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Hemolytic Activity in Enterotoxigenic and Non-Enterotoxigenic strains of *Escherichia coli*

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We screened 223 strains of *Escherichia coli* belonging to serotypes previously associated with the production of enterotoxin for hemolytic activity, using horse erythrocytes in liquid and in agar media. Thirty-eight were hemolytic. They belonged to nine different serotypes; most (65.8%) belonged to one serotype, O6: H−. Additionally, all 38 strains were specifically assayed for a filterable, heat-labile hemolytic activity previously associated with a hemolysin plasmid. A comparison of hemolytic activity and enterotoxicity showed that none of 32 strains hemolytic in both media was enterotoxigenic; 28 of the 32 expressed heat-labile hemolytic activity. Four of the six strains hemolytic in only one of the media were enterotoxigenic; none of these six expressed heat-labile hemolytic activity. Of 223 strains, 176 that were of human origin and isolated in the United States were further assayed for three traditionally plasmid-mediated characteristics: heat-labile enterotoxin, heat-stable enterotoxin, and colonization factors. The interrelationships of these characteristics, including hemolytic activity, may reflect varying degrees of plasmid compatibility.

Enterotoxigenic *Escherichia coli* (ETEC) have been implicated in sporadic and epidemic outbreaks of diarrhea in both infants and adults in many parts of the world. ETEC produce one or both of two plasmid-mediated enterotoxins (14, 30): a heat-stable enterotoxin (ST) and a heat-labile enterotoxin (LT). Possession of an enterotoxin plasmid (Ent), however, is not sufficient in itself to make ETEC pathogenic (27, 31). To delineate pathogenic mechanisms in ETEC, it is useful to study other traditionally plasmid-mediated virulence characteristics associated or compatible with Ent. Although each plasmid may be an independent unit of replication and transmission, the total plasmid "profile" or "complex" of a pathogenic organism has important implications for its ability to cause disease. Other plasmids often associated with Ent may also contribute to the disease potential of an organism in the presence or the absence of Ent (31).

One plasmid-mediated characteristic that may be associated with pathogenicity is a specifically defined hemolytic activity. At least four types of hemolysin (15) may be produced by *E. coli*, each giving rise to a clear zone of hemolysis (beta hemolysis) on blood agar. In this investigation, all hemolytic activities of *E. coli* will be characterized as either type 1 (HLY1) or type 2 (HLY2). HLY1 activity is produced by an extracellular, filterable, heat-labile macromolecule (19, 20, 24, 28, 29), a portion of which is protein (28, 32, 35). The ability to produce HLY1 is mediated by a plasmid (13, 22), termed Hly. HLY2 denotes any and all types of hemolytic activity other than HLY1. Only HLY1 has been shown to be plasmid mediated.

As part of a broad project dealing with the phenotypic and genotypic characterization of ETEC, we used both screening and specific assays to determine the presence of HLY1 in 223 strains of *E. coli* belonging to serotypes previously associated with enterotoxin production. To delineate possible associations among different plasmid-mediated characteristics, we correlated hemolytic activity with data from assays for ST, LT, and colonization factors (CFs).

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MATERIALS AND METHODS

All strains of *E. coli* used in this investigation were received and serotyped (7) by the Center for Disease Control between 1960 and 1978 and were stocked in paraffin-corked blood agar base slants at room temperature. These strains were recently selected from the culture collection of the Enteric Section, Bureau of Laboratories, Center for Disease Control, for enterotoxin, hemolysin, and CF testing. All stock cultures were rejuvenated by subculturing on tryptic soy agar

plates before transfer and maintenance on blood agar base slants at room temperature. A total of 223 strains of *E. coli* were screened for hemolytic activity. The strains belonged to serotypes twice reported to contain ETEC. Of these 223 strains, 176 were of human origin and were isolated in the United States; these were also tested for ST and LT production and for CFs.

Screening tests for hemolytic activity were done in liquid medium and in agar plates according to the basic procedures of Cooke (1) and Short and Kurtz (28). Defibrinated horse blood (Granite Diagnostics) was centrifuged at 500 × *g* for 20 min, the supernatant was removed, and the erythrocytes were washed four times in tris(hydroxymethyl)aminomethane-buffered saline [0.01 M tris(hydroxymethyl)aminomethane-0.135 M NaCl, pH 7.4] and centrifuged at 500 × *g* for 10 min after each wash.

The liquid medium consisted of 15-ml volumes of peptone-saline (1% peptone [Difco Laboratories]-0.85% NaCl, pH 7.4) containing 2% (vol/vol) washed horse erythrocytes in sterile, round, glass scintillation vials. Vials were inoculated with *E. coli* test strains, taken from fresh, overnight cultures on tryptic soy agar plates, and incubated for 48 h at 36 ± 1°C. Visual readings for hemolysis were taken after 24 h of incubation. The vials were then shaken, and final readings were taken after an additional 24 h.

For testing in plates containing 5% horse erythrocyte agar, the erythrocytes were washed as for the liquid medium. Washed, packed erythrocytes (30 ml) were added to 570 ml of sterile, cooling blood agar base (Difco) before 20 ml of erythrocyte agar was poured into plastic petri plates (100 by 15 mm). Six *E. coli* test strains per plate were stab inoculated. Plates were incubated at 36 ± 1°C and visually read for a clear zone of hemolysis surrounding the stab at 8 and 24 h after inoculation.

The specific assay for HLY1 followed the basic procedures of Smith (29), Short and Kurtz (28), and Kurtz and Short (18). Strains that were positive for hemolytic activity, on one or both screening media, were tested specifically for HLY1. These strains were inoculated into duplicate screw-capped tubes (16 by 100 mm) containing 6 ml of alkaline extract broth, pH 7.4 (18), and incubated for 18 and 42 h at 37 ± 1°C in a roller drum (5 rpm). After incubation, the broth cultures were centrifuged (3,500 × *g*, 30 min), the supernatants were removed by pipette (not filtered), and 1 ml from each supernatant was put into two test tubes. One drop of 50-mg/ml sodium azide was put in each tube. One tube was heated at 98°C (steam bath) for 1 h; the second tube was held at room temperature. One milliliter of 2% washed horse erythrocytes in tris(hydroxymethyl)aminomethane-buffered saline with 20 mM calcium chloride (28) was added to both the heated and unheated tubes. These were then incubated for 2 h at 37°C in a water bath. After incubation, both tubes were centrifuged (500 × *g*, 10 min), and the supernatants were transferred by Pasteur pipette and diluted 1:3 in water; the diluted filtrates were read for hemoglobin content at 550 nm in a spectrophotometer (Bausch and Lomb, Inc., Spectronic 20). An absorbance of ≥0.13 was considered positive for HLY1. All negative tests had an absorbance of ≤0.08.

The assays for ST, LT, and CFs have been described in detail elsewhere (4). The assays for ST and LT consisted of the infant mouse assay (3, 12) and the Y1 adrenal cell assay (6, 26), respectively. The assays for CFs consisted of hemagglutination tests (8–10, 23), with human and bovine erythrocytes, and a microprecipitation test (C. F. Deneke, G. M. Thorne, and S. L. Gorbach, Abstr. 15th Jt. Conf. Cholera, U.S.-Japan Coop. Med. Sci. Prog. 1979, p. 35–36), with specific antipili sera (5), supplied by Grace M. Thorne, New England Medical Center, Boston, Mass.

RESULTS

The origins of the 223 strains of *E. coli* tested for hemolytic activity are listed in Table 1. Of the strains from blood, urine, and miscellaneous wounds, from 35 to 50% were hemolytic, but few strains from stools were hemolytic. Hemolytic strains belonged to nine different serotypes (Table 2), but one serotype, O6:H-, contained most (65.8%) of the hemolytic strains. The 38 strains found to be hemolytic in one or both of the screening media and the spectrophotometric readings (in absorbance) resulting from the specific assays for HLY1 are shown in Table 2.

Of the 223 strains tested, 184 were nonhemolytic in either screening medium. Of the 38 hemolytic strains listed in Table 2, 28 expressed HLY1, and all 28 were positive in both screening media; 10 of the hemolytic strains expressed HLY2, and 6 of these 10 were hemolytic in only one screening medium (3 in liquid and 3 in agar).

Hemolytic activity and enterotoxigenicity are compared in Table 3. None of the 32 strains, hemolytic in both screening media, was enterotoxigenic; 28 of these 32 expressed HLY1. Four of the six strains found to be hemolytic on only one of the two screening media and expressing HLY2 only were enterotoxigenic. These four strains were: (i) O15:H-, LT; (ii) O20:H-, LT and ST; (iii) O149:H7, ST; and (iv) O128:H7, ST.

A total of 126 *E. coli* strains belonging to ETEC serotypes were phenotypically characterized by ST, LT, HLY1, and CFs and yielded the data in Table 4. Strains were found with only 10 of a possible 16 combinations or patterns of these four plasmid-mediated characteristics. Of these 176 strains, 150 (85%) contained at least one

TABLE 1. *Hemolytic E. coli listed by origin*

Origin	No. hemolytic/no. tested	%
Stool	8/110	7.3
Blood	7/14	50.0
Urine	7/20	35.0
Miscellaneous wounds	8/23	34.8
Unknown	8/56	14.3

TABLE 2. *Thirty-eight strains of E. coli found hemolytic by testing in liquid peptone-saline medium, in erythrocyte agar plates, and in filtrates of alkaline extract broth*

Strain	Serotype	Time (h) until positive for hemolysis		Absorbance of filtrates after treatment				Type of hemolytic activity[b]
				18 h		42 h		
		Liquid medium	Agar plates	Unheated	Heated[a]	Unheated	Heated[a]	
2154-71	O6:H-	24	8	0.15	0.00	0.41	0.00	HLY1
3927-71	O6:H-	24	8	0.46	0.04			HLY1
4111-71	O6:H-	24	8	0.47	0.05			HLY1
4625-71	O6:H-	24	8	0.46	0.02			HLY1
4966-71	O6:H-	24	8	0.19	0.00	0.34	0.00	HLY1
0042-72	O6:H-	24	8	0.47	0.04			HLY1
5769-72	O6:H-	24	8	0.42	0.02			HLY1
1223-74	O6:H-	24	8	0.44	0.02			HLY1
1765-74	O6:H-	24	8	0.43	0.02			HLY1
2155-74	O6:H-	24	8	0.07	0.01	0.41	0.00	HLY1
2156-74	O6:H-	24	8	0.10	0.00	0.38	0.00	HLY1
3903-74	O6:H-	24	8	0.42	0.04			HLY1
0048-75	O6:H-	24	8	0.56	0.07			HLY1
0337-75	O6:H-	24	8	0.52	0.05			HLY1
0840-75	O6:H-	24	8	0.40	0.02			HLY1
0938-76	O6:H-	24	8	0.45	0.04			HLY1
0403-77	O6:H-	24	8	0.40	0.02			HLY1
0405-77	O6:H-	24	8	0.46	0.04			HLY1
0406-77	O6:H-	24	8	0.42	0.00			HLY1
0549-77	O6:H-	24	8	0.12	0.02	0.16	0.02	HLY1
1326-77	O6:H-	24	8	0.12	0.01	0.14	0.02	HLY1
2614-77	O6:H-	24	8	0.16	0.02	0.19	0.03	HLY1
3052-77	O6:H-	24	8	0.39	0.04			HLY1
2000-69	O15:H-	24	8	0.13	0.02	0.12	0.00	HLY1
3383-72	O148:H2	48	8	0.02	0.00	0.15	0.03	HLY1
0814-73	O25:H-	48	8	0.00	0.02	0.17	0.01	HLY1
1125-74-1	O6:H16	48	24	0.00	0.02	0.17	0.01	HLY1
0856-76	O20:H-	24	8	0.56	0.03			HLY1
1125-74-2	O6:H16	48	24	0.00	0.02	0.06	0.02	HLY2
2614-75	O20:H-	24	8	0.01	0.01	0.06	0.03	HLY2
2615-75	O20:H-	24	8	0.02	0.00	0.03	0.01	HLY2
3216-76	O6:H-	24	8	0.00	0.00	0.00	0.00	HLY2
1519-70	O15:H-		24	0.00	0.02	0.02	0.01	HLY2
2694-73	O149:H7		24	0.00	0.00	0.08	0.03	HLY2
0404-75	O6:H-		24	0.01	0.01	0.03	0.02	HLY2
5605-70	O20:H-	24		0.02	0.02	0.01	0.05	HLY2
5619-70	O8:H9	48		0.00	0.04	0.01	0.03	HLY2
2372-77	O128:H7	24		0.00	0.00	0.02	0.01	HLY2

[a] Filtrates heated at 98°C (steam bath) for 60 min.

[b] HLY1, Type 1 hemolytic activity (unheated filtrate from alkaline extract broth possessed hemolytic activity; no hemolytic activity remained after filtrate was heated at 98°C [steam bath] for 60 min); HLY2, type 2 hemolytic activity (hemolytic activity only in liquid or on agar screening medium or both; no hemolytic activity detected in unheated filtrates of alkaline extract broth).

plasmid-mediated characteristic. These are listed by serotype in Table 5.

DISCUSSION

A review of the literature (1, 2, 29, 33, 34) has shown that the percentage of *E. coli* reported to be hemolytic is dependent on many variables. Percentages vary with animal species, site of isolation, assay method, and type of hemolysin. Our finding (Table 1) that hemolytic strains were isolated more often from blood, urine, and miscellaneous wounds than from stools agrees

TABLE 3. *Enterotoxin production in hemolytic E. coli*

Hemolytic in:		No. hemolytic	No. enterotoxigenic	Type of hemolytic activity		% Enterotoxigenic
Agar	Liquid			HLY1[a]	HLY2[b]	
+	+	32	0	28	4	0
+	−	3	2	0	3	67
−	+	3	2	0	3	67

[a] Alkaline extract broth filtrate possessed hemolytic activity; no activity after filtrate was heated 98°C for 60 min.

[b] Hemolytic activity only in liquid peptone or blood agar or both; no hemolytic activity detected in filtrates.

TABLE 4. *Pattern combinations of plasmid-mediated characteristics in 176 strains of E. coli*

Phenotypic pattern				Strains found
ST	LT	CF	HLY[a]	0
ST	LT	CF	—	6
ST	LT	—	HLY	0
ST	—	CF	HLY	0
—	LT	CF	HLY	0
ST	LT	—	—	9
ST	—	CF	—	10
ST	—	—	HLY	0
—	LT	CF	—	10
—	LT	—	HLY	0
—	—	CF	HLY	11
ST	—	—	—	25
—	LT	—	—	7
—	—	CF	—	42
—	—	—	HLY	14
—	—	—	—	42

[a] HLY, Type 1 hemolytic activity.

with earlier reports, in which hemolytic strains occurred more commonly in specimens from patients with peritonitis, appendicitis, and urinary tract infections than in fecal specimens from ill or well patients (1, 11, 35). Hemolysin activity may reflect a potent mechanism of pathogenesis at extraintestinal sites (2, 21).

The data presented in Tables 2 and 3 show that both the liquid and the agar screening media detected most of the hemolytic strains. None of 32 strains that were hemolytic on both screening media was enterotoxigenic, but 4 of 6 strains that were hemolytic in only one screening medium were enterotoxigenic ($\chi^2 > 17$, $P < 0.0001$). Therefore, an efficient screening procedure to correlate hemolytic activity and enterotoxin would require the use of both media. The data of Cooke (1) led her to a similar conclusion.

Table 3 shows that serotype O6:H- is the principal ETEC serotype expressing HLY1. Although no enterotoxigenic strains were found in this study, this *E. coli* serotype has been associated with ulcerative colitis (1) and may be especially pathogenic for the urinary tract (25). None of 27 O6:H- strains tested was found positive for ST or LT; however, a number of O6:H- strains expressing HLY1 apparently contain an intestinal CF (manuscript in preparation). Although *E. coli* hemolytic activity in general may be associated with pathogenicity at extraintestinal sites (2, 21), Smith and Linggood (31) found an association of hemolysin plasmids (Hly) with porcine ETEC. There was no evidence that the Hly plasmid participated in the diarrheal disease process (31). Minshew et al. (21) have suggested that there may be a fundamental difference in the plasmid-mediated hemolysin and the hemolysin produced by extraintestinal isolates.

The combinations of four traditionally plasmid-mediated characteristics of *E. coli* are presented as probable patterns in Table 5. The ETEC serotype strains in this study harbored only 10 of a possible 16 patterns. A comparison of these 10 patterns with the 6 not found show that all 6 of the missing patterns involve HLY1 in combination with LT or ST or both.

Investigators have recognized that closely related (i.e., incompatible) plasmids cannot replicate or exist in the same host cell at the same time. Relationships are such that the presence of one plasmid(s) prevents the cell from acquiring or maintaining a second, similar plasmid. For example, the acquisition of certain antibiotic resistance plasmids has been shown to result in the loss of colicin plasmids (16, 17); this may hold true for Ent and Hly plasmids in human ETEC serotypes.

Although 4 of 10 strains that expressed HLY2 were enterotoxigenic, no data are available to suggest that HLY2 activity is plasmid mediated. No method of cell disruption has been found that will liberate HLY2 into a liquid medium (27, 28). In addition, Short and Kurtz (28) interpreted the inhibition of HLY2 (beta) by several compounds that inhibit nucleic acid synthesis, protein synthesis, or aerobic metabolism to mean that beta-HLY2 is dependent on the metabolic status of the organism. This might explain strains expressing HLY2 in only one screening medium and others in both.

Phenotypic patterns involving ST, LT, and CF agree with the data of Evans and Evans (8), which reported the ST-LT-CF, ST-CF, and LT-CF patterns. In addition, we found 48 strains of *E. coli* (Table 5), belonging to a number of different serotypes, that appear to possess a CF in the absence of ST and LT. This latter observation is important in the light of an earlier

TABLE 5. *Strains of E. coli listed by phenotypic pattern and serotype*[a]

Serotype	Phenotype pattern[b]									No. of strains phenotyped
	ST LT CF —	— LT CF —	— — CF HLY	ST — CF —	— LT — —	ST — — —	ST LT — —	— — CF —	— — — HLY	
O128:H7				4		2				9
O128:H12								3		9
O15:H-	1	1	3		1		1	16	1	28
O15:H11	4	2		1	1		3	4		17
O20:H-					1		1	1		7
O25:H-			1		3		2			9
O27:H20				1		5				10
O6:H-			4					2	13	22
O6:H16			1	2		2		1		6
O78:H11		4			1					6
O78:H12	1						1		2	6
O78:H-		1						6		11
O8:H9								6		10

[a] Only those serotypes for which six or more strains were available are included in the table.
[b] HLY, Type 1 hemolytic activity.

study (31) in which mild diarrhea developed in pigs that received CF^+ (i.e., $K88^+$) Ent^- Hly^- *E. coli*. In pigs, at least, certain serotypes of *E. coli* possessing a CF in the absence of ST and LT Ent plasmids are capable of inducing diarrhea. It is also interesting that 59 of 110 *E. coli* strains possessing traditionally plasmid-mediated characteristics possessed no detectable H antigens (Table 5).

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ADDENDUM

Preliminary genotypic data from our laboratory show that of 10 *E. coli* strains expressing HLY1, 9 possess a plasmid with a molecular weight of $\geq 60 \times 10^6$. Seven strains expressing HLY2 all possess a plasmid between 20×10^6 and 56×10^6. The HLY2-associated plasmids may arise from deletions in HLY1 plasmids which affect both the range of hemolytic activity (e.g., HLY2 is less active than HLY1) and the incompatibility category.

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