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Spontaneous Deletions and Flanking Regions of the Chromosomally Inherited Hemolysin Determinant of an *Escherichia coli* O6 Strain

J. HACKER, S. KNAPP, AND W. GOEBEL*

Institut für Genetik und Mikrobiologie, Universität Würzburg, D-8700 Würzburg, West Germany

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The hemolytic *Escherichia coli* strain 536 (O6) propagates spontaneous hemolysin-negative mutants at relatively high rates (10^{-3} to 10^{-4}). One type of mutant (type I) lacks both secreted (external) and periplasmic (internal) hemolysin activity (Hly_{ex}^-/Hly_{in}^-) and in addition shows no mannose-resistant hemagglutination (Mrh^-), whereas the other type (type II) is Hly_{ex}^-/Hly_{in}^+ and Mrh^+. The genetic determinants for hemolysin production (*hly*) and for mannose-resistant hemagglutination (*mrh*) of this strain are located on the chromosome. Hybridization experiments with DNA probes specific for various parts of the *hly* determinant reveal that mutants of type I have lost the total *hly* determinant, whereas those of type II lack only part of the *hlyB* that is essential for transport of hemolysin across the outer membrane. Using a probe that contains the end sequence of the plasmid pHly152-encoded *hly* determinant (adjacent to *hlyB*), we determined that a related sequence flanks also the *hlyB*-distal end of the chromosomal *hly* determinant of *E. coli* 536. In addition several other similar or even identical sequences are found in the vicinity of the *hlyC*- and the *hlyB*-distal ends of both the chromosomal and the plasmid *hly* determinants.

Hemolysin produced by *Escherichia coli* is an extracellular protein that is considered to be a virulence factor in *E. coli* causing extraintestinal infections (7, 17, 27, 28; J. Hacker, C. Hughes, H. Hof, and W. Goebel, submitted for publication). The genetic determinant for hemolysin synthesis and secretion (22) is located either on transmissible plasmids (10, 11, 21) or on the chromosome (2, 17; D. Müller, C. Hughes, and W. Goebel, in press).

Plasmid-inherited and chromosomally inherited hemolysin determinants (*hly*) have been cloned (2, 9, 18), and their complex structure and function have in part been elucidated (19). Hybridization studies performed with both types of *hly* determinants show extended sequence homologies, and therefore both probably derive from a common origin.

Most hemolytic *E. coli* strains isolated from extraintestinal infections also form fimbriae that cause mannose-resistant hemagglutination (Mrh) (7, 12; C. Hughes, J. Hacker, A. Roberts, and W. Goebel, in press). These protein fimbriae (adhesins) seem to be responsible for adhesion of *E. coli* to the target cells (14, 26).

All attempts to transpose plasmid or chromosomal *hly* determinants onto a small plasmid have failed (D. Müller and W. Goebel, unpublished data). Here we show that at least one end of the plasmid pHly152 inherited *hly* determinant and also of a chromosomal *hly* determinant is flanked by a sequence that behaves like an insertion sequence (IS) element. Similar or even identical sequences are also located in the vicinity of the other end of the *hly* determinant. There is evidence that these sequences lead to the loss of either the total *hly* determinant or part of it. Deletions that remove the *hly* determinant entirely also eliminate the Mrh phenotype.

MATERIALS AND METHODS

Bacteria. The *E. coli* strain 536, isolated from a patient suffering from a urinary tract infection, was obtained from the Institut für Hygiene und Mikrobiologie, Universität Würzburg, West Germany. This strain exhibits the following characteristics: O6, Mrh, Vb (hemagglutination with bovine erythrocytes and delayed hemagglutination with human and guinea pig erythrocytes). For transformation we used the *E. coli* K-12 strain 5K (*resK rpsL*). The *E. coli* strain carrying the Hly plasmid pHly152 was described previously (18). The recombinant plasmids used here are listed in Table 1.

Media, chemicals, and enzymes. Cultures were grown in enriched nutrient broth or in alkaline broth extract. For hemagglutination tests we used CFA plates as described previously (8). Radiochemicals were purchased from New England Nuclear Corp., Boston, Mass.; antibiotics used were a gift from Bayer, Leverkusen, West Germany. All other chemicals were obtained from Merck, Darmstadt, West

TABLE 1. Recombinant plasmids used in this study

Plasmid	*hly* cistrons and plasmid resistance markers	Source or reference
pANN312	*hlyA hlyB* Ap^r	9
pANN215	part of *hlyA* Cm^r	18
pANN202	*hlyC* part of *hlyA* Cm^r	18
pANN205-222	*hlyB* Ap^r	Goebel (unpublished data)
pANN250	part of *hlyB* Tc^r	18
pANN202-312	*hlyC hlyA hlyB* Cm^r	9
pANN202-3123	*hlyC hlyA* Cm^r	9
pANN206	part of *hlyB* Ap^r	Knapp (unpublished data)
pANN801	*mrh* Vb Ap^r	2

Germany. Restriction enzymes and T4 ligase were purchased from Bio-Rad Laboratories, Richmond, Calif.

Isolation of chromosomal DNA. Cells from a stationary culture grown in nutrient broth were centrifuged, washed once in 0.15 M NaCl–0.1 M EDTA (pH 8), and suspended in 25% sucrose–10 mM Tris-hydrochloride (pH 7.5). After the addition of 0.05 volume of 0.25 M EDTA (pH 8), the cells were treated with lysozyme (10 mg/ml) followed by 1% sodium dodecyl sulfate, both for 30 min at 37°C. The resulting lysate was shaken with pronase (100 μg/ml) for 3 h until clear and then shaken with the same volume of isoamyl alcohol-chloroform (1:24) for 40 min. The two phases were separated by low-speed centrifugation; after the addition of 2 volumes of absolute ethanol (4°C), the DNA was isolated from the aqueous solution by subsequent spinning onto a glass rod. The DNA was then dried, dissolved in 10 mM Tris-hydrochloride (pH 7.5), and purified once in a CsCl gradient.

Isolation of plasmid DNA. Plasmid DNA from clones carrying recombinant DNA was screened by the cleared lysate procedure (3), and preparative DNA isolation was achieved as described previously (13).

Nick translation. Plasmids were labeled by nick translation with a mixture of all 4 α-^{32}P-labeled dinucleotide triphosphates as described previously (20) and purified on a Sephadex G50 column.

Cleavage with restriction enzymes and electrophoresis of chromosomal DNA. The chromosomal DNA was treated with the restriction enzymes *Eco*RI and *Hin*dIII, and the resulting fragments were separated by agarose gel electrophoresis in 1% agarose as described previously for plasmid DNA (6).

Hybridization and autoradiography. The transfer of DNA fragments from agarose gels to nitrocellulose filters and the washing and autoradiography of the filters after hybridization were performed as described previously (24). The filters were hybridized in 50% formamide at 43°C for 3 days. Stringent conditions were used for the washing procedure.

Transformation. *E. coli* K-12 strains were transformed by the $CaCl_2$ method (4). The 536 strains were transformed by a modified $CaCl_2$ procedure for wild-type strains (16).

Hemolysin production. Erythrocyte lysis was detected on meat agar plates containing washed human erythrocytes and was confirmed by a liquid assay (22).

Hemagglutination. The hemagglutination test with *E. coli* 536 and the K-12 Mrh^+ transformants were performed with bovine erythrocytes. Agglutination, sensitive and resistant to 1% mannose, was assayed in phosphate-buffered saline and classified after incubation on ice for 2 min as described previously (8).

RESULTS

Formation of spontaneously occurring hemolysin-negative mutants of *E. coli* 536 (O6). The *E. coli* strain 536 belongs to the serotype O6 and produces hemolysin encoded by the chromosome (2; Müller et al., in press). We noticed when plating this strain on blood agar that mutant colonies arose which no longer formed clear lysis zones around the colonies. These colonies retained other strain-specific phenotypes, like resistance to streptomycin and the O6 character, which indicate that they derive from the original Hly^+ strain by spontaneous mutations. The mutation rate is about 10^{-3} to 10^{-4}, unusually high for a spontaneously occurring chromosomal mutation. Two types of hemolysin-negative mutants were found, those that did not form any lysis zones (type I) and those that showed tiny lysis zones around the growing colony (type II). None of the previously cloned cistrons (*hlyC*, *hlyA*, or *hlyB*) of the plasmid (pHly152) *hly* determinant alone was able to restore hemolysin formation when transformed into type I mutants. It has been recently shown that the corresponding plasmid and chromosomal *hly* cistrons *A*, *B*, and *C* can be functionally exchanged. These data will be published elsewhere (D. Müller, J. Hacker, and W. Goebel, manuscript in preparation).

Hemolysin synthesis and secretion was observed, however, when pANN202-312, a recombinant plasmid carrying the total plasmid (pHly152)-encoded *hly* determinant, was introduced into these mutants by transformation. This indicates that no other chromosomal functions interfering with synthesis or secretion of hemolysin were impaired in type I mutants. These mutants exhibited neither periplasmic (internal) nor external hemolysin activity (Hly_{ex}^-/Hly_{in}^-) (19). Type II mutants produced internal hemolysin at a normal level, indicating that *hlyC* and *hlyA* must be functional. This was confirmed by the complementation of these mutants to full hemolytic activity (Hly_{ex}^+/Hly_{in}^+) by pANN312, which is $hlyA^+$ $hlyB^+$ (9), or by pANN205-222, a recombinant plasmid that carries *hlyB* under the control of the *lac* promoter (Goebel, unpublished results). Type II mutants can be therefore considered as *hlyB* mutants, which are phenotypically Hly_{ex}^-/Hly_{in}^+ (19).

Type I but not type II mutants have also lost mannose-resistant hemagglutination properties

(**Mrh Vb**). *E. coli* 536 (O6) forms adhesion pili which cause mannose-resistant hemagglutination of bovine erythrocytes. All type I mutants were found to have lost this ability, but type II mutants retained mannose-resistant hemagglutination at its normal level. To test whether the *mrh* Vb determinant was directly or indirectly affected by this mutation, the recombinant cosmid pANN801 (Ap^r, Mrh^+), which carries this determinant (2), was transformed into type I mutants. All transformants selected on ampicillin-containing agar again showed mannose-resistant hemagglutination of bovine erythrocytes. No alteration of the O antigen or any other recognizable phenotype was observed in these mutants. The characteristic properties of type I and type II mutants are summarized in Table 2.

Hybridization of chromosomal DNA of type I and II mutants with probes specific for the *hly* determinants. Nick-translated ^{32}P-labeled pANN202 (*hlyC*, part of *hlyA*) hybridized with *Hin*dIII-cut chromosomal DNA of the wild-type *E. coli* 536 in a single *Hin*dIII fragment of 10.3 kilobases (kb) (Fig. 1). No hybridization occurred with this probe in *Hin*dIII-cleaved chromosomal DNA of mutants of type I, suggesting that this part of the chromosomal *hly* determinant is completely lost. The ^{32}P-labeled probe pANN215 (part of *hlyA*) hybridized in the DNA of the wild-type strain with the same *Hin*dIII fragment as did pANN202, suggesting that *hlyC* and *hlyA* are located on the same *Hin*dIII fragment. Again, no hybridization with pANN215 was observed with *Hin*dIII-cut chromosomal DNA of type I mutants. ^{32}P-labeled pANN250 (part of *hlyB*) hybridized, as expected, with the *Hin*dIII-cut DNA of the wild type in two *Hin*dIII fragments (2; Müller et al., in press) of 1.6 and 10.3 kb. These data suggest that this class of mutants has deleted all three cistrons of the *hly* determinant. The two strains 25 and 114 belong to type II mutants. Chromosomal DNAs of both mutants were also digested with *Hin*dIII and hybridized with the three probes pANN202, pANN215, and pANN250 (data not shown). *Hin*dIII-cleaved DNA of both mutants hybridized with pANN202 in a *Hin*dIII fragment of 10.3 kb and with pANN215 and pANN250 in two *Hin*dIII fragments of 10.3 and 1.6 kb. This suggests that at least that part of the *hly* determinant that is contained in these fragments, i.e., *hlyC*, *hlyA*, and part of *hlyB*, is still retained in these mutants.

Hybridization of chromosomal DNA from the wild type and mutants with a recombinant plasmid containing the total *hly* determinant. To test whether the mutants have also lost regions of the *hly* determinant which are not included in the recombinant DNA probes described above we used pANN202-312, a recombinant plasmid that contains the total *hly*-determinant of pHly152 and, downstream of *hlyB*, some additional sequences of pHly152 (9). Unexpectedly, *Hin*dIII-cleaved chromosomal DNA of the wild-type strain 536 hybridized with ^{32}P-labeled pANN202-312 in several fragments (Fig. 2) that did not hybridize with the former three probes. Since the vector DNA (pACYC184) used for the construction of this recombinant DNA did not hybridize at all with the chromosomal DNA of this strain (Fig. 1), the hybridization had to be specific for inserted DNA. Using the entire ^{32}P-labeled pHly152 plasmid DNA as the hybridization probe, we obtained a very similar hybridization pattern with *Hin*dIII-cleaved chromosomal DNA of the wild-type strain (Fig. 2) and with pANN202-312, which indicates that probably only the *hly*-specific part of pHly152 is causing hybridization of the various chromosomal *Hin*dIII fragments of this strain. From the above data we could readily identify the strongest hybridizing fragments, C3 (10.3 kb) and C9 (1.6 kb), as belonging to the *hly* determinant. The other hybridizing *Hin*dIII fragments (C3a, C4, C5, C6, C8, C10, and C11) exhibited a less

TABLE 2. Mutant strains and transformants of *E. coli* 536 (O6)[a]

Strain	Transformed plasmid[b]	Hemolysin production[c]		Mrh antigen
		Hly_{in}	Hly_{ex}	
536 (wild type)		2.54	1.11	Vb
536/114	None	1.43	0.13	Vb
536/114	pANN312	1.84	1.20	Vb
536/114	pANN205-222	1.83	1.23	Vb
536/114	pANN202-3123	1.94	0.21	Vb
536/25	None	1.79	0.11	Vb
536/25	pANN312	2.10	1.12	Vb
536/25	pANN205-222	2.24	1.24	Vb
536/25	pANN202-3123	1.92	0.13	Vb
536/21	None	0.0	0.0	
536/21	pANN312	0.0	0.0	
536/21	pANN202-3123	0.0	0.0	
536/21	pANN202-312	2.02	1.20	
536/21	pANN801	0.0	0.0	Vb

[a] All mutants retained the wild-type properties, O6 and *rpsL*.

[b] The recombinant plasmids used for complementation of the Hly^- mutants were described previously (2, 9): pANN312 is $hlyA^+$ $hlyB^+$; pANN202-3123 is $hlyC^+$ $hlyA^+$; pANN205-222 is $hlyB^+$; pANN202-312 is $hlyC^+$ $hlyA^+$ $hlyB^+$; pANN801 is *mrh* Vb^+.

[c] The values indicate the amount of released hemoglobin (measured as optical density at 536 nm) after incubation of human erythrocytes with internal or external hemolysin preparations in a standard assay (22).

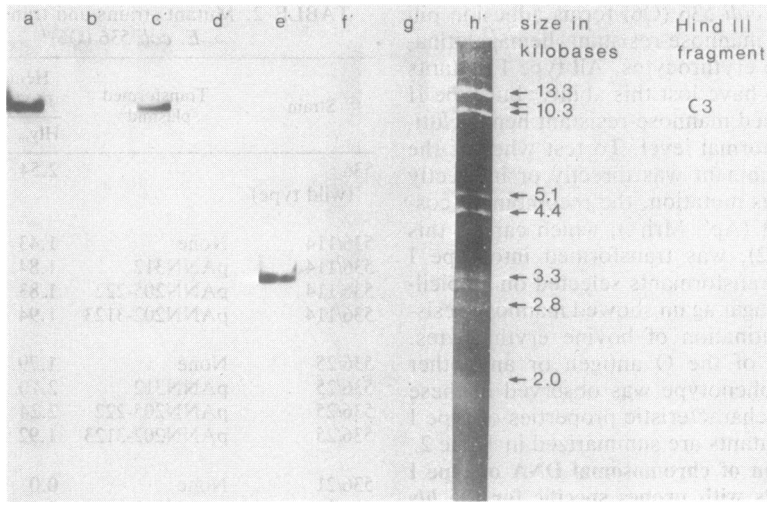

FIG. 1. Hybridization patterns of *Hin*dIII-cleaved (lanes a', b', c', d', and g') and *Eco*RI-cleaved (lanes e' and f') chromosomal DNA from the *E. coli* 536 wild-type strain (lanes a', c', e', and g') and mutant 111 (lanes b', d', and f'). The DNA fragments of lanes a' through g' were hybridized with nick-translated, ^{32}P-labeled DNA of pANN202 (lanes a' and b'), pANN215 (lanes c' and d'), and pANN250 (lanes e' and f') and pACYC184 vector DNA (lane g'). As size markers, *Eco*RI fragments of pHly152 were used (lane h') (18).

pronounced hybridization with pANN202-312 (Fig. 2). These fragments, with the possible exception of C5, hybridized also with pANN206, which contains the *Eco*RI-*Bgl*II fragment of pANN202-312 downstream of *Eco*RI-G (Knapp, unpublished data). This fragment is known to carry the residual part of *hlyB*, missing on *Eco*RI-G (Goebel, unpublished data). The corresponding part of *hlyB* of the chromosomal *hly* determinant of *E. coli* 536 is located on C10 and C11 (see below). A probe carrying C10 alone did not hybridize with these fragments, and there is no reason to assume that the residual part of *hlyB* located on C11 would hybridize with chromosomal *Hin*dIII fragments that are entirely unrelated to the *hly* determinant, such as C3a, C4, C6, and C8. It is therefore more likely that these latter *Hin*dIII fragments (C3a, C4, C6, and C8) hybridize with a sequence that lies next to *hlyB* on pHly152 and marks one end of the *hly* determinant of pHly152. C3a seems to be a neighboring fragment of C11 (Fig. 2) and possibly marks the *hlyB*-adjacent end of the *hly* determinant of *E. coli* 536. C4 and C6 are also located on the *hlyB* distal part of the *hly* determinant (Knapp, unpublished data), but it is not yet known whether they represent continuous fragments next to C3a or whether they are separated by other *Hin*dIII fragments. C8 is located on the other side (*hlyC*-adjacent end) of the *hly* determinant. Again, it is unknown whether C8 borders directly on C3 or whether there are *Hin*dIII fragments in between. However, it is evident that the chromosomal *Hin*dIII fragments that hybridize with the "end sequence" of the plasmid *hly* determinant are located in the vicinity of both ends of the chromosomal *hly* determinants of *E. coli* 536.

The plasmid *hly* determinant seems to be directly flanked by these sequences only on one end (adjacent to *hlyB*) since the *Hin*dIII-*Eco*RI fragment that is located adjacent to *hlyC* (upstream of *hlyC*) did not hybridize with pANN206. Hybridization of pHly152 with pANN206 occurred, however, in *Eco*RI-C and *Eco*RI-J, which are in the vicinity of the *hlyC*-distal end of this plasmid *hly* determinant (19), indicating that there are at least two such sequences on the plasmid pHly152 surrounding the *hly* determinant. When *Hin*dIII-cut chromosomal DNAs of type I mutants were hybridized with pANN202-312, hybridization was still observed with C6 and C8, but in addition to the previously recognized fragments C3 and C9, the fragments C10 and C11 and the less extensively hybridizing *Hin*dIII fragments C4 and C3a were found to be missing in the *Hin*dIII fragment pattern of these mutant DNAs (Fig. 2). This indicates that the deletion occurring in these mutants removes obviously a larger region of the chromosome which includes the total hemolysin determinant and probably part or all of the determinant for mannose-resistant hemagglutination. Otherwise, mutant 112 differs slightly from the two other type I mutants analyzed in that *Hin*dIII fragment C8 is missing, but another fragment C5a appears which probably derives from C8 (Knapp, unpublished data), indicating

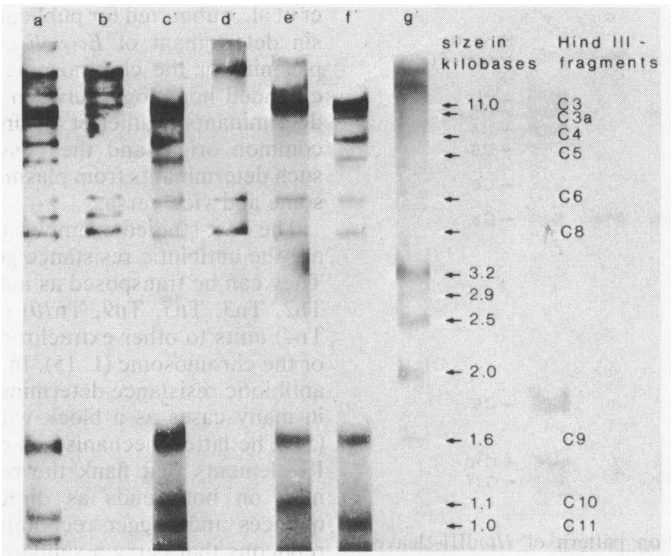

FIG. 2. Hybridization patterns of *Hin*dIII-cleaved chromosomal DNA from *E. coli* 536 wild-type strain (lanes a′ and c′), mutant 111 (lanes b′ and d′), mutant 114 (lane e′), and mutant 25 (lane f′). The DNA fragments of lanes a′ through f′ were hybridized with nick-translated, ^{32}P-labeled DNA of pHly152 (lanes a′ and b′) and pANN202-312 (lanes c′ through f′). As a control, *Hin*dIII-cleaved pHly152 DNA was hybridized with nick-translated, ^{32}P-labeled DNA of the same plasmid (lane g′).

that the deletion occurring in this mutant, which is phenotypically identical to mutants 111 and 113, differs somewhat from the latter. As expected, the same hybridization pattern as with pANN202-312 was obtained when *Hin*dIII-cut chromosomal DNA of these mutants was hybridized with the recombinant DNA, pANN206, carrying the *hlyB*-distal "end fragment" of the plasmid *hly* determinant (Fig. 3).

*Hin*dIII-cut chromosomal DNAs of type II mutants were also hybridized with pANN202-312 and pANN206. Mutant 114 hybridized with pANN202-312 in the *Hin*dIII fragment C3 and C9 and in addition with C6, C8, and C10, whereas C3a, C4, and C11 did not hybridize (Fig. 2). The recombinant plasmid, pANN206, as expected, did not hybridize with C3, but did so with all other *Hin*dIII-fragments, which hybridized with pANN202-312. The mutant 25 showed hybridization in *Hin*dIII fragments C3, C3a, C4, C6, C8, and C9 when pANN202-312 was used as hybridization probe and in the same *Hin*dIII fragments, except C3, when pANN206 was taken as probe. Another fragment that showed hybridization resembles C11 in size, but is more likely to be the deleted C10 fragment (Knapp, unpublished results). Since both mutants are defective in *hlyB* function and C10 and C11 are located next to *Eco*RI-G, which carries part of *hlyB* (18), it seems reasonable to assume that the deletion of C11 (in mutant 114) and C10 (in mutant 25) is responsible for the Hly_{ex}^- phenotype. It further suggests that all of C10 and at least part of C11 are necessary for functional transport of hemolysin across the outer membrane, in agreement with recent results on the plasmid pHly152 *hly* determinant (Goebel, unpublished data).

Estimation of the size of the *hlyB*-distal end sequence of the *hly* determinant. The size of the *Eco*RI-*Bgl*II fragment of pHly152 in pANN206 which carries this sequence is 2.3 kb. From the deletions of the two analyzed type II mutants it is evident that the complete C11 fragment (800 base pairs) and part of the C10 fragment (900 base pairs) are still required for the *hlyB* function. Both are included within the *Eco*RI-*Bgl*II fragment. There is no *Bgl*II site within the other chromosomal *Hin*dIII fragments of *E. coli* 536 or in the *hlyC*-distal part of pHly152 that hybridized with pANN206 (2; Müller et al., in press). In addition, no hybridization occurred in the chromosomal *Hin*dIII fragments that hybridized with pANN206, when a probe was used that contained the *Bgl*II-*Hin*dIII fragment adjacent to the fragment in the pANN206 probe. This seems to suggest that this abundant sequence is located entirely within pANN206. The size of this sequence therefore cannot be longer than 1,200 base pairs and cannot be shorter than 500 base pairs. Based on the location within the chromosome of the *Hin*dIII fragments that hybridize with pANN206, we propose the following mechanism for the generation of the deletion

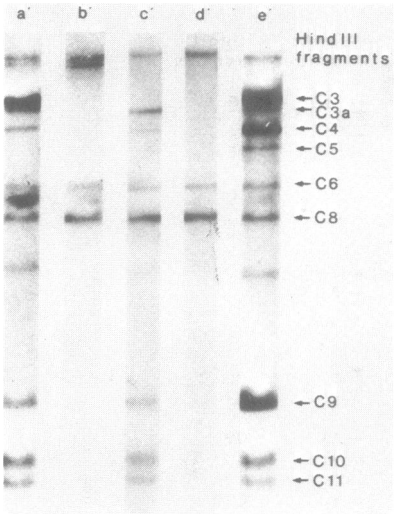

FIG. 3. Hybridization pattern of *Hin*dIII-cleaved chromosomal DNA of the *E. coli* wild-type strain (lanes a', c', and e') and mutant 111 (lanes b' and d'). The fragments were hybridized with nick-translated, ^{32}P-labeled DNA of pANN205-222 (lanes a' and b'), pANN206 (lanes c' and d'), and pANN202-312 (lane e').

mutants that affect adhesin and hemolysin formation or hemolysin formation alone in *E. coli* 536 (Fig. 4).

DISCUSSION

The hemolysin determinant (*hly*) is widespread among *E. coli* strains. In particular, strains that cause extraintestinal infections in humans are often hemolytic. The frequent association of the Hly phenotype with specific O serotypes and hemagglutination antigens has been described previously (12, 17), and it has been suggested that the combination of these factors may be essential for virulence (7, 28; Hughes et al., in press). Yet, this statistical evidence does not exclude the possibility that properties such as hemolysin production and others are fortuitous characters of a pathogenic clone, the pathogenicity of it being determined by totally different, unknown properties. The cloning method has provided the possibility to isolate individual virulence factors and study their contribution to virulence in suitable animal systems.

The cloning of hemolysin determinants and hemagglutination antigens from *E. coli* strains causing urinary tract infections has been described previously (2, 9, 18), and recent investigations with isolated *hly* determinants from various sources have provided evidence for the direct contribution of hemolysin to the virulence of *E. coli* in extraintestinal infections (27; Hacker et al., submitted for publication). The hemolysin determinant of *E. coli* can be located on plasmids or the chromosome (10, 11, 21). The extended homology between both types of *hly* determinants (Müller et al., in press) suggests a common origin and the possible transition of such determinants from plasmids to the chromosome and vice versa.

The best-studied examples of such transitions are the antibiotic resistance genes of R-factors. They can be transposed as individual (i.e., Tn*1*, Tn*2*, Tn*3*, Tn*5*, Tn*9*, Tn*10*) or combined (i.e., Tn*4*) units to other extrachromosomal elements or the chromosome (1, 15). In addition, the total antibiotic resistance determinant can recombine in many cases as a block with other replicons (5). The latter mechanism is caused by specific IS elements that flank the resistance determinant on both ends as directly repeated sequences and trigger recombination with other replicons that carry a similar IS element as the site of recombination. A number of experiments performed to demonstrate the direct transposition of the *hly* determinant from various sources onto small replicons such as ColE1 or pSC101 failed completely (D. Müller and W. Goebel, unpublished data), but the observation of the frequent loss of chromosomal *hly* determinants from various UTI strains of *E. coli* suggested a specific structure. Two types of spontaneously occurring mutants were selected and described here, those that had lost hemolysin synthesis completely and those that failed to transport hemolysin across the outer membrane, but nevertheless retained normal levels of internal (periplasmic) hemolysin (18, 22). Both types of mutants are generated by deletions, as shown by hybridization of their chromosomal DNAs with recombinant plasmids that carry various parts or all of the plasmid *hly* determinant. All spontaneous mutants of the hemolysin synthesis-negative type were also deficient in the formation of the strain-specific hemagglutination antigen (Mrh-Vb), which suggested that these mutants have lost, in addition to the Hly-specific cistrons (19), neighboring sequences including part or all of the genes required for Mrh or its regulation. The other type of mutants showed smaller deletions that affected only that region of the *hly* determinant which was recently recognized (18) as being essential for the transport of hemolysin across the outer membrane (*hlyB*). None of these mutations affected the adhesin formation. Since the deletions of the latter mutants were all starting in the *hlyB*-adjacent part of the chromosome the genes for Mrh must be located on the opposite end of the *hly* determinant (e.g., upstream of *hlyC*; 9, 19). As possible sites that trigger these deletions, we identified a common sequence present in several restriction fragments of the

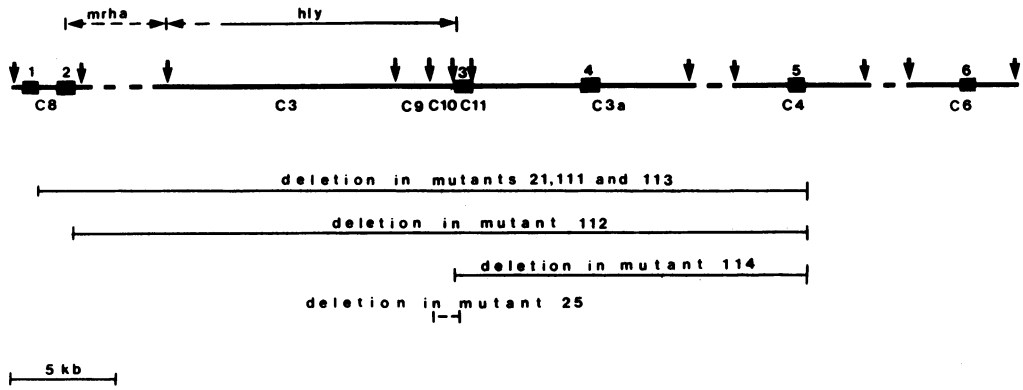

FIG. 4. Proposed mechanism for the DNA region involved in the hemolysin (*hly*) and adhesin (*mrh*) production of the *E. coli* strain 536. The main line represents part of the chromosome of strain 536 with *Hin*dIII fragments C3 through C11. The thin lines represent the lengths of the deletions, and the dotted lines indicate regions of unknown distance. The black boxes represent copies of the 500 to 1,200 base-pair sequence of pANN206.

chromosomal DNA of this UTI strain. These fragments were recognized by hybridization with a sequence of 600 to 1,200 base pairs that flanks the *hly* determinant of the plasmid pHly152 at the *hlyB*-distal end. The common sequence appears to be located on the chromosome of the UTI strain analyzed in the vicinity of both ends of the *hly* determinant. The large deletions that affect both hemolysin and adhesin synthesis could be caused by recombination between two such sites located on fragments C6 and that on C8 followed by the excision of the enclosed DNA region. The frequent loss of the *mrh* determinant together with the *hly* determinant during this deletion suggests a close linkage between both determinants on the chromosome, at least in the strain analyzed. The *hly* determinant of *E. coli* strains isolated from animal sources is frequently found on transmissible plasmids, whereas most UTI strains carry the hemolysin determinant on the chromosome. Recent studies on pHly152 and other Hly plasmids have provided evidence that all of these Hly plasmids seem to contain IS-like elements close to the *hly* determinant (30) which may be identical with the sequence recognized in this study. Similar or identical sequences have been also shown to surround the chromosomal *hly* determinants of other *E. coli* strains belonging to different O serotypes (Müller et al., in press). Whether the sequence identified here is an IS element (15, 25) is yet unknown.

The flanking by IS*1* elements has been shown for the heat-stable enterotoxin gene (23). Other IS-like sequences have been recently reported to flank heat-stable and heat-labile enterotoxin genes (29). The comparison of the partial nucleotide sequence obtained from the sequence of pHly152 (Goebel, unpublished data) with published IS sequences of IS*1*, IS2, IS*3*, and IS*4* does not show any homology. Thus, this sequence may be a specific element flanking hemolysin determinants.

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LITERATURE CITED

1. **Berg, C. M., and D. E. Berg.** 1982. Bacterial transposons, p. 107–116. *In* D. Schlessinger (ed.), Microbiology—1981. American Society for Microbiology, Washington, D.C.
2. **Berger, H., J. Hacker, A. Juarez, C. Hughes, and W. Goebel.** 1982. Cloning of the chromosomal determinants encoding hemolysin production and mannose-resistant hemagglutination in *Escherichia coli*. J. Bacteriol. **152**:1241–1247.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recominant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:2110–2114.
5. **Cohen, S. N., and D. J. Kopecko.** 1976. Structural evolution of bacterial plasmids: role of translocating genetic elements and DNA sequence insertions. Fed. Proc. **35**:1031–1036.
6. **De la Cruz, F., D. Müller, J. M. Ortiz, and W. Goebel.** 1980. Hemolysis determinant common to *Escherichia coli* hemolytic plasmids of different incompatibility groups. J. Bacteriol. **143**:825–833.
7. **Evans, D. T., D. G. Evans, C. Höhne, M. H. Noble, E. V. Haldane, H. Lior, and L. S. Young.** 1981. Hemolysin and K-antigens in relation to serotype and hemagglutination type of *Escherichia coli* isolated from extraintestinal infections. J. Clin. Microbiol. **13**:171–178.
8. **Evans, D. J., D. G. Evans, L. S. Young, and J. Pitt.** 1980. Hemagglutination typing of *Escherichia coli*: definition of seven hemagglutination types. J. Clin. Microbiol. **12**:235–242.
9. **Goebel, W., and J. Hedgpeth.** 1982. Cloning and characterization of the entire plasmid-encoded hemolysin determinant of *Escherichia coli*. J. Bacteriol. **151**:1290–1298.
10. **Goebel, W., B. Royer-Pokora, W. Lindenmaier, and H. Bujard.** 1973. Plasmids controlling synthesis of hemolysin

in *Escherichia coli*. I. Molecular properties. J. Bacteriol. **118**:964–973.

11. **Goebel, W., and H. Schrempf.** 1971. Isolation and characterization of supercoiled circular deoxyribonucleic acid from β-hemolytic strains of *Escherichia coli*. J. Bacteriol. **106**:311–317.
12. **Green, C. P., and V. L. Thomas.** 1981. Hemagglutination of human type O erythrocytes, hemolysin production, and serogrouping of *Escherichia coli* isolates from patients with acute pyelonephritis, cystitis, and asymptomatic bacteruria. Infect. Immun. **31**:309–315.
13. **Grinstedt, J., P. M. Bennett, S. Higginson, and M. H. Richmond.** 1978. Regional preference of insertion of Tn501 and Tn802 into RP1 and its derivatives. Mol. Gen. Genet. **166**:313–320.
14. **Hagberg, L., U. Jodal, T. Korhonen, G. Lidin-Janson, U. Lindberg, and C. Svanborg-Eden.** 1981. Adhesion, hemagglutination and virulence of *Escherichia coli* causing urinary tract infections. Infect. Immun. **31**:564–570.
15. **Kleckner, N.** 1981. Transposable elements in prokaryotes. Annu. Rev. Genet. **15**:341–404.
16. **Lederberg, E. M., and S. N. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119**:1072–1074.
17. **Minshew, B. H., J. Jorgensen, G. W. Counts, and S. Falkow.** 1978. Association of hemolysin production, hemagglutination of human erythrocytes and virulence for chicken embryos at extraintestinal *Escherichia coli* isolates. Infect. Immun. **20**:50–54.
18. **Noegel, A., U. Rdest, and W. Goebel.** 1981. Determination of the functions of hemolytic plasmid pHly152 of *Escherichia coli*. J. Bacteriol. **145**:233–247.
19. **Noegel, A., U. Rdest, W. Springer, and W. Goebel.** 1979. Plasmid cistrons controlling synthesis and excretion of the exotoxin α-hemolysin of *E. coli*. Mol. Gen. Genet. **175**:343–350.
20. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
21. **Smith, H. W., and S. Halls.** 1967. The transmissible nature of the genetic factor in *Escherichia coli* that controls haemolysin production. J. Gen. Microbiol. **47**:153–167.
22. **Springer, W., and W. Goebel.** 1980. Synthesis and secretion of Hemolysin by *Escherichia coli*. J. Bacteriol. **144**:53–59.
23. **So, M., F. Heffron, and B. J. McCarthy.** 1979. The *E. coli* gene encoding heat-stable toxin is a bacterial transposon flanked by inverted repeats of IS1. Nature (London) **277**:453–456.
24. **Southern, E. M.** 1975. Detection of specific sequences among DNA-fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
25. **Starlinger, P., and H. Saedler.** 1976. IS-elements in microorganisms. Curr. Top. Microbiol. Immunol. **63**:112–152.
26. **Svanborg-Eden, C., B. Eriksson, and L. A. Hanson.** 1977. Adhesion of *Escherichia coli* to human uroepithelial cells in vitro. Infect. Immun. **18**:767–774.
27. **Welch, R. A., E. P. Dellinger, B. Minshew, and S. Falkow.** 1981. Haemolysin contributes to virulence of extraintestinal *E. coli* infections. Nature (London) **294**:665–667.
28. **Van den Bosch, J. F., P. Postma, J. de Graaff, and D. M. MacLaren.** 1981. Haemolysis by urinary *Escherichia coli* and virulence in mice. J. Med. Microbiol. **14**:321–331.
29. **Yamamoto, T., and T. Yokota.** 1981. *Escherichia coli* heat-labile enterotoxin genes are flanked by repeated deoxyribonucleic acid sequences. J. Bacteriol. **145**:850–860.
30. **Zabala, J. C., F. de la Cruz, and J. M. Ortiz.** 1982. Several copies of the same insertion sequence are present in alpha-hemolytic plasmids belonging to four different incompatibility groups. J. Bacteriol. **151**:472–476.