

## Occurrence of *hlyA* and *sheA* Genes in Extraintestinal *Escherichia coli* Strains

Monika Kerényi,<sup>1\*</sup> Heather E. Allison,<sup>2</sup> István Bártai,<sup>3</sup> Ágnes Sonnevend,<sup>4</sup> Levente Emödy,<sup>1</sup>  
Nóra Plaveczy,<sup>1</sup> and Tibor Pál<sup>1,4</sup>

Departments of Medical Microbiology and Immunology<sup>1</sup> and Anesthesiology and Intensive Therapy,<sup>3</sup> Medical School, University of Pécs, Hungary; School of Biological Sciences, Division of Microbiology and Genomics, University of Liverpool, Liverpool, United Kingdom<sup>2</sup>; and Department of Medical Microbiology, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al-Ain 17666, United Arab Emirates<sup>4</sup>

Received 10 August 2004/Returned for modification 3 September 2004/Accepted 3 February 2005

**The association of a hemolytic phenotype with the carriage of the  $\alpha$ -hemolysin gene (*hlyA*) and/or the silent hemolysin gene (*sheA* or *clyA*) among 540 extraintestinal clinical isolates of *Escherichia coli* and 110 fecal isolates from healthy individuals was investigated. Though HlyA is an important virulence factor in extraintestinal *E. coli* infection, the role of SheA is not completely clarified. Two hemolytic *sheA*<sup>+</sup> *E. coli* strains that lacked *hlyA* and possessed no other hemolysin genes were identified. No *hlyA*<sup>+</sup> *sheA*<sup>+</sup> strains were identified, suggesting that there is possible incompatibility between *hlyA* and *sheA* in the chromosome of *E. coli*.**

*Escherichia coli* strains that cause extraintestinal infections possess numerous virulence factors, including hemolysin production. *E. coli*  $\alpha$ -hemolysin (HlyA) produces large, clear zones of hemolysis around colonies on blood agar. The hemolysin is present in cell-free filtrates and is the best characterized member of the RTX (repeat in toxin) toxin family (25). HlyA lyses cells by the creation of pores in the target cell membrane and affects erythrocytes, leukocytes (4, 5), and renal tubular cells (12). Its activity on polymorphonuclear granulocytes liberates leukotrienes, histamine, and ATP (13) and is neutralized by specific antiserum.

Cytolysin A or “silent hemolysin” (SheA) causes hemolysis when its gene, *sheA* (also known as *clyA* or *hlyE*), is present on high-copy-number plasmids; when certain regulator genes, *mprA* and *slyA*, are overexpressed (8); or when the transcription of the chromosomal *sheA* is derepressed (i.e., in *hms* mutant *E. coli* strains) (26). The activities of these two *E. coli* hemolysins differ (Table 1) (1). It has been proven that SheA is cytotoxic to human and mouse macrophages and is capable of inducing apoptosis, and so SheA has been implicated as a factor contributing to the pathogenicity of some *E. coli* strains (14).

The *sheA* gene is present in certain nonpathogenic *E. coli* strains (K-12), in the pathogenic Shiga toxin-producing *E. coli* strain O157:H7 (9), and in other enteropathogenic (enteroinvasive, enteroaggregative, and enterotoxigenic) *E. coli* strains (16), but its presence in *E. coli* strains associated with extraintestinal infections has not been well studied. We examined the distribution of *sheA* and *hlyA* genes among 540 *E. coli* strains isolated from human extraintestinal infections (isolated in the diagnostic laboratory at the Department of Medical Microbiology, University of Pécs, from urinary, genital, and respiratory tract infections and at the University Teaching Hospitals from

bloodstream and abdominal wound infections during 2001–2002); 110 strains isolated from the feces of different healthy subjects, aged 20 to 55 years, who had received no antibiotics in the previous 6 months or suffered urinary tract infections (including asymptomatic bacteriuria) or diarrheagenic disease within the previous 6 months; the 72-member ECOR collection (18); and 5 laboratory K-12 strains, namely, HB101 (22), MC4100 (24), J53 (7), DH5- $\alpha$  (27), and XL1-Blue (6).

The presence or absence of a hemolytic phenotype was determined for all strains following growth on Columbia blood agar plates containing 5% (wt/vol) defibrinated ox erythrocytes following incubation for 24 h at 37°C. Using the primer sets described in Table 2, hemolysin genes (*sheA*, *hlyA*, *ehx*, and *ehly*) were detected by PCR. Briefly, a single bacterial colony was suspended in 100  $\mu$ l sterile ultrapure water, and 1  $\mu$ l of this suspension was added to a 10- $\mu$ l reaction mixture [REDTaq [3 U *Taq* DNA polymerase, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP] and 40 pmol of each primer]; Sigma). The template was denatured for 2 min at 94°C followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 52°C (*sheA* and *ehx*), 58°C (*hlyA*), or 61°C (*ehly*) for 60 s, and extension at 72°C for 60 s. All products were analyzed by 1% agarose gel electrophoresis. The strains DH5- $\alpha$  (K-12) carrying pCFP60 (9), J96 (O4) (10), and EDL933 (O157:H7) (21) served as positive controls for the presence of *sheA*, *hlyA*, and phage-associated enterohemolysin (*ehly*) and plasmid-borne enterohemolysin (*ehx*) genes, respectively. The results are given in Table 3. No *E. coli* K-12 strains tested carried the *hlyA* gene. Nine members of the ECOR collection carried the *hlyA* gene, supporting results of a previous study (15), while 198 extraintestinal clinical isolates and only 8 normal fecal isolates carried *hlyA*. The *sheA* gene was identified in all tested K-12 strains, 55 of the ECOR strains, 241 of the extraintestinal clinical isolates, and 94 of the fecal isolates from healthy individuals (Table 3). Of the *hlyA*<sup>+</sup> strains from the ECOR group (nine strains) and the fecal isolates (eight strains), two strains from each group did not possess hemolytic activity. Of the 198 *hlyA*<sup>+</sup> strains from extraintestinal infections, 26 strains were nonhemolytic, as were

\* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, Medical School, University of Pécs, Pécs, Szegedi u. 12. H-7624, Hungary. Phone: 36 72 536001. Fax: 36 72 536253. E-mail: monika.kerenyi@aok.pte.hu.

TABLE 1. Comparison of HlyA and SheA<sup>a</sup>

Property	Characteristic of:	
	HlyA	SheA
Molecular weight	~110 kDa	~34 kDa
Pore formation	5–20 molecules, Ca <sup>2+</sup> dependent	1,000–2,000 molecules, Ca <sup>2+</sup> ; independent
Pore size	1.5–2.5 nm	2.5–3 nm
Secretion system	Type 1	Membrane vesicles?
Genes	Chromosome (human pathogens), plasmid (animal pathogens), requires HlyB, HlyD, and TolC	Chromosome
GC content	~40%	~40%
Target cells	RBC, granulocytes, monocytes, lymphocytes, endothelial cells, renal epithelial cells	RBC, macrophages, monocytes
Effects	Cytolytic, cytotoxic, cytokine production, leukotriene production, superoxide production	Cytolytic, cytotoxic

<sup>a</sup> RBC, red blood cells.

2 members of the ECOR group and 2 of the fecal isolates. We identified two strains from the extraintestinal infection group that lacked *hlyA*, *ehx* (enterohemolysin associated with some virulent *E. coli* strains) (3), and *ehly* but possessed *sheA* and were hemolytic.

The most common extraintestinal infections caused by *E. coli* are urinary tract infections. The  $\alpha$ -hemolysin is present in about 25 to 56% of isolated strains from urinary tract infections (17, 19), similar to our rate of 35%. The presence of the *sheA* gene in normal fecal strains is higher (85.4%;  $P < 0.001$ ) than in the isolates from urinary tract infection (47.1%). In our study, the occurrence of *hlyA* gene in normal fecal isolates was 7.3%, similar to that previously reported (11). Though it is accepted that the  $\alpha$ -hemolysin is an important virulence factor to the pathogenic profile of *E. coli*, the role played by the silent hemolysin SheA in disease is unknown.

This study focused on correlating the hemolytic phenotype of extraintestinal *E. coli* isolates with the presence of *hlyA* and *sheA* genes. The absence of a hemolytic phenotype in the presence of *hlyA* has been well characterized and can be due to defects in the *hlyBCD* genes or to defects in the transcriptional activator *rfaH* (2). We detected 174 hemolytic *E. coli* strains

out of our pool of 540 extraintestinal clinical isolates. Two of these isolates did not possess the *hlyA* gene and also lacked the other common *E. coli* hemolysin, enterohemolysin (*ehx*). However, these two strains did possess the *sheA* gene (Table 3). Oscarsson et al. reported that the bacteriophage-associated Ehly determinant does not encode enterohemolysins but causes the release of SheA or ClyA cytolytic (20). Although this *ehly* was not detected, it may be that *sheA* is responsible for the hemolytic phenotype for these two strains. The observed hemolytic phenotype was identical to the HlyA-mediated phenotype. Further studies are required to determine if this hemolytic phenotype is due to SheA and the role SheA plays in the pathogenic profile of these strains. The *sheA* gene was deleted in the 14 uropathogenic *E. coli* (UPEC) strains examined by Ludwig et al. in a recent study (16). Their 14 UPEC strains, including the well-known *hlyA*<sup>+</sup> J96 and 536 strains, were assayed for the presence of an *hlyA* fragment by Southern blot hybridization, but it was identified in only 10 of the 14 strains (28, 29). In our study, we found that 47.1% of the 486 UPEC strains contained the *sheA* gene (Table 3). The smaller number of UPEC strains in the first two studies may explain the differences in *sheA* gene carriage. The surprising finding of

TABLE 2. PCR primers for the identification of hemolysin genes

Gene and description	Product size (bp)	Primer name	Primer sequence
<i>hlyA</i> , from the 5' region of <i>hlyA</i> from J96	561	hlyA1 hlyA2	5'-GTC TGC AAA GCA ATC CGC TGC AAA TAA A-3' 5'-CTG TGT CCA CGA GTT GGT TGA TTA G-3'
<i>sheA</i> , from the 5' region of <i>sheA</i> from pCFP60	920	she1 she2	5'-GAG GCG AAT GAT TAT GAC TG-3' 5'-ACT TCA GGT ACC TCA AAG AG-3'
<i>ehly</i> , from the chromosome of O26 <i>E. coli</i> strain (phage coding)	808	ehly1 ehly2	5'-TCG CAA TCA CAT CAC AAC C-3' 5'-CCA GCA GTT CGT CAT CAT CTG AA-3'
<i>ehx</i> , from the plasmid pO157 carried by EDL933	1,518	ehx1 ehx2	5'-CAG TGA CGC ACA TAC AG-3' 5'-TCG GGA TAT ATA ATC ATC C-3'

TABLE 3. Distribution of the hemolysin genes *hlyA* and *sheA* and relevant phenotypes<sup>a</sup>

Strain (total no.)	No. of strains:		
	Carrying <i>hlyA</i>	Carrying <i>sheA</i>	Lacking <i>hlyA</i> and <i>sheA</i>
K-12 (5)	0	5 [0]	NA
ECOR (72)	9 [7]	0	NA
	0	55 [0]	NA
	0	0	8 [0]
Extraintestinal clinical isolates (540)			
Urinary tract infection (486)	169 [148]	0	NA
	0	229 [2]	NA
	0	0	88 [0]
Genital tract infection (8)	5 [5]	0	NA
	0	1 [0]	NA
	0	0	2 [0]
Respiratory tract infection (21)	12 [7]	0	NA
	0	1 [0]	NA
	0	0	8 [0]
Blood culture (19)	7 [7]	0	NA
	0	10 [0]	NA
	0	0	2 [0]
Abdominal wound (6)	5 [5]	0	NA
	0	0	1 [0]
Subtotal	198 [172]	241 [2]	101 [0]
Fecal isolates (110)	8 [6]	0	NA
	0	94 [0]	NA
	0	0	8 [0]
Total (727)	215 [185]	395 [2]	117 [0]

<sup>a</sup> Brackets indicate the number of isolates with a hemolytic phenotype. NA, not applicable.

this study was that out of the 727 *E. coli* strains tested, 610 possessed either the *hlyA* or the *sheA* gene and none carried both of them. The complete absence of *hlyA*<sup>+</sup> *sheA*<sup>+</sup> individuals within a sample of 540 individuals is sufficiently compelling to make additional statistical confirmation redundant (however, if we look at the subset of individuals with one of these two genes and calculate the expected proportion of *hlyA*<sup>+</sup>, *sheA*<sup>+</sup>, and *hlyA*<sup>+</sup> *sheA*<sup>+</sup> individuals that would exist by random assortment, the probability that the actual frequencies match this null hypothesis is vanishingly small [ $P < 0.0001$ ; chi square = 439]). The *sheA* gene in Shiga toxin-producing *E. coli* (enterohemorrhagic *E. coli*) can be found in *ehx* gene-positive isolates (9, 16), even though the plasmid-encoded *ehx* gene exhibits 60 to 65% sequence homology to *hlyA* (23). The reasons for the mutual exclusivity between *sheA* and *hlyA* in the chromosome found in this study are not understood. It might be that the complex mechanisms regulating expression of *hlyA* and *sheA* interfere with each other, posing a selective pressure against their coexistence in the same cell. Further studies will shed light on these questions.

We thank Gyula Mestyán and Anita Novák for their help in collecting the clinical isolates, Gabor Nagy for the *hlyA* primers, and Mike Speed (University of Liverpool) for his help with statistical analyses.

This work was supported by grant OTKA T037833 from the Hungarian Science Foundation and a Bolyai scholarship from the Hungarian Academy of Sciences.

## REFERENCES

- Atkins, A., N. R. Wyborn, A. J. Wallace, T. J. Stillman, L. K. Black, A. B. Fielding, M. Hisakado, P. J. Artymiuk, and J. Green. 2000. Structure-function relationships of a novel bacterial toxin, hemolysin E. The role of alpha G. *J. Biol. Chem.* **275**:41150–41155.
- Bailey, M. J., V. Koronakis, T. Schmolli, and C. Hughes. 1992. *Escherichia coli* HlyT protein, a transcriptional activator of haemolysin synthesis and secretion, is encoded by the *rfaH* (*sfjB*) locus required for expression of sex factor and lipopolysaccharide genes. *Mol. Microbiol.* **6**:1003–1012.
- Beutin, L., J. Prada, S. Zimmermann, R. Stephan, I. Orskov, and F. Orskov. 1988. Enterohemolysin, a new type of hemolysin produced by some strains of enteropathogenic *E. coli* (EPEC). *Zentbl. Bakteriol. Mikrobiol. Hyg. A* **267**:576–588.
- Bhakdi, S., S. Greulich, M. Muhly, B. Eberspacher, H. Becker, A. Thiele, and F. Hugo. 1989. Potent leukocidal action of *Escherichia coli* hemolysin mediated by permeabilization of target cell membranes. *J. Exp. Med.* **169**:737–754.
- Bhakdi, S., M. Muhly, S. Korom, and G. Schmidt. 1990. Effects of *Escherichia coli* hemolysin on human monocytes. Cytocidal action and stimulation of interleukin 1 release. *J. Clin. Investig.* **85**:1746–1753.
- Bullock, W. O., J. M. Fernandez, and J. M. Short. 1987. X11-Blue: a high efficiency plasmid transforming *recA* *Escherichia coli* strain with beta-galactosidase selection. *BioTechniques* **5**:376–378.
- Clowes, R., and D. Rowley. 1954. Some observations on linkage effects in genetic recombination in *Escherichia coli* K-12. *J. Gen. Microbiol.* **11**:250–260.
- del Castillo, F. J., S. C. Leal, F. Moreno, and I. del Castillo. 1997. The *Escherichia coli* K-12 *sheA* gene encodes a 34-kDa secreted haemolysin. *Mol. Microbiol.* **25**:107–115.
- del Castillo, F. J., F. Moreno, and I. del Castillo. 2000. Characterization of the genes encoding the SheA haemolysin in *Escherichia coli* O157:H7 and *Shigella flexneri* 2a. *Res. Microbiol.* **151**:229–230.
- Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow. 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. *Infect. Immun.* **33**:933–938.
- Johnson, J. R. 1991. Virulence factor in *Escherichia coli* urinary tract infection. *Clin. Microbiol. Rev.* **4**:80–128.
- Keane, W. F., R. Welch, G. Gekker, and P. K. Peterson. 1987. Mechanism of *Escherichia coli* alpha-hemolysin-induced injury to isolated renal tubular cells. *Am. J. Pathol.* **126**:350–357.
- Konig, B., A. Ludwig, W. Goebel, and W. Konig. 1994. Pore formation by the *Escherichia coli* alpha-hemolysin: role for mediator release from human inflammatory cells. *Infect. Immun.* **62**:4611–4617.
- Lai, X. H., I. Arencibia, A. Johansson, S. N. Wai, J. Oscarsson, S. Kalfas, K. G. Sundqvist, Y. Mizunoe, A. Sjostedt, and B. E. Uhlin. 2000. Cytocidal and apoptotic effects of the ClyA protein from *Escherichia coli* on primary and cultured monocytes and macrophages. *Infect. Immun.* **68**:4363–4367.
- Lai, X. H., S. Y. Wang, and B. E. Uhlin. 1999. Expression of cytotoxicity by potential pathogens in the standard *Escherichia coli* collection of reference (ECOR) strains. *Microbiology* **145**:3295–3303.
- Ludwig, A., C. von Rhein, S. Bauer, C. Hüttinger, and W. Goebel. 2004. Molecular analyses of cytolysin A (ClyA) in pathogenic *Escherichia coli* strains. *J. Bacteriol.* **186**:5311–5320.
- Minshew, B. H., J. Jorgensen, M. Swanstrum, G. A. Grootes-Reuvecamp, and S. Falkow. 1978. Some characteristics of *Escherichia coli* strains isolated from extraintestinal infections of humans. *J. Infect. Dis.* **137**:648–654.
- Ochman, H., and R. K. Selander. 1984. Standard reference strains of *Escherichia coli* from natural populations. *J. Bacteriol.* **157**:690–693.
- Opal, S. M., A. S. Cross, P. Gemski, and L. W. Lyhte. 1990. Aerobactin and alpha-hemolysin as virulence determinants in *Escherichia coli* isolated from human blood, urine, and stool. *J. Infect. Dis.* **161**:794–796.
- Oscarsson, J., M. Westermark, L. Beutin, and B. E. Uhlin. 2002. The bacteriophage-associated Ehly1 and Ehly2 determinants from *Escherichia coli* O26:H<sup>-</sup> strains do not encode enterohemolysins per se but cause release of the ClyA cytolysin. *Int. J. Med. Microbiol.* **291**:625–631.
- Perna, N. T., G. Plunkett III, V. Burland, B. Mau, J. D. Glasner, D. J. Rose, G. F. Mayhew, P. S. Evans, J. Gregor, H. A. Kirkpatrick, G. Posfai, J. Hackett, S. Klink, A. Boutin, Y. Shao, L. Miller, E. J. Grobeck, N. W. Davis, A. Lim, E. T. Dimalanta, K. D. Potamouis, J. Apodaca, T. S. Anantharaman, J. Lin, G. Yen, D. C. Schwartz, R. A. Welch, and F. R. Blattner. 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* **409**:529–533.
- Sambrook, J. E., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schmidt, H., L. Beutin, and H. Karch. 1995. Molecular analyses of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infect. Immun.* **63**:1055–1061.
- Silhavy, T. J., M. L. Berman, L. W. Enquist, and Cold Spring Harbor

- Laboratory. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. Welch, R. A., C. Forestier, A. Lobo, S. Pellett, W. Thomas, Jr., and G. Rowe. 1992. The synthesis and function of the *Escherichia coli* hemolysin and related RTX exotoxins. *FEMS Microbiol. Immunol* **5**:29–36.
26. Westermarck, M., J. Oscarsson, Y. Mizunoe, J. Urbonaviciene, and B. E. Uhlin. 2000. Silencing and activation of ClyA cytotoxin expression in *Escherichia coli*. *J. Bacteriol.* **182**:6347–6357.
27. Woodcock, D. M., P. J. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S. S. Smith, M. Z. Michael, and M. W. Graham. 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res.* **17**:3469–3478.
28. Zingler, G., M. Ott, G. Blum, U. Falkenhagen, G. Naumann, W. Sokolowska-Köhler, and J. Hacker. 1992. Clonal analysis of *Escherichia coli* serotype O6 from urinary tract infection. *Microb. Pathog.* **12**:299–311.
29. Zingler, G., G. Blum, U. Falkenhagen, I. Orskov, F. Orskov, J. Hacker, and M. Ott. 1993. Clonal differentiation of uropathogenic *Escherichia coli* isolates of serotype O6:K5 by fimbrial antigen typing and long-range mapping techniques. *Med. Microbiol. Immunol.* **182**:13–24.