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Cloned Hemolysin Genes from *Escherichia coli* That Cause Urinary Tract Infection Determine Different Levels of Toxicity in Mice

JÖRG HACKER,[1]* COLIN HUGHES,[1] HERBERT HOF,[2] AND WERNER GOEBEL[1]

Institut für Genetik und Mikrobiologie[1] and Institut für Hygiene und Mikrobiologie,[2] Universität Würzburg, D-8700 Würzburg, West Germany

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After intraperitoneal injection of mice with *Escherichia coli* strains isolated from patients with urinary tract infections, the mortality due to hemolytic (Hly^+) and nonhemolytic (Hly^-) isolates was 77 and 40%, respectively. Deletion of the chromosomal hemolysin (*hly*) determinant in an *E. coli* O6:K15:H31 urinary tract infection strain led to a significant reduction in toxicity for mice, and its reintroduction on a recombinant plasmid partially restored the original toxicity. Although introduction of the cloned plasmid pHly152-encoded *hly* determinant into the Hly^- *E. coli* O6 mutant strain increased toxicity by only a marginal degree, transformation with the cloned chromosomal *hly* determinants from two *E. coli* strains of serotypes O18ac:K5:H^- and O75:K95:H? resulted in markedly greater toxicity, even exceeding that of the original Hly^+ *E. coli* O6 wild-type strain.

Hemolytic (Hly^+) *Escherichia coli* strains may be isolated from a variety of sources. In humans, a low percentage (up to 12%) of the normal fecal *E. coli* strains are Hly^+ (16, 18) compared with 35 to 50% of *E. coli* strains causing extraintestinal infections, such as bacteremia, septicemia, and urinary tract infections (UTI) (17, 23).

Hly^+ *E. coli* strains isolated from humans usually carry the *hly* determinant on the chromosome (18, 19, 24; D. Müller, J. Hacker, and C. Hughes, unpublished results), whereas earlier reports examining Hly^+ *E. coli* strains from animal sources had shown that the genes for hemolysin formation are located on large transmissible plasmids (4, 12, 29, 33). The plasmid and chromosomal *hly* determinants share high sequence homology (4, 24), and all comprise four genes involved in the synthesis of the active hemolysin (*hlyA* and *hlyC*) and its transport ($hlyB_a$ and $hlyB_b$) (11, 25, 26, 38).

Among *E. coli* strains causing UTI, the production of hemolysin is often associated with other factors assumed to contribute to virulence, i.e., mannose-resistant hemagglutination (Mrh^+) caused by specific protein pili (7) and specific O- and K-antigens (14, 27, 28). These additional factors cause adherence of the bacteria to epithelial cells and resistance to serum and phagocytosis (20, 31, 32). The frequent occurrence of the Hly^+ phenotype among these *E. coli* strains suggests that hemolysin may also contribute to virulence in extraintestinal *E. coli* infections, and investigations with Hly^+ and nonhemolytic (Hly^-) *E. coli* strains in different animal models (5, 8–10, 22, 23, 34–37) and cell cultures (3) seem to support this view. In most cases, however, data have been obtained from clinical studies or with strains mutagenized in an uncontrolled way or differing by the presence or absence of Hly plasmids of more than 60 kilobases (kb). Welch et al. (39) first demonstrated the direct contribution of hemolysin to *E. coli* virulence by cloning the chromosomal region containing the *hly* determinant and measuring its influence in a rat peritonitis model.

In this paper we first present epidemiological and genetic data to emphasize the association of *hly* carriage with higher levels of toxicity for mice among *E. coli* causing UTI. We then demonstrate the causal relationship by exact isolation of chromosomal *hly* genes from strains of the predominant UTI serotypes O6, O18ac, and O75. In addition, we show that these precisely subcloned determinants, which have small differences in *hlyA*, encode levels of toxicity markedly different to each other and to that coded for by a cloned plasmid *hly* determinant.

MATERIALS AND METHODS

***E. coli* wild-type strains.** The 69 *E. coli* strains tested for toxicity were isolated from patients attending the UTI clinic at Charing Cross Hospital, London, En-

gland and were identified by standard methods. Additional characterization of the strains, i.e., hemolysin synthesis, colicin V production (Cva^+), and the carriage of K-, O-, and hemagglutination (HA)-antigens have been described previously (17). Strains from which hly^+ clones were derived are listed in Table 1.

Mutants, transformants, and recombinant DNAs. *E. coli* strain 536, isolated from a patient suffering from UTI, was obtained from the Institut für Hygiene und Mikrobiologie, Universität Würzburg, Germany. This strain belongs to the serotype O6:K15:H31 and is Mrh Vb (causing mannose-resistant hemagglutination of bovine erythrocytes and a delayed HA with human and guinea pig erythrocytes).

The spontaneous mutant Nr.21 of strain 536 has been described elsewhere and has lost the ability to produce hemolysin and the Mrh^+ phenotype (Hly^-, Mrh^-) owing to a deletion in the chromosome of more than 30 kb (15; S. Knapp, personal communication). Strain 536/21 was transformed with hly^+ recombinant plasmids by a modified $CaCl_2$ procedure for wild-type strains (21) grown in Luria broth. In all cases, these recombinant DNAs contain *Bam*HI-*Sal*I fragments carrying *hlyA*, $hlyB_a$, and $hlyB_b$ of various chromosomal and plasmid *hly* determinants cloned into pANN202, which is pACYC184 bearing *hlyC* of the plasmid pHly152 (Table 1) (1, 11, 25). The construction of these recombinant plasmids is summarized in Fig. 1.

Isolation of plasmid DNA. Plasmid DNA from transformants carrying recombinant DNA was screened by the cleared lysate procedure (2). Preparative DNA isolation was achieved as described previously (13).

Toxicity tests. The toxicity of bacteria for mice was assessed by a modification of the test described by Van den Bosch et al. (36). Cells from overnight cultures (grown in enriched nutrient broth) were harvested, washed twice in phosphate-buffered saline, and suspended in phosphate-buffered saline to ca. 10^9 viable cells per ml. Ten female specific-pathogen-free NRMI mice (each weighing ca. 16 g; Zentral-Institut für Versuchstiere, Hannover, Germany) were injected via the intraperitoneal route with 0.2 ml of the suspension described above. The number of mice killed after 24 h was recorded. The toxicity of the *E. coli* O6 wild-type strain 536, the *E. coli* O6 deletion mutant 536/21, and the transformed strains carrying the recombinant plasmids was quantified by injecting 7×10^7 to 8×10^8 *E. coli* cells into the mouse peritoneal cavity. In all toxicity tests, at least 10 mice were used per strain. To test whether multiplication of the bacteria occurred, the spleens of several killed mice were removed and homogenized, and viable bacteria were counted. The properties of the reisolated bacteria were controlled by examining 100 colonies of each strain. Strains 536 and 536/21 were tested with endotoxin-resistant C3H/He mice (Zentral-Institut für Versuchstiere).

Screening of transformants and strains reisolated from mice. Transformed strains and those isolated from the spleens of dead or surviving mice were checked for the presence of covalently closed circular DNA and markers on the recombinant DNA (chloramphenicol resistance and hemolysin production). The 536/21 derivatives were further tested for chromosomal streptomycin resistance and production of K15- and O6-antigens.

The K15-antigens were detected by slide agglutination with K15 antiserum (kindly provided by G. Schmidt, Max-Planck-Institut für Immunobiologie, Freiburg, Germany). O6 antibodies were raised against an O6 standard strain (kindly provided by I. Ørskov and F. Ørskov, Statens Servminstitut, Copenhagen, Denmark), and dilutions were used to assay the O6 titer of strain 536 and its derivatives. In each case the standard strain was used as a control (16).

TABLE 1. Recombinant plasmids

Recombinant plasmid	Cloned *hly* genes	Reference
pANN202	*hlyC* from pHly152[a]	(25)
pANN202-312	*hlyC*, *hlyA*, $hlyB_a$, and $hlyB_b$ from pHly152[a]	(11)
pANN5211-O6	*hlyC* from pHly152,[a] *hlyA*, $hlyB_a$, and $hlyB_b$ from *E. coli* 536[b] (O6:K15:K31)	(1)
pANN5311-O18ac	*hlyC* from pHly152,[a] *hlyA*, $hlyB_a$, and $hlyB_b$ from *E. coli* 764[b] (O18ac:K5:H^-)	(1)
pANN5411-O75	*hlyC* from pHly152,[a] *hlyA*, $hlyB_a$, and $hlyB_b$ from *E. coli* 341[b] (O75:K95:H?)	(1)

[a] Plasmid-borne genes.
[b] Chromosomal genes.

RESULTS

Toxicity of Hly^+ and Hly^- *E. coli* strains causing UTI. We assessed the toxicity of clinical isolates in a mouse mortality test, i.e., the ability of high doses of living bacteria to kill mice without extensive multiplication. In our experiments, the mice did not die from an *E. coli* infection but rather from toxicity. The mice died 6 to 20 h after injection of the bacteria without extensive multiplication. From an inoculum of more than 10^8 bacteria, only 10^5 to 10^4 and 10^3 to 10^2 bacteria could be isolated after 3 and 24 h, respectively, from the spleens of dead and surviving animals.

Of the 69 *E. coli* UTI strains tested, 46 were derived from symptomatic infections, and 23 were derived from asymptomatic infections (17). Of the Hly^+ strains, 90% killed 6 or more of 10 mice within 24 h compared with 35% of the Hly^- isolates (Fig. 2). In all cases in which no (or only one or two) mice were killed, the test strain was Hly^-. The difference between the mean number of deaths caused by Hly^+ and Hly^- strains, 7.7 and 4.0 (10 mice tested), respectively, was significant (χ^2, 89.3 and $P < 0.01$), and the standard deviations of the means, 2.0 and 3.9, respectively, are a measure of the constant toxicity levels determined by Hly^+ strains and of the relatively

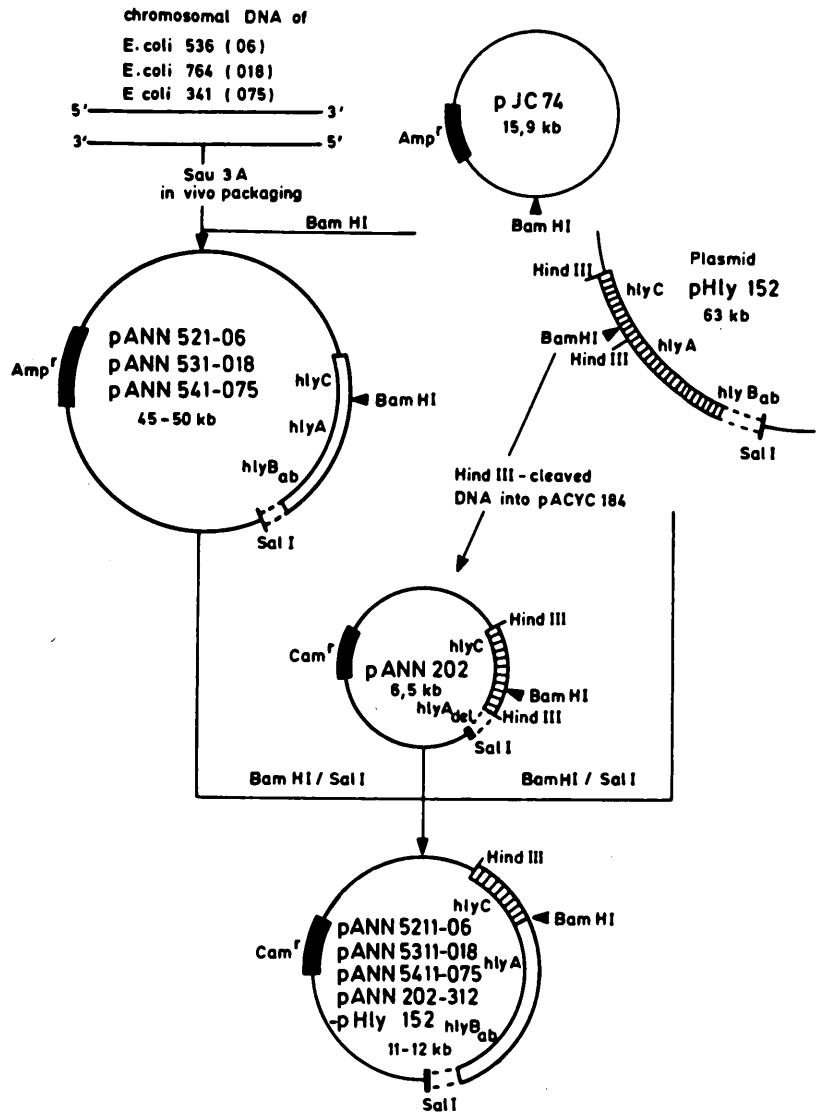

FIG. 1. Construction of the recombinant DNAs. The open boxes indicate chromosomally encoded *hly* cistrons, the hatched boxes indicate plasmid-encoded *hly* cistrons, and the broken lines indicate the 2-kb DNA segments between the distal ends of $hlyB_b$ and the *Sal*I sites, which encode no gene products (see text).

heterogeneous degree of toxicity associated with their Hly^- counterparts. Within the different O serogroups, the Hly^+ strains were more toxic than the Hly^- strains. Higher toxicity for mice seems to be independent of the HA-, K1-, and K5-antigens and colicin V production. These findings support the view that hemolysin exerts a toxic effect. To analyze this hypothesis further, genetic manipulation of *hly* determinants was undertaken.

Toxicity of Hly^+ *E. coli* 536 (O6) and spontaneous Hly^- deletion mutants. As described elsewhere (15), the hemolytic *E. coli* strain 536, which carries a chromosomal *hly* determinant, gives rise to nonhemolytic mutants at a frequency of 10^{-3} to 10^{-4}. From hybridization studies, it is clear that all mutants tested, including mutant 536/21 used here, result from deletions in the region containing *hly* and the determinant which codes for mannose-resistant hemagglutination (*mrh*). The toxicity of the Hly^+ wild-type strain 536 and that of the Hly^- mutant 536/21 were compared, and strain 536 killed 30% of the NMRI mice at titers of 7×10^7 with a 100% lethal dose of 5×10^8 (Fig. 3). The toxicity of the deletion mutant 536/21, which still retains the

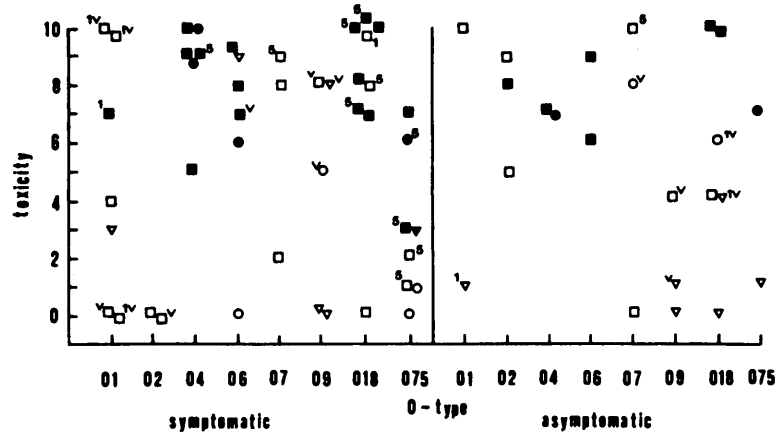

FIG. 2. Toxicity for mice of *E. coli* strains causing symptomatic and asymptomatic UTI in relation to hemolysin production and other presumptive virulence factors. The closed symbols represent Hly^+ strains, and the open symbols represent Hly^- strains. Symbols: V, colicin V production; 1, K1-antigen; 5, K5-antigen; ■ and □, Mrh^+; ▼ and ▽, mannose-sensitive HA (Msh^+) and HA type III, not common type I; ● and ○, no HA (HA type IVb).

K15-antigen and shows the same O6-antigen titer as the wild-type parent, dropped considerably. A titer of 8×10^8 bacteria killed only 45% of the NMRI mice, and no mice were killed at titers between 7×10^7 and 8×10^8. Analogous results were obtained when C3H/He endotoxin-resistant mice were used. At titers of 10^7 and 10^8 bacteria, no mice and 80% of the mice, respectively, were killed by the Hly^+ wild-type isolate 536. At both titers, no mice died after injection of the Hly^- 536/21 derivative. At titers of 10^9 bacteria all C3H/He mice were killed by both strains. Further tests were carried out with NMRI mice. The deletion mutant 536/21 was used for further analysis, as it provided an extremely suitable system to study the effects of isolated *hly* genes.

Toxicity of the Hly^- *E. coli* 536/21 (O6) mutant carrying cloned *hly* determinants. By using the cosmid system, chromosomal *hly* determinants were isolated (1) from *E. coli* strains belonging to three serogroups (O6, O18ac, and O75) predominant and typically Hly^+ in UTI (17). The determinants, first isolated on the cosmid clones pANN 521-O6, pANN531-O18ac, and pANN541-O75 (see Fig. 1), were then subcloned in such a way that the effects on toxicity and virulence of the hemolysins produced could be assessed and compared directly, i.e., by minimizing the presence of non*hly* DNA. This was done by introducing *Bam*HI-*Sal*I fragments carrying *hlyA*, $hlyB_a$, and $hlyB_b$, which encode synthesis of the *hly* precursor and transport of the processed product out of the cell, into the

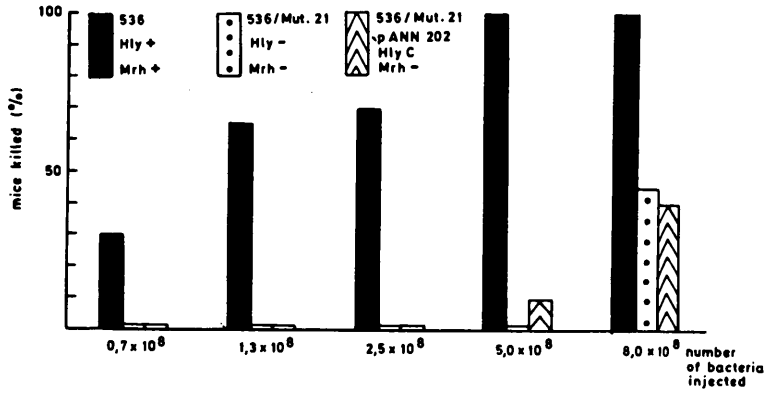

FIG. 3. Toxicity for mice of the Hly^+ *E. coli* wild-type strain (O6:K15:H31), the Hly^- deletion mutant 536/21, and 536/21 transformed with the plasmid pANN202.

clone pANN202. pANN202 is the vector pACYC184 carrying *hlyC* (from the plasmid pHly152), which is responsible for processing of the precursor (24, 25; M. Härtlein, S. Schiessl, U. Rdest, J. Kreft, and W. Goebel, submitted for publication). The *hly* determinant of the plasmid pHly152 was cloned in the same way; therefore, the four *hly* determinants examined are directly comparable.

As a preliminary test, the common vector pANN202 (pACYC183:*hly*C) was transformed into *E. coli* 536/21, and the toxicity of the Hly$^-$ mutant was unaltered (Fig. 3). This was plainly not the case, however, after introduction of the four *hly*$^+$ recombinant DNAs, as all increased the toxicity of the Hly$^-$ deletion mutant (Fig. 4). Furthermore, the resulting levels of toxicity were significantly different. The plasmid-borne *hly* determinant (pANN202-312) contributed only marginally to toxicity; i.e., at a dose of 2.5×10^8, only 7% of the mice were killed, and the 50% lethal dose was 8×10^8. The lowest contribution to toxicity of the chromosomal *hly* determinants was observed with that of *E. coli* O6 (pANN5211-O6). Titers of 2.5×10^8 and 5×10^8 killed 30 and 70% of the mice, respectively.

In contrast, the 536/21 mutant carrying the *hly* determinants from the strains O18ac (pANN5311-O18ac) and O75 (pANN5411-O75) killed 90 and 100% of the mice, respectively, at a titer of 2.5×10^8. The 50% lethal dose of the nontransformed strain 536/21 was ca. 8×10^8. After introduction of the cloned *hly* determinants from *E. coli* 764 (O18ac:K5:H$^-$) and *E. coli* 341 (O75:K95:H?), the 50% lethal dose dropped to ca. 7×10^7; i.e., the *hly* determinants increased the toxicity of the strains by a factor of ten. Interestingly, after introduction of both the O18ac and the O75 *hly* determinants the 536/21 strain showed a higher toxicity than wild-type parent strain 536 (Fig. 3 and 4). All strains isolated after 24 h still had streptomycin resistance, the K15-antigen, and the same O6-antigen titer. The endotoxin thus appears to have no influence on the change in toxicity measured in this test. A total of 90% or more of the strains still carried the recombinant plasmid DNA, as indicated by the retention of chloramphenicol resistance (Cm^r) and Hly$^+$ markers and also by gel electrophoresis of cleared cell lysates. Strains which had lost the recombinant *hly*$^+$ DNAs but still retained the O6-antigen (and also the K15-antigen) showed the same level of toxicity as the original 536/21 mutant strain (data not shown). As described for the urinary tract isolates, no extensive multiplication of the strains could be observed. From an inoculum of more than 10^8 bacteria only 10^{-5} to 10^{-4} bacteria were isolated from spleens after 1 h, and this titer decreased to 10^{-3} to 10^{-2} after 24 h. These data strongly suggest that the increased toxicity is determined by the cloned *hly* cistrons.

DISCUSSION

Among the *E. coli* strains causing extraintestinal infections, hemolysin production is especially common (6, 17, 23) and is associated with higher levels of toxicity for mice after intraperitoneal injection. The view that it contributes to *E. coli* virulence in such infections, e.g., those of the urinary tract, is supported by the use of in vivo models (3, 5, 8–10, 23, 36, 37). Data from studies on clinical isolates or genetic variants of bacterial strains which have been mutagenized or have lost or received large plasmids (8, 9, 22, 23, 34–37) are, nevertheless, indicative rather than conclusive. The genetic background of the

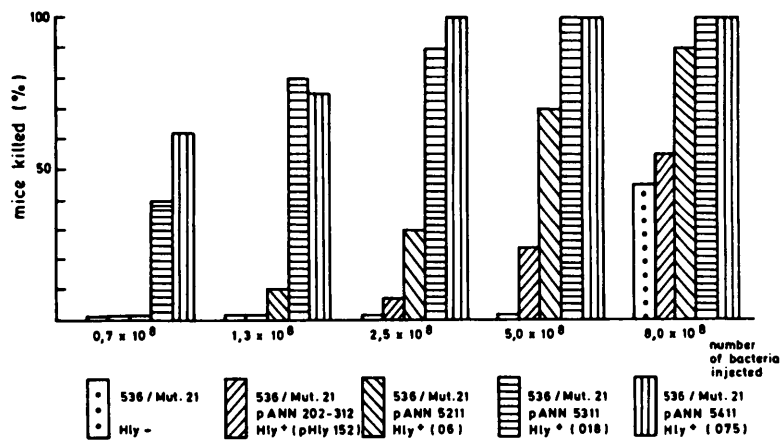

FIG. 4. Influence of the transformed cloned *hly* determinants on the toxicity for mice of the Hly$^-$ *E. coli* deletion mutant 536/21.

organisms examined, particularly the immediate environment of the *hly* determinants involved, is, to various degrees, undefined, and as the extraintestinal pathogenesis of *E. coli* strains appears to be a multifactorial phenomenon (7, 17, 23), the specific contribution of hemolysin itself cannot be readily deduced.

The one previous use of recombinant DNA techniques to resolve this problem compared virulence in a rat peritonitis model of an *E. coli* fecal strain after introduction of recombinant DNAs carrying chromosomal (pSF4000) (39) and plasmid (pANN202-312) (described above and in reference 11) *hly* determinants cloned into pACYC184. The size of the chromosomal insert found to increase virulence, 11.7 kb, was twice that required for hemolysin synthesis (11, 25), and the additional DNA was uncharacterized. Since we have observed that chromosomal *hly* determinants may be located in the vicinity of genes encoding HA (15) and serum resistance (C. Hughes, J. Hacker, S. Knapp, and W. Goebel, manuscript in preparation), factors assumed to influence urinary tract virulence (31, 32), our primary aim was to study the effects of precisely defined hly^+ recombinant DNAs derived from different serotypes in a well-characterized genetic background. This was made possible by the use of the well-defined UTI O6 derivative strain 536/21 and the cloning strategy described above.

The endotoxin appears to have no influence on the change in toxicity for mice because all derivatives of the 536/21 strain tested showed the same amount of O6-antigen as did the wild-type parent. When the 536 Hly^+ wild-type strain and the 536/21 Hly^- mutant were injected, the same results were found with endotoxin-resistant and -sensitive mice. Strains cured of hemolysin production but still retaining the O6-antigen lost the ability to increase toxicity for mice.

The cloning of the various *hly* determinants was achieved by subcloning *Bam*HI-*Sal*I fragments known (1, 11) to carry the *hlyA*, $hlyB_a$, and $hlyB_b$ cistrons of *hly* determinants into the vector pACYC184 carrying *hlyC* (pANN202) (Fig. 1). This resulted in the foreign DNA being restricted almost exclusively to the *hly* determinant and its normal regulatory regions. There is virtually no additional DNA between the N-terminal (*hlyC*) end and pACYC184. Approximately 2 kb of chromosomal DNA, known from Southern blots to have no homology with *hly* and *mrh* genes (S. Knapp, personal communication) and to encode no gene products in either mini- or maxicells, is located at the C-terminal end distal to $hlyB_b$ (Härtlein et al., submitted for publication).

These recombinant DNAs determined an increase in the toxicity of an Hly^- mutant of an O6 UTI isolate which lacks its *mrh* and *hly* genes owing to a well-characterized chromosomal deletion (15). Since pANN202 does not influence toxicity, the toxic effects must be assumed to derive from the gene products of the *Bam*HI-*Sal*I fragments. It has been shown by complementation tests (Müller, D., J. Hacker, and W. Goebel, manuscript in preparation) that the transport functions of the different *hly* determinants tested are structurally and functionally closely related, if not identical. Despite the fact that the cloned *hly* determinants are located on multicopy plasmids, the level of the secreted hemolysins is the same as that in the wild-type strains from which the *hly* determinants were derived (1, 11, 26, 30). These findings indicate that the different contributions to toxicity do not result from quantitative differences in the expression or the transport of the cistrons $hlyB_a$ and $hlyB_b$. Rather, it seems likely that the small variations previously observed in the nucleotide sequence of the *hlyA* cistrons of these four recombinant DNAs (1) influence in a marked manner the toxicity of the resulting hemolysin proteins, presumably by involving changes in either overall conformation or in an as yet undetermined active site. Thus, although hemolysin production may be regarded as a marker of UTI virulence among *E. coli* (17), the phenotype may not indicate a constant level of toxicity. Such small differences in structure have already been noted among *E. coli* toxins produced during intestinal infections (40).

To avoid the term "virulence" in connection with our results we refer to "toxicity" in describing the reduction in the lethal dose for mice caused by the *hly* determinants. The virulence of uropathogenic strains comprises the properties of adherence, multiplication, and interference with the host defense system. After attachment and colonization, the toxic or cytotoxic potential of the bacteria may be essential for the progression of UTI, e.g., by damaging kidney cells or destroying leukocytes (3, 9, 10). The existence of defined hly^+ recombinant DNAs derived from strains of different serotypes should now allow a more precise appraisal of the specific action of hemolysin in the development of UTI.

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