanking the intact *clyA* genes of the different *E. coli* strains proved to be very similar to those flanking $clyA_{K\text{-}12}$. In several cases (STEC strain ST2415/01; EIEC strains 12860, W7062, 76-5, 4608/58, and 309-36/85; EAEC strains 17-2, 5477/94, and OPA065; ETEC strains 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 (78 to 84 and 102 to 107 bp upstream from the translational start codon of *clyA*) 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.

```
                    10         20         30         40         50         60
E. coli K-12        MTEIVADKTVEVVKNAIETADGALDLYNKYLDQVIPWQTFDETIKELSRFKQEYSQAASV
STEC 3817/96        MTEIVADKTVEVVKNAIETADGALDLYNKYLDQVIPWQTFDETIKELSRFKQEYSQAASV
EPEC 212/87         MTEIVADKTVEVVKNAIETADGALDLYNKYLDQVIPWQTFDETIKELSRFKQEYSQAASV
ETEC 284/97         MTEIVADKTVEVVKNAIETADGALDLYNKYLDQVIPWQTFDETIKELSRFKQEYSQAASV
ETEC 297/87         MTEIVADKTVEVVKTQSKPQMEH*

                    70         80         90         100        110        120
E. coli K-12        LVGDIKTLLMDSQDKYFEATQTVYEWCGVATQLLAAYILLFDEYNEKKASAQKDILIKVL
STEC 3817/96        LVGNIKTLLMDSQDKYFEATQTVYEWCGVATQLLAAYILLFDEYNEKKASAQKDILIKVL
EPEC 212/87         LVGDIKTLLMDSQDKYFEATQTVYEWCGVATQLLAAYIFLFDEYNEKKASAQKDILIKVL
ETEC 284/97         LVGDIKTLLMDSQDKYFEATQTVYEWFGVATQLLAAYILLFDEYNEKKASAQKDILIKVL

                    130        140        150        160        170        180
E. coli K-12        DDGITKLNEAQKSLLVSSQSFNNASGKLLALDSQLTNDFSEKSSYFQSQVDKIRKEAYAG
STEC 3817/96        DDGITKLNEAQKSLLVSSQSFNNASGKLLALDSQLTNDFSEKSSYFQSQVDKIRKEAYAG
EPEC 212/87         DDGITKLNEAQKSLLVSSQSFNNASGKLLALDSQLTNDFSEKSSFSSHR*
ETEC 284/97         DDGITKLNEAQKSLLVSSQSFNNASGKLLALDSQLTNDFSEKKQLFPVTGR*

                    190        200        210        220        230        240
E. coli K-12        AAAGVVAGPFGLIISYSIAAGVVEGKLIPELKNKLKSVQNFFTTLSNTVKQANKDIDAAK
STEC 3817/96        AAAGVVAGPFGLIISYSIAAGVVEGKLIPELKNKLKSVQSFFTTLSNTVKQANKDIDAAK

                    250        260        270        280        290        300
E. coli K-12        LKLTTEIAAIGEIKTETETTRFYVDYDDLMLSLLKEAAKKMINTCNEYQKRHGKKTLFEV
STEC 3817/96        LKLTTEIAPSGR*

                    303
E. coli K-12        PEV*
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FIG. 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.

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 DEF52 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 $clyA_{K\text{-}12}$ (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 1 and 3). Deletion I generally comprised the 493-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 $clyA_{K\text{-}12}$. 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.

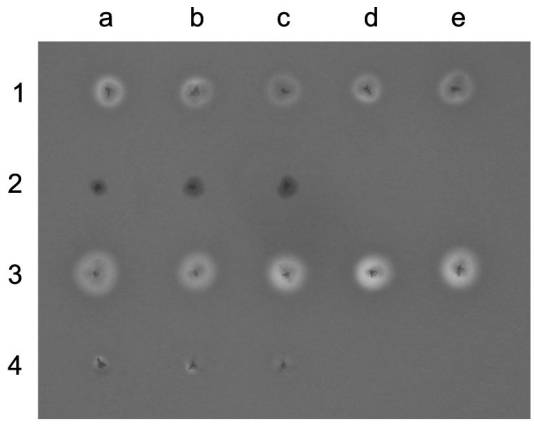

FIG. 3. Hemolytic phenotypes of *E. coli* DH5α clones harboring the following plasmids: pAL202 (1a), pCLYA3232/96 (1b), pCLYA12860 (1c), pCLYA5477/94 (1d), pCLYAG1253 (1e), pCLYA212/87 (2a), pCLYA284/97 (2b), pCLYA297/87 (2c), pAL202 and pAL108 (3a), pCLYA3232/96 and pAL108 (3b), pCLYA12860 and pAL108 (3c), pCLYA5477/94 and pAL108 (3d), pCLYAG1253 and pAL108 (3e), pCLYA212/87 and pAL108 (4a), pCLYA284/97 and pAL108 (4b), and pCLYA297/87 and pAL108 (4c). A single colony of each strain was picked onto blood agar containing 100 μg of ampicillin/ml. The agar plate was photographed after overnight incubation at 37°C. Phenotypes identical to those shown here were observed in at least three independent experiments.

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 $clyA^+$ 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 $clyA_{K\text{-}12}$ on plasmid pAL202 (Fig. 3). As shown in Fig. 4, these recombinant DH5α clones also produced amounts

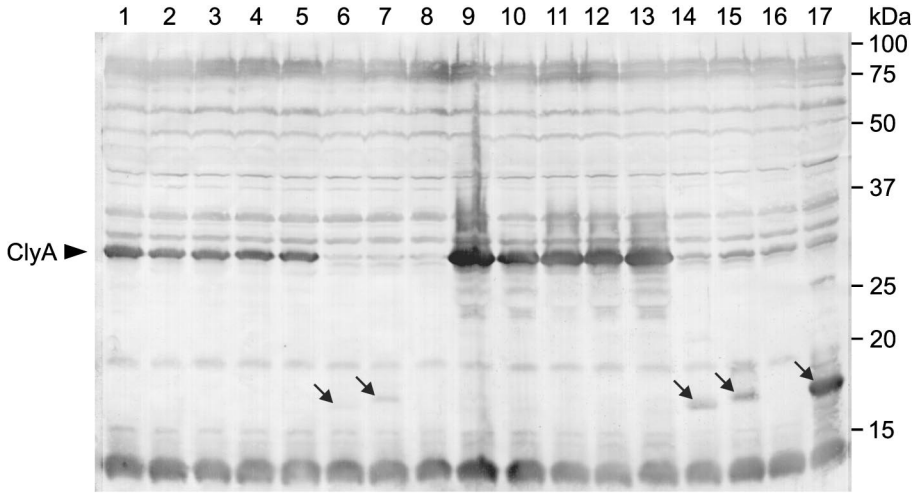

FIG. 4. Western blot analysis of the expression of cloned *clyA* genes from clinical *E. coli* isolates in *E. coli* DH5α, using a polyclonal anti-ClyA antiserum. The production of ClyA was determined in *E. coli* DH5α clones carrying the following plasmids: pAL202 (lane 1), pCLYA3232/96 (2), pCLYA12860 (3), pCLYA5477/94 (4), pCLYAG1253 (5), pCLYA212/87 (6), pCLYA284/97 (7), pCLYA297/87 (8), pAL202 and pAL108 (9), pCLYA3232/96 and pAL108 (10), pCLYA12860 and pAL108 (11), pCLYA5477/94 and pAL108 (12), pCLYAG1253 and pAL108 (13), pCLYA212/87 and pAL108 (14), pCLYA284/97 and pAL108 (15), pCLYA297/87 and pAL108 (16), and pCLYA284/97A (17). Whole-cell proteins from 25 μl of an overnight culture (grown for 18 h) were analyzed in each case. The immunoreactive bands were visualized by chromogenic detection. The arrows in lanes 6, 7, 14, 15, and 17 point to truncated ClyA derivatives. Positions of marker proteins are shown on the right. The data presented are representative of three separate experiments.

of the 34-kDa ClyA protein similar to amounts produced by *E. coli* DH5α/pAL202. Transformation of a $slyA_{K\text{-}12}$-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 $clyA_{K\text{-}12}$ (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 ($clyA_{212/87}$), ETEC 284/97 ($clyA_{284/97}$), and ETEC 297/87 ($clyA_{297/87}$) 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 $clyA_{K\text{-}12}$ gene (Fig. 3 and 4). Proteins corresponding in size to the predicted products of $clyA_{212/87}$ (19.03 kDa) and $clyA_{284/97}$ (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 $clyA_{212/87}$ and $clyA_{284/97}$ are positively controlled by SlyA (Fig. 4). Nevertheless, in the absence as well as in the presence of pAL108 the cellular levels of $ClyA_{212/87}$ and $ClyA_{284/97}$ 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 $clyA_{284/97}$ under control of the *lacZ* promoter, produced rather large amounts of $ClyA_{284/97}$ (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 $clyA_{297/87}$ (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* 12860Δ*clyA*) remained nonhemolytic under the same conditions, demonstrating that this hemolytic phenotype is *clyA* dependent (Fig. 5A).

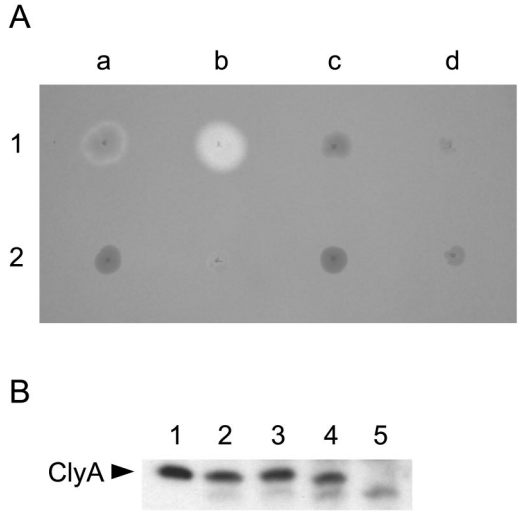

FIG. 5. (A) Phenotypes of the EIEC strains 12860 and 4608-58 and of derivatives of these strains on blood agar. Shown are *E. coli* 12860 (1a), *E. coli* 12860/pAL115 (1b), *E. coli*12860Δ*clyA* (1c), *E. coli* 12860Δ*clyA*/pAL115 (1d), *E. coli* 4608-58 (2a), *E. coli* 4608-58/pAL115 (2b), *E. coli* 4608-58Δ*clyA* (2c), and *E. coli* 4608-58Δ*clyA*/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*12860Δ*clyA*. In lanes 2 to 5, 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* 12860Δ*clyA* (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 $slyA_{K\text{-}12}$-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 (12860Δ*clyA* and 4608-58Δ*clyA*) 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^r) were nonhemolytic on blood agar and retained this phenotype after introduction of $slyA_{K\text{-}12}$-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^r), 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 (AAGCATTCGCCATAATGACATT and AAGCATCCGCCCAGAAAGACATT) 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 Oscarsson 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 $clyA_{K\text{-}12}$, 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 cytolysin 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 cytolysin 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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