Genetic Conservation of *hlyA* Determinants and Serological Conservation of HlyA: Basis for Developing a Broadly Cross-Reactive Subunit *Escherichia coli* Alpha-Hemolysin Vaccine

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The HlyA determinant among *Escherichia coli* isolates from patients with symptomatic urinary tract infection was compared in this report with a prototype HlyA encoded by pSF4000 by DNA-DNA hybridization tests with 20-base synthetic oligonucleotides and monoclonal antibody binding and neutralization assays. Hybridization results demonstrated that 349 (98%) of 357 definitive reactions among 54 hemolytic strains shared homology with seven DNA probes spanning many HlyA regions corresponding to residues (R) 41 to 47, 55 to 61, 248 to 254, 306 to 312, 336 to 343, 402 to 408, and 929 to 935. Genetic divergence was identified by lack of hybridization signals among 17 to 76% of the hemolytic strains within the distal portion of a predicted hydrophobic region corresponding to R491 to 319 and within a predicted hydrophilic region corresponding to R491 to 497 and R532 to 538. Serological studies demonstrated that 26 (81%) culture supernatants of 32 hemolytic strains were bound by all 12 monoclonal anti-HlyA antibodies. Among five of six remaining strains, the culture supernatants were bound by 3 to 11 monoclonal antibody preparations. There was only one hemolytic culture supernatant that failed to be bound by any monoclonal antibody, although the strain hybridized with nine hemolysin DNA probes. In addition, hemolytic activity of all 24 different culture supernatants tested was reduced by at least twofold by one monoclonal antibody specific for R2-161. These data extend and support previous views that the HlyA determinant is conserved among *E. coli* strains and suggest that a broadly cross-reactive HlyA subunit vaccine can be developed.

Escherichia coli alpha-hemolysin (HlyA) is representative of a gram-negative bacterial membrane pore-forming cytolytic toxin with glycine-rich repeats (viz., a member of the RTX gene toxin family [37]) (6). Biologically active HlyA is an important virulence determinant for extraintestinal *E. coli* disease (6, 8, 16, 35, 39, 40, 42, 43) and contributes to the severity of experimental pyelonephritis in rodents (4, 8, 16, 35). In addition, sublytic concentrations of HlyA have been demonstrated in vitro to inhibit binding of chemotactic factors to neutrophils (44). Diminished host cellular responses by neutrophils and macrophages to invading hemolytic bacteria in vivo might allow for greater tissue injury or damage and systemic septic effects. Strategies to modulate the noxious consequences of HlyA in clinical conditions warrant consideration. Preliminary evidence demonstrates the efficacy of a denatured HlyA vaccine that is encoded by pSF4000 to modulate the severity of experimental murine pyelonephritis (35). Vaccine recipients of denatured HlyA had lower death rates and less renal damage after intravesicular challenge with a homologous hemolysin-producing wild-type strain than similarly challenged mice that were immunized previously with either lipopolysaccharide homologous to the challenge strain or saline (35). Native HlyA is highly immunogenic (6, 7, 19, 21, 35, 37, 38), and anti-HlyA neutralizing antibodies have been reported after natural infections and under experimental conditions (7, 21, 37, 38).

These data suggest that certain antibody responses to HlyA might be protective.

Many studies suggest conservation of *E. coli* HlyA at genetic, protein, and serological levels. *hlyA* gene determinants located on the chromosome or a plasmid appear to be highly conserved (4, 10, 14, 17, 42, 43, 45). DNA-DNA and RNA-DNA hybridization studies employing endonuclease-digested fragments of >500 bases indicate similar *hlyA* gene determinants under stringent conditions among chromosomal and plasmid-encoded alpha-hemolytic strains, respectively (1, 6, 36, 42, 43). Restriction endonuclease fragment analysis with a limited number of enzymes within the hemolysin region of chromosomal and plasmid determinants indicates that the majority of restriction sites are shared among different hemolysin operons (43). This elegant study demonstrates also that there were different restriction site patterns among these operons as well, despite the use of only a few enzymes (43). Alpha-hemolytic *E. coli* isolates secrete a 107- to 120-kDa protein exhibiting hemolytic activity (6, 7, 10, 18, 19, 30, 42). Antibodies elicited after natural infection by alpha-hemolytic strains and after immunization with the purified putative HlyA in animals bind and precipitate the HlyA produced by homologous and heterologous strains (7, 8, 35, 38). Murine anti-HlyA monoclonal antibodies (MAbs) have also been raised, and they react against most HlyA preparations (19, 21, 37). However, there is an example in which one anti-HlyA MAb did not react to all HlyA preparations (19).

The extent and location of diversity within HlyA are not known. This information is obviously important for rational

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TABLE 1. Synthetic *hlyA* oligonucleotide probes corresponding to the *hlyA* gene in pSF4000

Probe	Location of probe nucleotide sequences (bases) and amino acid residues (R) for HlyA ^a	Oligonucleotide sequence (5'→3') and amino acid sequence (NH ₂ → COOH)								Temp (°C) of hybridization	Comments ^b
HA 122	Bases 122 → 141	AA	ACC	CGC	AAT	GCG	GGA	AAC		57	Predicted immunogenic epitope and possibly a protective epitope
	R41 → 47	Q ^{°°}	T	R	N	A	G	N			
HA 163	Bases 163 → 182	AAA	GAT	TAT	AAA	GGG	CAG	GG		51	Predicted immunogenic epitope and possibly a protective epitope
	R55 → 61	K	D	Y	K	G	Q	G [°]			
HA 743	Bases 743 → 762	TA	TCT	GCG	ATT	TCA	GCA	AGT		51	Predicted hydrophobic transmembrane segment and nonimmunogenic
	R248 → 254	L [°]	S	A	I	S	A	S			
HA 917	Bases 917 → 936	CC	GGT	TTA	ATT	GCT	TCT	GTT		51	Predicted hydrophobic transmembrane segment and nonimmunogenic
	R306 → 312	A [°]	G	L	I	A	S	V			
HA 937	Bases 937 → 956	GTG	ACA	CTG	GCA	ATT	AGT	CC		55	Predicted hydrophobic transmembrane segment and nonimmunogenic
	R313 → 319	V	T	L	A	I	S	P [°]			
HA 1006	Bases 1006 → 1025	GAG	GAG	TAT	TCA	CAA	GGA	TT		51	Predicted hydrophilic segment between two hydrophobic transmembrane portions of hemolysin
	R336 → 342	E	E	Y	S	Q	R	F [°]			
HA 1204	Bases 1204 → 1223	ACG	GGG	ATA	ATT	TCA	GGC	AT		53	Predicted hydrophobic transmembrane segment and nonimmunogenic
	R402 → 408	T	G	I	F	S	G	I [°]			
HA 1471	Bases 1471 → 1490	AGA	AAT	GGA	GAC	AAA	ACA	CT		49	Predicted immunogenic epitope
	R491 → 497	R	N	G	D	K	T	L [°]			
HA 1594	Bases 1594 → 1613	AC	CTT	TCT	GAC	AGC	AAA	TCT		51	Predicted immunogenic epitope
	R532 → 538	D [°]	L	S	D	S	K	S			
HA 2786	Bases 2796 → 2905	TT	TTT	GAT	AAA	GAC	GGC	AGG		51	Predicted immunogenic epitope
	R929 → 935	I [°]	F	D	K	D	G	R			

^a Designations refer to the probe location of corresponding base and amino acid residue sequences within HlyA. The base numbers reflect the corresponding sequences in HlyA and not their positions in the operon (14).

^b See references (14, 21, 37) for rationale for these comments.

^c The ° superscript symbol indicates that only two of three bases per codon are included in the probe. The proximal or distal two bases in the sequence are included for partial codon usage in the probe.

vaccine development. The purpose of this study is to further assess at finer levels of resolution the genetic and antigenic diversity of HlyA. In this report, we compare the DNA hybridization pattern of hemolytic clinical isolates with a prototype *hlyA* by using 10 different synthetic oligonucleotides corresponding to predicted HlyA immunogenic epitopes or structural features crucial for function. These DNA-DNA hybridization assays provide a basis to screen clinical isolates for fine-structure homology with a prototype *hlyA*. In addition, a battery of 12 murine MAbs elicited to the expressed prototype *hlyA* was used to assess the antigenic diversity of HlyA within certain crucial structure-function regions of the polypeptide among hemolytic clinical isolates.

MATERIALS AND METHODS

Bacteria. A total of 72 wild-type *E. coli* strains was studied. Thirty-six isolates were obtained from individuals with radiologically documented, anatomically normal urinary tracts and clinically rigorous specific types of urinary tract infection (36). Nineteen were from women with acute pyelonephritis, 10 were from women with acute cystitis, and 7 were from men with acute urethritis. The remaining 36 urinary isolates were from adults that had radiologically undetermined urinary tract anatomy and indeterminant clinical syndromes of symptomatic urinary tract infection. Control *E. coli* strains (21) included strain J96, recombinant strain GEJ800, and strain HB101. Strains J96 and GEJ800 harbored the same chromosomally encoded hemolysin operon contained in pSF4000 (42). Strain HB101 was a nonhemolytic laboratory K-12 isolate. The bacterial isolates were stored at -70°C in Luria broth and glycerol (1:1) until needed.

Synthetic oligonucleotide probes. Ten different 20-base

oligonucleotides corresponding to DNA segments of the *hlyA* gene from pSF4000 (14) (hemolysin A [HA] probes) were synthesized (Table 1). The selection of oligonucleotides for synthesis was based on one of three criteria. Probes HA 122, HA 163, HA 1471, HA 1594, and HA 2786 correspond to sequences within potential antigenic or immunogenic epitopes of HlyA as predicted by the algorithm of Jameson and Wolf (20). Probes HA 743, HA 917, HA 937, and HA 1204 were selected because they correspond to linear amino acid sequences within the two predicted major hydrophobic segments of HlyA (14). Probe 6 corresponds to sequences within the predicted hydrophilic portion between the two hydrophobic segments (14). The oligonucleotides were synthesized by cyanoethyl chemistry on an Applied Biosystems 380A DNA Synthesizer (Foster City, Calif.) and purified on a reverse-phase high-performance liquid chromatography column. One hundred fifty nanograms of purified oligonucleotides were 5' end labelled with [γ -³²P]ATP (5,000 Ci/mmol) by using T4 polynucleotide kinase (Bethesda Research Laboratories, Bethesda, Md.) (31). The labelled oligonucleotides were eluted from Sep-Pak columns (Millipore Products, Bedford, Mass.) with acetonitrile in water (1:1), dried in vacuo, and resuspended in 30 μ l of 10 mM Tris-1 mM EDTA (pH 7.5). The specific radioactivity of the probes was approximately 10⁹ cpm/ μ g.

Colony hybridization. The bacterial colonies were replica plated onto nitrocellulose filters and screened for homology with the pSF4000 *hlyA* probes by in situ hybridization (3). Bacterial DNA was fixed to nitrocellulose by a standard technique (31), and debris was removed from the filters by three washes in prehybridization solution (3). The filters were then incubated for 8 h at 65°C in fresh prehybridization solution and then incubated in hybridization solution (3) with

5×10^6 to 10×10^6 cpm of radiolabelled oligonucleotide probe for 36 h at 5°C below the calculated temperature of dissociation (41) (Table 1). The filters were then washed three times at room temperature and washed finally for 5 min at the temperature of hybridization (41) for each probe. The filters were dried and exposed to X-ray film at -70°C for 2 days. Each filter included positive controls (e.g., strain J96 and strain GEJ800) and negative controls (i.e., strain HB101 and Trypticase soy broth [TSB]). Hybridizations for each probe were repeated at least two times to assess consistency of results. Positive reactions were read as dark or gray, like the positive controls; negative reactions were blank or faint, scattered gray, like the negative controls. If the reaction was not definitively positive or negative because of higher background noise, the test was designated as indeterminate. Additional studies were performed on a selected number of strains to assess the reliability of hybridization results by using the probes under less and more stringent conditions of hybridization. These studies utilized a different in situ hybridization procedure, as described previously (12).

Hemolysin assays. The hemolytic capacity of the bacterial strains was assessed both on agar plates and by a microtiter liquid assay. Alpha-hemolysis of sheep and human erythrocytes in agar by each *E. coli* strain was assessed after incubation for 18 h at 37°C. Strains were determined to be nonhemolytic by plate tests if alpha-hemolysis was not evident by a third serial passage. In addition, a modified liquid assay protocol described originally by Smith was used to assess the hemolytic activity of the strains (9). In brief, the culture supernatant centrifuged at $18,000 \times g$ and the filtrate from passage through a 0.2- μ m-pore-size polysulfone membrane (Gelman Sciences, Ann Arbor, Mich.) from a 5-h growth of bacteria in TSB were serially diluted twofold in CaCl_2 hemolysis assay buffer. These preparations were assessed for hemolytic activity on human erythrocytes. Each experiment was done in duplicate, and hemolytic titers were expressed as the reciprocal of the highest dilution at which hemolysis was observed. Controls included culture supernatants of strains J96 and GEJ800 (positive controls) and strain HB101 (negative control), heat-treated culture supernatants (100°C for 5 min) from each of the clinical isolates, and buffer alone.

Binding of murine anti-hemolysin MAbs to culture supernatants of different strains. Murine anti-GEJ800 HlyA MAbs preparations have been previously characterized (21). The binding of 12 different anti-GEJ800 HlyA MAbs to culture supernatants of 49 different *E. coli* strains was assessed by immunoblotting. In brief, bacteria were inoculated into TSB and incubated overnight at 37°C. One milliliter of this culture was inoculated into 30 ml of fresh TSB and incubated usually for 3 to 4 h at 37°C with constant shaking to maximize hemolysin production. When the optical density (wavelength, 600 nm) of the culture was 0.8, the bacteria and debris were pelleted by centrifugation ($18,000 \times g$) at 4°C. The culture supernatants were filtered through 0.2- μ m-pore-size polysulfone membranes (Gelman). Aliquots of each culture filtrate were assessed for hemolytic activity by liquid assay (see above), and 500 μ l of each culture filtrate was replica dotted onto 0.45- μ m-pore-size nitrocellulose membranes by using a 96-well Bio-Dot template (Bio-Rad, Richmond, Calif.). After washing the dots via suctioning through the template ports with 0.1 M phosphate-buffered saline (PBS; pH 7.4), supplemented with 0.02% Brij 35 (Sigma Chemical Co., St. Louis, Mo.), the filters were removed from the template and immersed in PBS supplemented with 0.05% gelatin for 1 h with gentle shaking. Blocked filters

were then washed three times with PBS-Brij and incubated for 1 h at room temperature with 1 of the 12 different MAB preparations diluted 1:1,000 in PBS (approximately 0.1 μ g of immunoglobulin G per ml). The filters were then washed three times with PBS-Brij and followed by incubation for 1 h at 37°C in horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Sigma) diluted 1:1,000 in PBS-Brij. The filters were washed three times in PBS-Brij, and binding antibody was detected by adding 4-chloro-1-naphthol and H_2O_2 . The reaction was stopped after 20 min by addition of 2 M H_2SO_4 and then immediately by washing with distilled water. The filters were air dried, photographed, and saved for permanent records. A positive reaction was represented by the color reaction observed for the culture supernatant from strain J96. The diluted J96 culture supernatant (positive control) contained sufficient hemolysin to produce a hemolytic reciprocal titer of 4. Negative binding was indicated by the absence of any immunoreactivity in a dot, represented by the blank observed for culture supernatant from strain HB101 (negative control). Strains that exhibited a negative reaction despite the presence of hemolytic activity in culture supernatants were cultured for an additional 2 h, and the culture supernatant was assessed again for antibody binding to ensure that sufficient amounts of hemolysin had been secreted.

Hemolysin neutralization assay. The capacity of murine anti-GEJ800 HlyA hemolysin MAbs 132 and 559 (21) (0.1 μ g of immunoglobulin G each) to inhibit hemolysis of 5-h TSB culture supernatants (50- μ l aliquots) was assessed for 24 different hemolytic *E. coli* strains by a standard hemolysin neutralization assay (7). Each culture supernatant of these strains was bound by MAbs 132 and 559 in the immunoblot assays as described above. Each experiment was done in triplicate, and the hemolysis inhibition range was expressed as the x -fold reduction in hemolysis from two separate experiments compared with that of a lysis buffer control.

RESULTS

Hemolytic status of the clinical isolates. Fifty-four (75%) of the 72 clinical strains exhibited alpha-hemolytic activity as determined by plate analysis. The zone of alpha-hemolysis was greater when human erythrocytes were used compared with when sheep erythrocytes were used. Fifty-three of 54 alpha-hemolytic strains on plates had clear zones of hemolysis after the first passage; 3 strains had lysis just underneath the surface colony, and 50 strains had lysis underneath and to various extents around the colony. The remaining hemolytic strain exhibited a scant zone of hemolysis underneath the surface colony after the second passage on plates, and the extent of hemolysis did not increase after a subsequent passage on agar. The culture supernatant preparations for 51 (94%) of 54 hemolytic strains on plates lysed human erythrocytes in a liquid assay. The range of hemolytic reciprocal titers was 2 to 64, with an average of 35.3. There were no differences in titers between filtered and nonfiltered culture supernatants from each strain and the extent of hemolysis for a particular strain from one passage to the next. Three of the 54 hemolytic strains on plates did not produce hemolysis in the liquid assay despite repeated testing. None of 18 nonhemolytic strains on plates or negative controls produced hemolysis in the liquid assay.

Assessment of positive hybridization. The 10 *hlyA* (HA) probes were employed to screen the 72 clinical isolates for homology by in situ hybridization assay (3). In general, the intensity of a positive hybridization signal for any probe

TABLE 2. Summary of hybridization results for HA probes with *E. coli* urinary tract isolates

Probe	No. of hemolytic strains (<i>n</i> = 54) that were:			No. of nonhemolytic strains (<i>n</i> = 18) that were:		
	Positive	Indeterminant	Negative	Positive	Indeterminant	Negative
HA 122	48	3	3	0	1	17
HA 163	51	3	0	6	3	9
HA 743	51	2	1	3	3	12
HA 917	50	3	1	0	0	18
HA 937	13	0	41	0	0	18
HA 1006	51	3	0	0	0	18
HA 1223	48	5	1	2	1	15
HA 1471	31	7	16	0	0	18
HA 1594	39	7	8	0	1	17
HA 2786	50	2	2	0	0	18
Summary ^a	432/540 (80%)	35/540 (6%)	73/540 (14%)	11/180 (6%)	9/180 (5%)	160/180 (89%)

^a Summary is expressed as the overall rate for a specific category/total number of hybridization tests among bacteria strains with a particular hemolytic status (percentage).

among the hemolytic isolates and positive control strains did not vary between tests and was usually dark at temperatures equivalent to the temperature of hybridization for both binding and washing. There were some hemolytic clinical isolates that exhibited less-strong signals (e.g., gray) for particular probes under these conditions. These gray signals were, however, easily distinguishable from the negative controls. Most nonhemolytic strains exhibited blank or light-gray scatter on the autoradiographs. In some circumstances, the signal for a particular strain and a random HA probe was indeterminant despite repeated testing. There was no positive hybridization signal observed with strain HB101 and any of the probes. In addition, another DNA-DNA in situ hybridization assay was employed to assess the specificity of the HA reactions by varying the washing temperatures (12). In these studies, hybridization signals were uniformly observed for hemolytic and nonhemolytic isolates after washes of 37 and 42°C for most HA probes. This indicates that the probes do bind nonspecifically to the dotted DNA samples under these conditions. These temperatures were, however, 7 to 15°C below the temperature of hybridization. After washes of ≥45°C and including 55°C, positive hybridization signals were readily distinguished among the majority of hemolytic strains from negative reactions of the nonhemolytic strains. These temperatures were no less than 2°C below the temperature of hybridization, and most were greater than 4°C above the temperature of hybridization. On the basis of these data, the positive hybridization reactions observed in our screening assay (3) were considered specific for strains harboring homologous DNA sequences.

Distribution of HA probes. Table 2 summarizes the in situ hybridization results (3) for the 72 clinical strains probed for homology to 10 different 20-mer-based synthetic oligonucleotide probes corresponding to *hlyA* sequences in pSF4000. Overall, 44 (6%) of 720 hybridization tests were indeterminant. Among the 54 hemolytic strains, 432 (86%) of 505 definitive colony hybridization tests (i.e., positive or negative signals) demonstrated homology between a HA probe and a clinical isolate. HA probes 122, 163, 743, 917, 1006, 1204, and 2786 hybridized definitively to more than 88% of the hemolytic strains. There was more variability in hybridization results when HA 937, 1471, and 1594 probes were assessed for homology among the hemolytic strains. HA 937 exhibited positive signals for 13 (24%) of 54 hemolytic strains, HA 1471 exhibited positive signal for 31 (57%) of 54

hemolytic strains, and HA 1594 exhibited positive signal for 39 (72%) of 54 hemolytic strains. In addition, analyses indicated that there was considerable conservation in this gene when homology was assessed across the 10 different HA probes. Forty (74%) of the 54 hemolytic strains, including the three hemolytic strains on plates that did not exhibit hemolytic activity in the liquid assay, hybridized with more than eight HA probes. There were only five (9%) hemolytic strains which failed to hybridize to more than five HA probes. Genetic conservation within this gene was further demonstrated by HA hybridization results from the 18 nonhemolytic strains. The HA probes failed to hybridize to the majority of nonhemolytic strains (i.e., no hybridization signal was detectable in 160 [94%] of 171 definitive tests among nonhemolytic strains). HA probes 163, 743, and 1204 hybridized to a total of six different nonhemolytic strains. One of these nonhemolytic strains (i.e., strain C153 from a woman with acute cystitis) hybridized with HAs 163, 743, and 1204.

Immunoblotting patterns for anti-GEJ800 hemolysin A MAbs with culture supernatants. Twelve anti-GEJ800 HlyA murine MAbs were used for immunoblotting assays assessing supernatants of 32 hemolytic strains and 17 nonhemolytic strains. There were no differences in antibody binding reactivity when either the culture supernatant or its filtrate from each strain was used to blot nitrocellulose membranes. Table 3 summarizes the overall rate of binding for each MAb to the culture supernatants of the strains. There was >84% reactivity between each MAb and the culture supernatants of hemolytic strains. There was only occasional reactivity between a MAb preparation and the culture supernatant of nonhemolytic strains. Table 4 summarizes the relationship of MAb reactivity patterns with HA probe profiles for these 49 strains. For 26 hemolytic strains, the culture supernatants were bound by each of the 12 MAbs. Among the remaining hemolytic strains, the culture supernatants for five of six strains were bound collectively by three or more MAbs. There was only one hemolytic strain in which none of the MAbs bound to its culture supernatant. There were only 6 (35%) of 17 nonhemolytic strains identified for which MAbs bound to their culture supernatants, and there was no MAb binding detectable for 11 of these strains. There was only one nonhemolytic strain in which culture supernatant preparations were bound collectively by three different MAbs (MAbs 132, 151, and 559); otherwise, two or usually one MAb bound to culture supernatants of these strains. Also, at

TABLE 3. Anti-GEJ800 alpha-hemolysin A (HlyA) MAbs binding to culture supernatants of *E. coli* strains

MAb ^a	MAb-HlyA binding specificity (Residues [R]/CnBr fragment ^b)	No. of MAb binding culture supernatants/no. tested	
		Hemolytic strains	Nonhemolytic strains
MAb 132	R2-160/II	30/32	1/17
MAb 145	R2-160/II	31/32	1/17
MAb 151	R425-892/V	30/32	3/17
MAb 326	R2-160/II	30/32	0/17
MAb 417	R2-160/II	27/32	1/17
MAb 424	R425-892/V	27/32	0/17
MAb 559	R425-892/V	28/32	3/17
MAb 659	R425-892/V	29/32	1/17
MAb 703	R425-892/V	29/32	0/17
MAb 835	R893-1023/VI	27/32	0/17
MAb 915	R2-160/II	28/32	0/17
MAb 942	R425-892/V	29/32	0/17

^a MAbs have been previously described (21).^b CnBr fragment refers to the cyanogen bromide fragment of HlyA that an MAb binds (21).

least eight of the MAbs bound to the culture supernatants of two of three hemolytic strains that did not exhibit hemolytic activity in the liquid assay. In addition, Table 4 demonstrates the lack of any specific correlation of MAb binding patterns with HA probe profiles among the strains. There were also no correlations among these features and whether the strain was originally isolated from the upper or lower urinary tract among 36 strains assessed (data not shown).

Anti-GEJ800 hemolysin A MAb 132 prevents hemolysis by culture supernatants from a variety of strains. The ability of MAb 132 to prevent hemolysis by culture supernatants from hemolytic strains was assessed. These results were compared with the hemolytic titers obtained when MAb 559 and buffer were tested in this assay. As mentioned previously, the prerequisite for screening a particular culture supernatant in this assay was prior confirmation by immunoblotting

that MAb 132 and MAb 559 bound the culture supernatant preparations (see above). The results demonstrated, as expected, that MAb 559 did not have any capacity to neutralize hemolysis by the 24 different culture supernatants (21). MAb 132 had the capacity to neutralize hemolysis by two- to eightfold for all supernatants screened: twofold for 3 clinical strains, between two- and fourfold for 8 clinical strains, and greater than fourfold for 13 clinical strains. The concentration of MAb 132 in these assays was sufficient to reduce the hemolytic capacity of control supernatants fourfold, from a hemolytic reciprocal titer of 64 to 16 for strain J96 culture supernatant and from 1,024 to 256 for strain GEJ800 culture supernatant.

DISCUSSION

This study demonstrates convincingly that the HlyA determinant of hemolytic *E. coli* strains is highly conserved. This conclusion was expected in view of original observations about the uniformity of this operon and earlier characterizations of HlyA attributes by other investigators (4, 10, 42, 43); however, this study extends these original observations by finer degrees of resolution because of the use of 20-mer gene probes and MAbs that bind to specific regions within HlyA. From the perspective of vaccine development, which is our focus of interest, these data suggest that a broadly cross-reactive immunogen could be identified in one or a limited number of HlyA prototypes. Analysis of our DNA-DNA hybridization experiments among hemolytic strains indicates that there is *hlyA* variation within certain regions of this gene. Genetic divergence is found in 76% of the strains within the distal portion of a predicted hydrophobic domain corresponding to R295 to 325 (14); less divergence occurs in the proximal portion of the predicted acidic alternating hydrophilic region between R491 to 538, accounting for genetic variability in 17 to 34% of the strains (14). These two regions are not likely to be candidates for subunit HlyA vaccines, since the major hydrophobic region is non-immunogenic and the proximal alternating hydrophilic re-

TABLE 4. Associations of anti-GEJ800 HA MAbs binding to culture supernatants and HA profiles of strains

Strain type and no. of strains	MAb ^a binding pattern	HA probe profile ^b
Hemolytic (<i>n</i> = 32)		
5	All 12 MAbs	All 10 HAs
11	All 12 MAbs	All HAs except HA 937
8	All 12 MAbs	All HAs except HAs 937 and 1471
1	All 12 MAbs	All HAs except HAs 122, 163, 1594, and 2786
1	All 12 MAbs	All HAs except HAs 937, 1471, and 1594
1	MAb 145, 559, and 659	All HAs except 937
1	All MAbs except MAbs 417, 424, 835, and 915	All HAs except HAs 917, 937, and 1594
1	All MAbs except MAbs 424, 559, 659, 703, and 835	All HAs except HA 937
1	All MAbs except MAb 559	All HAs except HA 937
1	All MAbs except MAbs 417 and 559	All HAs except HA 937
1	No MAbs	All HAs except HA 937
Nonhemolytic (<i>n</i> = 17)		
10	No MAbs	No HAs
1	All 12 MAbs	No HAs except HA 163
2	MAb 151	No HAs except HA 163
2	MAb 559	Strain 1, no HAs; strain 2, no HAs except 163 ^c
1	MAbs 417 and 659	No HAs except HA 163
1	MAbs 132, 151, and 559	No HAs

^a MAbs employed in these assays included: MAb 132, 145, 151, 326, 417, 424, 559, 659, 703, 835, 915, and 942 (21).^b HA probes employed in these assays included the 10 identified in Table 1.^c Designates two nonhemolytic strains in which one did not hybridize with any HA probes (number 1) and the other hybridized with HA 163 (number 2).

gion, although immunogenic, does not constitute an important HlyA neutralization domain (21, 37). Except for these regions, the *hlyA* determinant exhibits little diversity from one hemolytic strain to another on the basis of hybridization results from our limited array of DNA probes. Three hundred forty-nine (98%) of 357 definitive hybridization reactions among hemolytic strains share homology with seven different HA probes spanning this gene, including HAs 122 and 163, which correspond to a region that elicits greater neutralization antibodies than any other region in HlyA (21). This suggests that HlyA is representative of a virulence determinant that is unlikely to diverge despite selection pressure. This is ideal from the perspective of vaccine development, because emergence of immunological variants are not likely to occur once an efficacious vaccine has been formulated and utilized in high-risk populations. The highly conserved nature of the HlyA determinant is in marked contrast to the Gal-Gal *pilA* genes which were evaluated in a similar fashion, as was done for the *hlyA* gene in this report (12). Gal-Gal pilus operons are genetically linked with hemolysin production in some uropathogenic *E. coli* strains (25) and are important for projecting the PapEFG bacterial adhesin complex from the cell membrane for host uroepithelial attachment (28). The Gal-Gal *pilA* genes have more extensive intracistron genetic diversity than *hlyA* genes; greater than 2% of strains hybridize Gal-Gal *pilA* probes corresponding to the major antigenic domain for this protein, and overall 41% hybridize with seven probes spanning the region of a prototype *pilA* (12). This is an interesting situation in which there has been differential structural gene divergence between two genetically linked operons that are associated with uropathogenicity (6, 8, 16, 22, 35). This might be explained by the necessity that *hlyA* must encode for functionally or structurally essential domains so that HlyA can be transported within the cytoplasm or be active extracellularly, whereas there can be more structural diversity among Gal-Gal PilA proteins and crucial transport and adhesive functions can still be preserved. Also, the effect of selection on these genes might be different because one of these genes is typical of other genomes in this bacterial species and the other is not. *hlyA* was probably acquired from an organism not closely related to *E. coli* on the basis of the discrepancies of *hlyA* guanine-plus-cytosine content and codon usage frequency compared with other *E. coli* genes (10, 14, 23). In contrast, Gal-Gal *pilA* genes are typical of other *E. coli* genomes (11).

Serological HlyA variants were not frequently identified among our 32 hemolytic strains by using 12 different MAb preparations (21). Twenty-six (81%) of 32 culture supernatants of hemolytic strains tested were bound collectively by all 12 anti-HlyA MAbs. There were six hemolytic strains in which there was not uniform reactivity between the culture supernatant and a specific MAb. There was only one hemolytic strain in which none of the antibodies bound to its culture supernatant, whereas the other five hemolytic strains exhibited reactivity with 3 to 11 MAbs among their culture supernatants. The possibilities for this lack of antibody reactivity to the secreted hemolysins include production of too little hemolysin or the presence of immunological HlyA variants. The former situation is unlikely, since all culture supernatants screened exhibited hemolytic reciprocal titers of ≥ 4 in a liquid assay by 6 h; the latter situation is more plausible despite the fact that seven to nine HA probes hybridized with each of these six strains. It is possible that more extensive DNA-DNA hybridization experiments using more probes specific to regions for MAb reactivity or the

sequencing of *hlyA* genes in specific regions among these strains might elucidate a structural basis for these findings.

Antibody binding assays demonstrate that 94% of the hemolytic clinical isolates are bound by a prototype anti-HlyA MAb specific for R2-161 reactivity. In addition, antibody specific for the isolates' HlyA regions is sufficient to reduce at least twofold the hemolytic capacity of all hemolytic supernatants tested. It is important to note that the neutralization capacity afforded by this antibody is not known. It is clear that it does not interfere with HlyA binding to target lipid structure in host cell membranes. Current studies are assessing the epitope domain for this antibody by synthetic peptide strategies in attempts to identify an immunogenic neutralizing subunit HlyA vaccine candidate. In view of the genetic and serological data from this report and previous investigators (6, 7, 8, 10, 14, 19, 38, 41, 43), the expectation is that this prototype HlyA subunit vaccine will elicit broadly cross-reactive antibody for a majority of HlyA moieties produced by hemolytic *E. coli* strains.

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