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Multiple Copies of Hemolysin Genes and Associated Sequences in the Chromosomes of Uropathogenic *Escherichia coli* Strains

STEFAN KNAPP, JÖRG HACKER, IRENE THEN, DOROTHEE MÜLLER, AND WERNER GOEBEL*
Institut für Genetik und Mikrobiologie der Universität Würzburg, 8700 Wurzburg, Federal Republic of Germany

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The O6 serogroup *Escherichia coli* strain 536 carries two hemolysin (*hly*) determinants integrated into the chromosome. The two *hly* determinants are not completely identical, either functionally or structurally, as demonstrated by spontaneous deletion mutants carrying only one of them and by cloning each of the two determinants separately into cosmid vectors. Each *hly* determinant is independently deleted at a frequency of 10^{-4}, leading to variants which exhibit similar levels of internal hemolysin but different amounts of secreted hemolysin. The two *hly* determinants were also identified in the O4 *E. coli* strain 519. The three *E. coli* strains 251, 764, and 768, which belong to the serogroup O18, and the O4 strain 367 harbor a single chromosomal *hly* determinant, as demonstrated by hybridization with *hly*-gene-specific probes. However, a hybridization probe derived from a sequence adjacent to the *hlyC*-proximal end of the plasmid pHly152-encoded *hly* determinant hybridizes with several additional chromosomal bands in hemolytic O18 and O6 *E. coli* strains and even in *E. coli* K-12. The size of the probe causing the multiple hybridization suggests a 1,500- to 1,800-base pair sequence directly flanking *hlyC*. Spontaneous hemolysin-negative mutants were isolated from strains 764 and 768, which had lost the entire *hly* determinant but retained all copies of the *hlyC*-associated sequence. This sequence is not identical to a previously identified (J. Hacker, S. Knapp, and W. Goebel, J. Bacteriol. 154:1145–1154, 1983) somewhat smaller (about 850 base pairs) sequence flanking the other ($hlyB_b$-proximal) end of the plasmid pHly152-encoded *hly* determinant which, as shown here, exists also in multiple copies in these hemolytic *E. coli* strains and in at least two copies in *E. coli* K-12. In contrast to the plasmid-encoded *hly* determinant which is directly flanked at both ends by these two different sequences, the chromosomal *hly* determinants are not immediately flanked by such sequences.

A high percentage of *Escherichia coli* strains isolated from patients with pyelonephritis secrete hemolysin (10, 13, 17). The synthesis of this protein in *E. coli* is determined by genes which are either plasmid encoded or part of the chromosome (1, 7, 14, 22). Both types of *hly* determinant consist of four genes which we designated *hlyC*, *hlyA*, $hlyB_a$, and $hlyB_b$ (12, 20, 25). These determinants are structurally and functionally closely related (5, 18). Restriction analysis of several of the relatively large *hly* determinants (7 kilobases [kb] not including flanking sequences [1, 7, 9]) have been performed (1, 5, 18, 19).

Previous data indicated that the hemolytic *E. coli* strain 536, which carries the *hly* determinant on the chromosome, also contains several chromosomal copies of a short sequence which flanks the $hlyB_b$-proximal end of the plasmid-encoded *hly* determinant. Experimental evidence has been obtained which suggests (9) that these sequences cause frequently occurring deletions of the chromosomal *hly* determinant in *E. coli* 536 (9). It has also been shown that some of these deletions lead, in addition, to the loss at a rather high rate of mannose-resistant hemagglutination (*mrh*), a property associated with specific protein fimbriae which are considered as specific adhesins of uropathogenic *E. coli* strains, allowing the colonization of the bacteria on the epithelial cells of the urogenital tract (6, 11, 24). The common loss of these two virulence properties suggests a close genetic linkage of the determinants for hemolysin synthesis and adhesin formation (K. B. Low and S. Falkow, personal communication).

In this paper we present evidence for the existence of two copies of the entire *hly* determinant in the chromosomes of some hemolytic *E. coli* strains and of multiple copies of a sequence which is found at the *hlyC*-proximal end of the plasmid-encoded *hly* determinant. It is shown that this sequence of about 1,500 to 1,800 base pairs (bp) is not identical to the sequence flanking the other (*hly*B-distal) end of the plasmid *hly* determinant.

MATERIALS AND METHODS

Bacteria. The wild-type strains used are listed in Table 1. For transformation the *E. coli* K-12 strain HB101 (H. Boyer) was used. The recombinant plasmids used here are listed in Table 2.

Media, chemicals, and enzymes. Cultures were grown in enriched nutrient broth or in alkaline broth extract (Oxoid, Basingstoke, England). Radiochemicals were purchased from New England Nuclear Corp., Boston, Mass. Antibiotics used were a gift from Bayer, Leverkusen, Federal Republic of Germany. All other chemicals were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany. Restriction enzymes and T4 ligase were purchased from New England Biolabs, Beverly, Mass. DNA polymerase I was obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany.

Isolation of chromosomal and plasmid DNA. Chromosomal DNA was isolated as described earlier (9). Plasmid DNA from clones carrying recombinant DNA was screened by the cleared lysate procedure (2), and preparative DNA isolation was achieved as described (8).

Nick translation. Plasmids were labeled by nick translation with a mixture of all four [α-^{32}P]dNTPs as described previously (21) and were purified by ethanol precipitation.

Cleavage with restriction enzymes and electrophoresis of chromosomal DNA. The chromosomal DNA was treated with appropriate restriction enzymes, and the resulting fragments were separated by agarose gel electrophoresis with 0.7 to 1.0% gels as described previously (9).

* Corresponding author.

TABLE 1. Hemolytic *E. coli* wild-type strains and Hly⁻ mutants used in this study

Strain	Hemolysin production	O serogroup	Origin	Reference(s)
367	+	O4	UTI[a]	1
519	+	O4	UTI	This work
536	+	O6	UTI	1, 9
536-225	(+)[b]	O6	UTI	This work
536-14	(+)[b]	O6	UTI	This work
764	+	O18ac	Fecal	1
764-2	−	O18ac	Fecal	This work
768	+	O18ac	Fecal	18
768-2	−	O18ac	Fecal	This work
768-3	−	O18ac	Fecal	This work
251	+	O18ac	UTI	This work

[a] UTI, Urinary tract infection.
[b] Strains showing a reduced level of internal or external hemolysin or both.

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 (23). The filters were hybridized in 50% formamide at 43°C for 3 days. Stringent hybridization conditions were used for the washing procedure.

Transformation. *E. coli* K-12 strains were transformed by the $CaCl_2$ method. Wild-type strains were transformed by a modified $CaCl_2$ procedure (15).

Hemolysin production. Erythrocyte lysis was detected on meat agar plates containing washed human erythrocytes and confirmed in a liquid assay (25).

Cosmid cloning procedure. Recombinant DNA was packaged in vitro as described previously (1, 3, 4).

RESULTS

Identification of two functional *hly* determinants in the chromosome of uropathogenic *E. coli* strains. To determine the number of *hly* copies present in the chromosome of hemolytic *E. coli* strains, we cleaved chromosomal DNA of 13 uropathogenic *E. coli* strains with *Sal*I, which does not cleave any of the known *hly* determinants (1, 5, 18), and hybridized the DNA with the *hly*-specific probes pANN202 (which carries *hlyC*), pANN215 (which includes most of *hlyA*), and pANN250 (which carries the C-terminal half of *hlyA* and the functional $hlyB_a$ gene). These *hly* gene probes were derived from the Hly plasmid pHly152 (7, 20, 25). Although the DNA from 11 of these strains hybridized only in one *Sal*I fragment (data not shown), indicating a single *hly* determinant, the two strains 536 (O6) and 519 (O4) gave positive hybridization signals with all three probes in two large *Sal*I fragments which in the case of 536 were 20.2 and 15.6 kb long, indicating the presence of two *hly* determinants in the chromosome (Fig. 1, lanes 1, 5, 9, and 4, 8, 12). To test whether both are functionally active, we constructed a gene bank of strain 536 by cloning large partial *Sau*3A fragments (average size, 40 kb) into the cosmid vector pHC79. Among several thousand colonies, we identified 50 which were Hly⁺. From these Hly⁺ clones we obtained two types of recombinant cosmids exemplified by pANN522 and pANN523 (Fig. 2), which were found by DNA probe analysis to differ in the level of hemolysin expression (Table 2) and in small *Hin*dIII fragments measuring 1.0 and 1.1 kb, respectively. In addition, hybridization occurred in two larger *Hin*dIII fragments (10.3 and 1.6 kb) when *Hin*dIII-cleaved DNA of these recombinant cosmids and *Hin*dIII-cleaved chromosomal DNA from the wild-type strains were hybridized with a *hly*-specific probe (pANN260). This probe spans the region from the *Eco*RI site at 3.9 kb to the *Pst*I site at 8.1 kb (Fig. 3) of the *hly* determinant (25) and thus includes part of the structural *hlyA* gene, $hlyB_a$ and $hlyB_b$. *Hin*dIII-cleaved chromosomal DNA from strain 536 hybridized with the same probe in both small *Hin*dIII fragments (Fig. 2, lane 3) in addition to the two larger *Hin*dIII fragments.

As previously reported (9), strain 536 yields nonhemolytic mutants at a relatively high rate (about 10^{-3} to 10^{-4}), and these mutants have apparently lost both *hly* determinants since none of the *hly*-specific probes hybridized with the chromosomal DNA of these mutants.

Among several thousand colonies of strain 536, we detected yet another type of mutant which exhibited a reduced level of external hemolysin synthesis, as readily visualized by producing smaller hemolysis zones on blood agar. Some

TABLE 2. Recombinant plasmids used

Plasmid	*hly* cistrons and resistance markers encoded[a]	Reference
pANN104	*hlyC*, part of *hlyA* (pHly152), Cm^r	19
pANN202	*hlyC*, part of *hlyA* (pHly152), Cm^r	19
pANN215	Part of *hlyA* (pHly152), Cm^r	19
pANN250	Part of *hlyA*, $hlyB_a$ (pHly152), Tc^r	19
pANN260	Part of *hlyA*, $hlyB_a$, $hlyB_b$, Tc^r	Unpublished
pANN522	*hlyC*, *hlyA*, $hlyB_a$, $hlyB_b$ of 536 (O6), Ap^r (cosmid)	Unpublished
pANN5211	*hlyC* (pHly152), *hlyA*, $hlyB_a$, $hlyB_b$ of 536 (O6), Cm^r	1
pANN523	*hlyC*, *hlyA*, $hlyB_a$, $hlyB_b$ of 536 (O6), Ap^r (cosmid)	Unpublished
pANN205-222	$hlyB_a$, $hlyB_b$ (pHly152), Ap^r	12
pANN601	Part of *hlyC* (pHly152), Ap^r	Unpublished
pANN603	Sequence proximal to $hlyB_b$ (pHly152)	Unpublished

[a] Resistance markers: Ap^r, ampicillin; Cm^r, chloramphenicol; Tc^r, tetracycline.

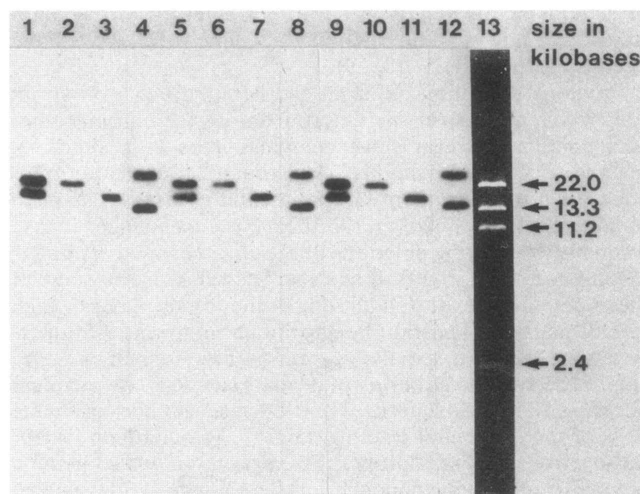

FIG. 1. Hybridization patterns of *Sal*I-cleaved chromosomal DNA from *E. coli* 536 (lanes 1, 5, 9), mutant 536-225 (lanes 2, 6, 10), mutant 536-14 (lanes 3, 7, 11), and wild-type strain 519 (lanes 4, 8, 12). The DNA fragments of lanes 1 through 12 were hybridized with nick-translated, ^{32}P-labeled DNA of pANN202 (lanes 1 through 4), pANN215 (lanes 5 through 8), and pANN250 (lanes 9 through 12). As size markers, *Bgl*II fragments of λ DNA were used (lane 13).

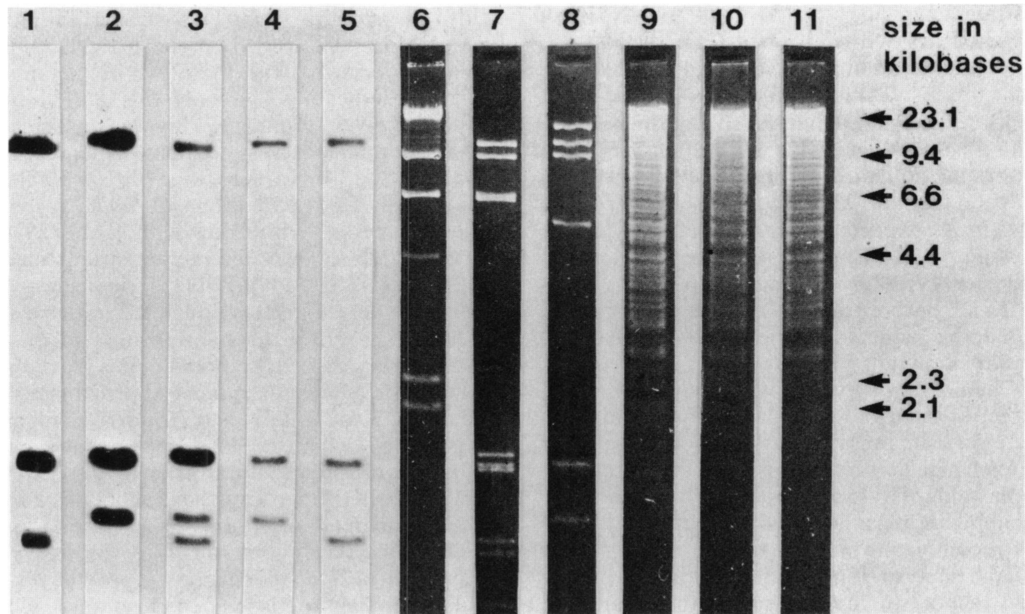

FIG. 2. Hybridization and restriction patterns of *Hin*dIII-cleaved chromosomal DNA of *E. coli* 536 (lanes 3 and 9), mutant 536-14 (lanes 4 and 10), mutant 536-225 (lanes 5 and 11), and *Hin*dIII-cleaved cosmid clones pANN522 (lanes 1 and 7) and pANN523 (lanes 2 and 8). As the hybridization probe, nick-translated, ^{32}P-labeled pANN260 (see text) was used. As size markers, *Hin*dIII fragments of λ DNA were used (lane 6).

of these mutants lacked either of the two *hly* determinants. This was demonstrated by hybridization of *hly*-specific probes with *Sal*I-cut chromosomal DNA. Chromosomal DNA of mutant 536-225 hybridized with all *hly*-specific probes (pANN250 and pANN215) only in the larger of the *hly*-specific *Sal*I-fragments of strain 536 (Fig. 1, lanes 2, 6, and 10), whereas mutant 536-14 hybridized only in the smaller *Sal*I fragment (Fig. 1, lanes 3, 7, and 11). When the chromosomal DNA from these two mutants was cleaved with *Hin*dIII and hybridized as described above with pANN260, hybridization was observed between chromosomal DNA from mutant 536-225 and the probe with the small *Hin*dIII fragment of 1.0 kb and between chromosomal DNA from mutant 536-14 and the probe with the slightly larger 1.1-kb fragment (Fig. 2, lanes 4 and 5). DNAs from both mutants hybridized again in the two larger chromosomal *Hin*dIII fragments (10.3 and 1.6 kb). These data indicate that the small 1.0-kb *Hin*dIII fragment belongs to the *hly* determinant which is cloned in the recombinant cosmid pANN522 and lost in mutant 536-14, whereas the 1.1-kb *Hin*dIII fragment belongs to the *hly* determinant of recombinant cosmid pANN523 which is deleted in mutant 536-225.

To test whether both *hly* determinants express the same level of hemolysin, we determined internal and external hemolysin levels from the mutants 536-14 and 536-225 and

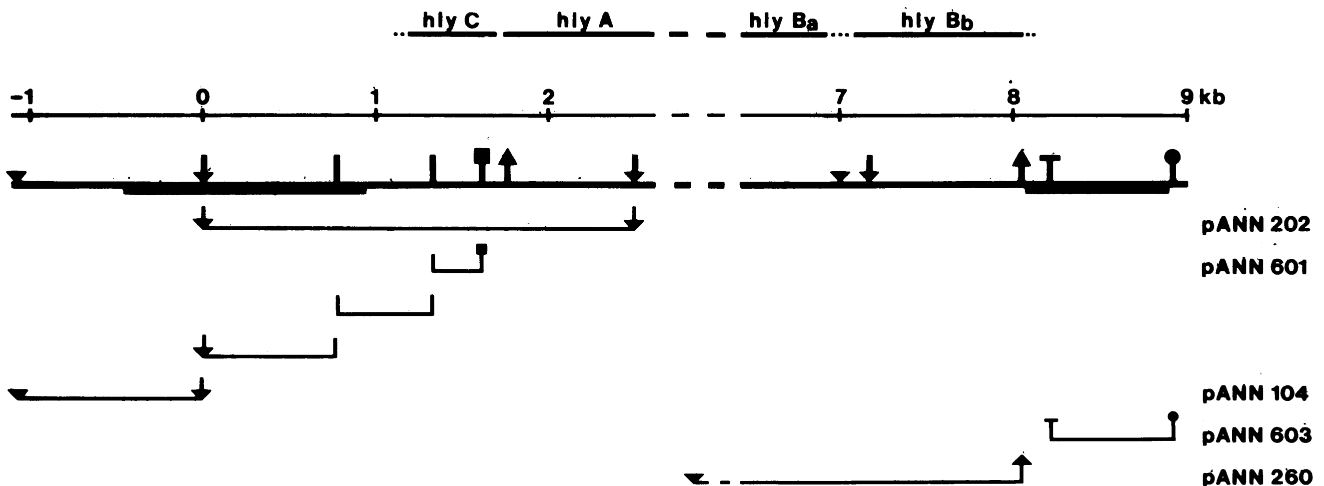

FIG. 3. Physical map of the *hlyC* and *hlyB*$_b$ proximal regions of the hemolysin determinant of pHly152. The coordinates of the *hly* determinant have been previously described (7). The thick bars represent the lengths and approximate locations of the flanking sequences. Symbols: ▼ (flag), *Bam*HI; ● (lollipop), *Bgl*II; ▼, *Eco*RI; |, *Hae*III; ↓, *Hin*dIII; ↑, *Pst*I; T, *Sau*3A.

from *E. coli* strains carrying the cosmids pANN522 or pANN523 in a liquid assay as described (25). In this test, mutant 536-225 exhibited amounts of internal and external hemolysin similar to those exhibited by the wild-type strain 536, although both strains differed in the size of the hemolysin zones on blood agar plates (Table 3). Mutant 536-14, in contrast, showed in the liquid test a considerably lower level of internal hemolysin than the wild-type strain and very little external hemolysin. As expected, *E. coli* K-12 carrying the recombinant cosmid pANN522 expressed the same level of internal and external hemolysin as mutant 536-225. Likewise, *E. coli* K-12 harboring the recombinant cosmid pANN523 yielded the same low amount of internal and external hemolysin as mutant 536-14.

Expression of hemolysin is primarily regulated by a promoter (p1) located in front of *hlyC* (25; A. Juarez and W. Goebel, J. Bacteriol., in press). This promoter region in pANN523 was exchanged with the corresponding promoter region of the plasmid pHly152-encoded *hly* determinant, yielding the recombinant plasmid pANN5211 (1). *E. coli* K-12 carrying this recombinant plasmid synthesizes a similar amount of internal hemolysin to that of the wild type, but the level of external hemolysin remains rather low (Table 3), suggesting that this *hly* determinant is also impaired in its functions required for excretion of hemolysin (25). We therefore complemented this strain with the recombinant plasmid pANN205-222, which as shown before carries two functions required for excretion of hemolysin (25). This complementation brings up the level of external hemolysin of *E. coli* pANN5211 almost to wild-type level (Table 3), indicating that one of the two *hly* determinants of strain 536 (cloned in pANN523) is impaired in *hlyB*$_a$ or *hlyB*$_b$ or both.

Multiple copies of an *hlyC*-adjacent sequence in the chromosome of uropathogenic *E. coli* strains. The hemolytic *E. coli* strains 251, 764, and 768 belong to serogroup O18 and carry the *hly* determinant, as most pyelonephritic *E. coli* strains do, on the chromosome (1, 10, 18). Chromosomal DNA from these strains cleaved with *Sal*I and hybridized with the *hlyC*-specific probe pANN202 gave positive signals from several *Sal*I fragments (Fig. 4). The hybridization patterns were rather similar in all three independently isolated *E. coli* strains. The only visible difference was a small *Sal*I fragment of 2.6 kb which hybridized in the DNA of strain 768 but not in that of strains 251 and 764. The *Sal*I fragment of 4.8 kb is probably a plasmid related to the vector (pACYC184) since vector DNA alone hybridized with this fragment but not with the others (Fig. 4). To test whether all other *Sal*I fragments which specifically hybridize with the *hlyC* probe represent complete *hly*-determinants or part of it, we hybridized *Sal*I-cut DNA of the three O18 strains with the *hlyA* probe pANN215 and the *hlyB*$_a$ probe pANN250. Only *Sal*I fragment 5 hybridized with these two probes in all three strains (Fig. 4), indicating that only this *Sal*I fragment carries a complete *hly* determinant. This was confirmed by the analysis of spontaneous Hly$^-$ mutants obtained from strains 764 and 768 (Fig. 4). In the three Hly$^-$ mutants tested (764-2, 768-2, and 768-3) *Sal*I fragment 5 was absent, but all other *Sal*I fragments hybridizing with pANN202 (Fig. 3) were retained. DNAs from the mutant strains failed to hybridize with pANN250 and pANN215, indicating that again the entire *hly* determinant was deleted in these strains.

TABLE 3. Amount of internal and external hemolysin produced by the wild-type strain 536 the mutants 536-14 and 536-225 and *E. coli* K-12 carrying the cloned *hly* determinants of this strain

Strain	Hemolysin activity (U)[a]	
	internal	external
E. coli 536	60	58
Mutant 536-225	60	53
Mutant 536-14	12	5
E. coli K-12(pANN522)	56	53
E. coli K-12(pANN523)	11	4
E. coli K-12(pANN5211)	61	4
E. coli(pANN5211) (pANN205-222)	61	48

[a] The determination of the hemolytic activity and the definition of hemolytic units were as described (25).

To test whether the hybridization in the other six *Sal*I fragments is caused by homology with the structural *hlyC* gene or the adjacent sequence approximately 1 kb upstream from *hlyC* also located on the *Hin*dIII-E fragment (19) cloned in pANN202, we used as a probe a fragment which carried only part of the structural gene for *hlyC* (pANN601). This *Hae*III-*Bam*HI fragment (Fig. 3) contains a 280-bp sequence located upstream of the *Bam*HI site, and thus it carries part of the structural gene for *hlyC* exclusively. As shown previously (12), *hlyC* encodes a protein of 18,000 daltons, and the *Bam*HI site is located close to the C-terminal end of *hlyC* (M. Vogel and W. Goebel, unpublished results). When this probe was used for hybridization to the *Sal*I-cleaved chromosomal DNA of the three strains, hybridization was observed only with *Sal*I fragment 5 (Fig. 5) and not with the other *Sal*I fragments hybridizing with pANN202. This suggests that the homology between the other six (7) *Sal*I fragments and pANN202 is not caused by homology with the structural *hlyC* gene.

When the 575-bp *Hae*III fragment located farther upstream from the 280-bp *Hae*III-*Bam*HI fragment (Fig. 3) was used as the hybridization probe, weak hybridization was observed in all fragments which also hybridized with pANN202 (Fig. 5), indicating that at least part of the homology between the chromosomal *Sal*I fragments and pANN202 is located on this *Hae*III fragment. The next *Hae*III-*Hin*dIII fragment upstream (750 bp; Fig. 3) adjacent to the 575-bp *Hae*III fragment of pANN202 yielded strong hybridization with all chromosomal *Sal*I fragments except *Sal*I fragment 5, which as shown above carries the *hly* determinant of these strains (Fig. 5). The size of the sequence which causes this multiple hybridization was further narrowed down by hybridization of the *Sal*I-cleaved chromosomal DNA with the adjacent 1,060-bp *Hin*dIII-*Eco*RI fragment (Fig. 3) from pHly152 (19). This fragment showed again strong hybridization in the same *Sal*I fragments as the 750-bp *Hae*III-*Hin*dIII probe, but an *Eco*RI-*Sau*3A fragment which originates 250 bp downstream from the *Eco*RI site no longer hybridized with these chromosomal *Sal*I fragments (data not shown). This indicates that the sequence ends at least 250 bp before the *Eco*RI site. The described data suggest that a sequence outside of the structural *hlyC* gene but close to *hlyC* on plasmid pHly152, and hence at one end of the *hly* determinant of plasmid pHly152, is causing the hybridization of multiple chromosomal fragments from the hemolytic O18 *E. coli* strains. The failure of *Sal*I fragment 5, which carries the *hly* determinant (see above), to hybridize with the sequence-specific 750-bp *Hin*dIII-*Hae*III fragment strongly suggests that this sequence does not directly flank the chromosomal *hly* determinant of the O18 strains, as seems to be the case for the plasmid pHly152 *hly* determinant. Since the 575-bp *Hae*III fragment hybridizes weakly with *Sal*I fragment 5 and the other pANN202-homologous *Sal*I fragments, one can conclude that this fragment carries,

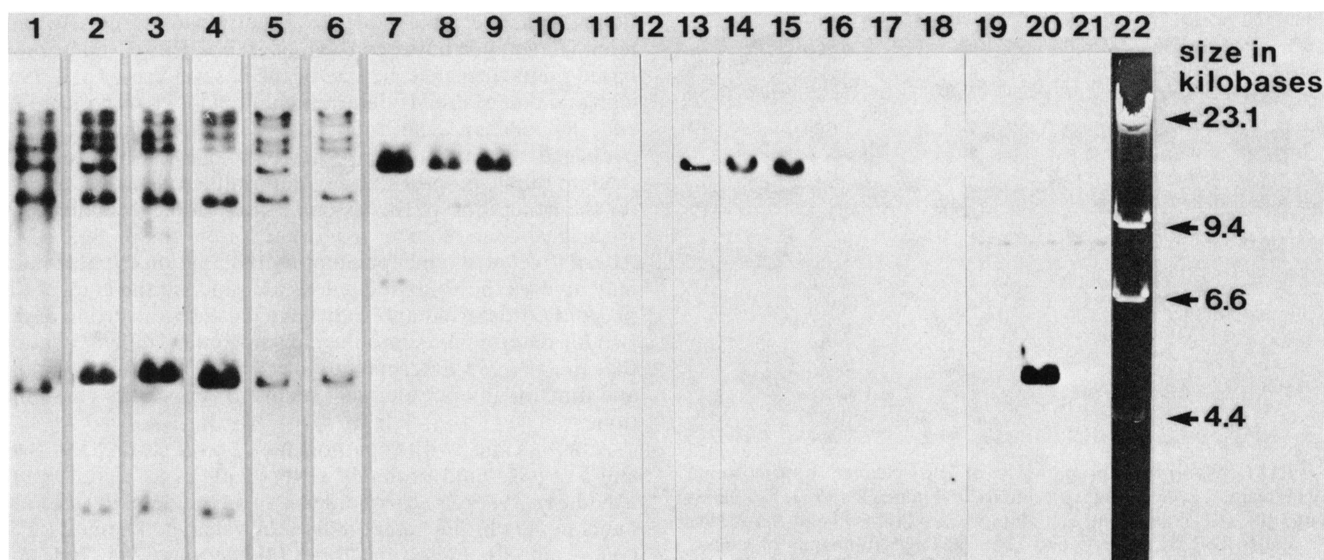

FIG. 4. Hybridization patterns of *Sal*I-cleaved chromosomal DNA of *E. coli* 251 (lanes 1, 7, 13, 19), 768 (lanes 2, 8, 14, 20), 764 (lanes 5, 9, 15, 21), and mutants 768-2 (lanes 3, 10, 16), 768-3 (lanes 4, 11, 17), and 764-2 (lanes 6, 12, 18). The DNA fragments in lanes 1 through 21 were hybridized with nick-translated, ^{32}P-labeled DNA of pANN202 (lanes 1 through 6), *Hin*dIII fragment C from pANN215 (lanes 7 through 12), and *Eco*RI fragment G from pANN250 (lanes 13 through 18) and vector pACYC184 (lanes 19, 20, 21). As size markers, *Hin*dIII fragments of λ DNA were used (lane 22).

as expected, the N-terminal end of *hlyC* and one end of the sequence which yields the multiple hybridization with the chromosomal DNA of the O18 strains. The sizes of the hybridizing fragments together with the hybridization intensities observed in the chromosomal *Sal*I fragments with these probes suggest a size in the order of 1,600 to 1,800 bp for this sequence.

We have previously reported that a sequence of about 850 bp located at the other ($hlyB_b$-proximal; Fig. 3) end of the plasmid pHly152-encoded *hly* determinant also shows multiple hybridization with the chromosomal DNA of the hemolytic O6 *E. coli* 536 (9). This sequence seems to be involved in the deletion events which affect the two *hly* determinants of this strain (9; see also above). Hybridization of the $hlyB_b$-proximal sequence with the above identified *hlyC*-proximal sequence does not occur (data not shown), indicating that the two sequences, although flanking the ends of the same *hly* determinant (in plasmid pHly152), are unrelated.

We next asked whether both end sequences of the pHly152-derived *hly* determinant occur in the chromosome of the described hemolytic strains in multiple copies. For this we used the two O18 strains, which (as shown above) contain multiple copies of the *hlyC*-proximal sequence; the O6 strains 401 and 536 were used as controls. We have already demonstrated the multiple occurrence of the $hlyB_b$-proximal sequence for strain 536 (9). Chromosomal DNAs of these strains were cut with *Pst*I (the two sequences do not contain internal *Pst*I sites) and hybridized with probes carrying internal fragments of one or the other sequence.

The data shown in Fig. 6 demonstrate the following. (i) All tested hemolytic *E. coli* strains carry several copies of the *hlyC*-proximal and the $hlyB_b$-proximal sequence. Probes of these two proximal sequences hybridize with different chromosomal fragments, which shows again that the two sequences are unrelated. (ii) Both sequences occur in the chromosome of *E. coli* K-12 in six copies for the *hlyC*-proximal sequence and two copies for the $hlyB_b$-proximal sequence. These copy numbers are based on the assumption that the sequences in *E. coli* K-12 also do not contain *Pst*I sites. (iii) In contrast to the deletion of the *hly* determinants in *E. coli* 536, in which the involvement of the $hlyB_b$-proximal sequence has been demonstrated (9), no such involvement can be shown for the *hly* deletion mutants 768-2 and 764-2 since hybridization of the *Pst*I-cut chromosomal

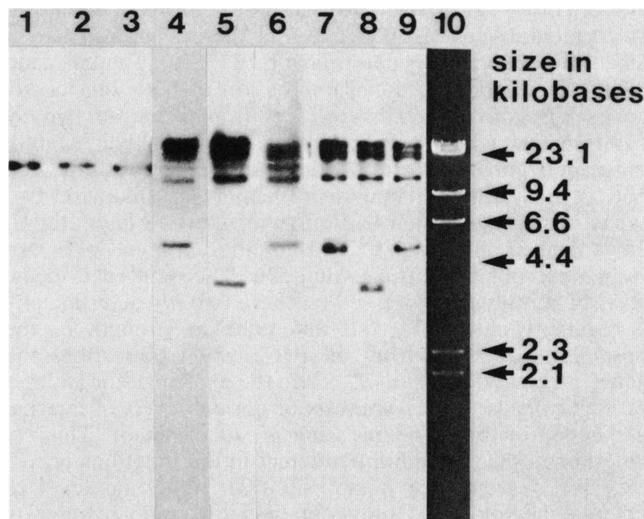

FIG. 5. Hybridization patterns of *Sal*I-cleaved chromosomal DNA from *E. coli* 251 (lanes 1, 4, 7), 768 (lanes 2, 5, 8), and 764 (lanes 3, 6, 9). The DNA fragments of lanes 1 through 9 were hybridized with nick-translated, ^{32}P-labeled DNA of the 280-bp *Bam*HI-*Hae*III fragment of pANN202 (lanes 1 through 3), the adjacent 575-bp *Hae*III fragment (lanes 4 through 6), and the next 750-bp *Hae*III fragment (lanes 7 through 9). As size markers, *Hin*dIII fragments of λ DNA were used (lane 10). Explanations for the hybridization probes are given in the text.

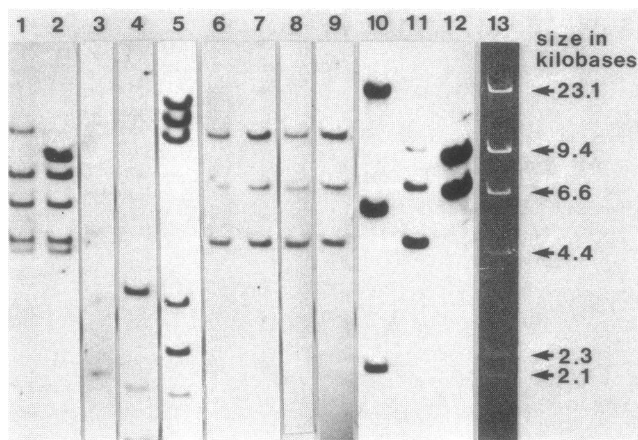

FIG. 6. Hybridization patterns of *Pst*I-cleaved chromosomal DNA from *E. coli* 768 (lanes 1 and 6), 764 (lanes 2 and 7), 536 (lanes 3 and 10), 401 (lanes 4 and 11), mutants 768-2 (lane 8) and 764-2 (lane 9), and *E. coli* 5K (lanes 5 and 12). The DNA fragments of lanes 1 through 12 were hybridized with nick-translated, ^{32}P-labeled DNA of the *Eco*RI-*Hin*dIII fragment in pANN104 (Fig. 3) (lanes 1 through 5) and the *Sau*3A-*Bgl*II fragment from pANN603 (Fig. 3) (lanes 6 through 12). As size markers, *Hin*dIII-cleaved λ DNA was used (lane 13).

DNA from both mutants with the probe specific for the *hlyB*$_b$-proximal sequence yields the same pattern as the corresponding wild-type strains.

DISCUSSION

The results presented in this report indicate that hemolytic *E. coli* strains with chromosomal *hly* determinants can carry more than one hemolysin determinant integrated in the chromosome. At least two *hly* determinants were detected in two different *E. coli* isolates, one belonging to serogroup O6 and the other to O4. We chose *Sal*I-cut chromosomal DNA since previous results indicated that there is no *Sal*I site in any of the analyzed *hly* determinants (1, 7, 19). There is good evidence for the functional expression of both sets of *hly* genes in *E. coli* 536. We have cloned from this strain two *hly* determinants which can be distinguished by their slightly different *Hin*dIII patterns, and we could demonstrate that both express different amounts of hemolysin. Likewise, two types of mutants which had either of the two *hly* determinants deleted and which expressed different levels of hemolysin were obtained from strain 536. The difference in the level of hemolysin expressed by these two *hly* determinants is probably caused by the difference in strength of the promoter located in front of *hlyC*, which transcribes the genes *hlyC*, *hlyA*, and *hlyB*$_a$, since the exchange for another promoter leads to the synthesis of normal levels of internal hemolysin in the low-expressing *hly* determinant. This *hly* determinant has in addition a defect in the functions necessary for excretion of hemolysin (25); a normal level of external hemolysin is only obtained by complementation with a recombinant plasmid carrying the genes required for excretion. Both determinants are lost at roughly the same frequency. We assume, therefore, that the deletion is caused in both cases by a similar mechanism. Since both *hly* determinants are highly homologous in their nucleotide sequence, homologous recombination could lead to the loss of either one (all wild-type strains studied are Rec$^+$).

The distance between the two *hly* determinants on the chromosome is not yet known. No cosmid clone was obtained which carried both *hly* determinants, suggesting a minimal distance between them of 20 kb. This assumption is based on the fact that the size of the cosmid vector pHC79 is 6 kb and that of the *hly* determinant about 7 kb (not including end sequences). The total size of DNA which can be packaged into the phage λ head is 45 kb. This makes a tandem duplication of one *hly* determinant as a mechanism for the generation of the second rather unlikely, contrary to what has been shown for the cholera toxin gene (16). Alternatively, two independent recombination events which may involve the short sequences identified at the ends of the plasmid *hly* determinants may have led to the insertion of the two *hly* determinants into the chromosome. The observation that the two *hly* determinants of strain 536 are structurally and functionally not identical seems to support this assumption.

Although the two uropathogenic *E. coli* strains 536 (O6) and 519 (O4) (and probably several others as well; unpublished observations) carry at least two complete *hly* determinants in the chromosome, most other analyzed hemolytic *E. coli* strains (including the three O18 strains, 764, 768, and 251 and the O4 strain 367, which were described here in detail) possess only a single *hly* determinant. However, all analyzed hemolytic strains show hybridization in several chromosomal *Sal*I fragments to probes which carry fragments adjacent to the two proximal *hly* genes, i.e., *hlyC* and *hlyB*$_b$ (25).

As shown here, the hybridization of chromosomal DNA with a *hlyC*-proximal probe is caused by a sequence of 1,500 to 1,800 bp located directly upstream of *hlyC* on plasmid pHly152. A somewhat smaller sequence (about 850 bp) was shown previously (9) to be located on the other (*hlyB*$_b$-proximal) end of the plasmid pHly152-encoded *hly* determinant. Both sequences occur in multiple copies in the chromosome of the *E. coli* hemolytic wild-type strains and even in *E. coli* K-12. Comparing the restriction patterns and the partial DNA sequences which have been obtained (M. Vogel, G. Michel, and W. Goebel, unpublished results) from both sequences, no similarities to the known insertion sequence elements of *E. coli* have been detected (I. Then and S. Knapp, unpublished observations). The newly identified sequence at the *hlyC*-proximal end of the plasmid-encoded *hly* determinant does not hybridize with the sequence at the *hlyB*-proximal end of the same *hly* determinant, indicating that both are entirely different. We have shown previously that most plasmid-borne hemolytic determinants studied exhibit strong homology in all four structural genes as well as in the flanking regions of the *hly* determinant, suggesting that in general, plasmid-encoded *hly* determinants are flanked by these two different short sequences. Their function is at present unknown, but the involvement in the deletion events which lead to the elimination of one or both *hly* determinants in *E. coli* 536 has been demonstrated (9). It is interesting to speculate that these sequences may be essential for transfer of the *hly* determinant between hemolytic plasmids and the chromosome or vice versa.

In contrast to the plasmid-encoded *hly* determinants, which seem to be directly flanked at both ends by these sequences, the chromosomal *hly* determinants do not carry these sequences directly at the ends of the proximal genes, e.g., *hlyC* or *hlyB*$_b$. This is indicated by the observation that hybridization with probes specific for these sequences does not occur in those chromosomal *Sal*I fragments which hybridize with *hly* gene-specific probes. However, at least some of the copies seem to be located relatively close to the *hly* determinants (S. Knapp, unpublished data). One of these

two end sequences flanking the plasmid-encoded *hly* determinant may well be identical with the as yet poorly characterized insertion sequence element IS*91* described by Zabala et al. (26). This sequence was identified on several hemolytic plasmids, including pHly152, and was shown to be transposable to pACYC184.

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ADDENDUM

Subsequent DNA sequence analysis of the *hlyC* flanking sequence revealed its identity with IS*2*.

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