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

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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 O4 *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-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, 8, 14, 22). Both types of hly determinant consist of four genes which we designated hlyC, hlyA, hlyB, and hlyB6 (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 hlyB6-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* strain 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 (hlyB-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 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-32P]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\textsuperscript{−} mutants used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hemolysin production</th>
<th>O serogroup</th>
<th>Origin</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>367</td>
<td>+</td>
<td>O4</td>
<td>UTI\textsuperscript{a}</td>
<td>1</td>
</tr>
<tr>
<td>519</td>
<td>+</td>
<td>O4</td>
<td>UTI</td>
<td>This work</td>
</tr>
<tr>
<td>536</td>
<td>+</td>
<td>O6</td>
<td>UTI</td>
<td>1, 9</td>
</tr>
<tr>
<td>536-225</td>
<td>(+)\textsuperscript{b}</td>
<td>O6</td>
<td>UTI</td>
<td>This work</td>
</tr>
<tr>
<td>536-14</td>
<td>(+)\textsuperscript{b}</td>
<td>O6</td>
<td>UTI</td>
<td>This work</td>
</tr>
<tr>
<td>764</td>
<td>+</td>
<td>O18ac</td>
<td>Fecal</td>
<td>1</td>
</tr>
<tr>
<td>764-2</td>
<td>−</td>
<td>O18ac</td>
<td>Fecal</td>
<td>This work</td>
</tr>
<tr>
<td>768</td>
<td>+</td>
<td>O18ac</td>
<td>Fecal</td>
<td>18</td>
</tr>
<tr>
<td>768-2</td>
<td>−</td>
<td>O18ac</td>
<td>Fecal</td>
<td>This work</td>
</tr>
<tr>
<td>768-3</td>
<td>−</td>
<td>O18ac</td>
<td>Fecal</td>
<td>This work</td>
</tr>
<tr>
<td>251</td>
<td>+</td>
<td>O18ac</td>
<td>UTI</td>
<td>This work</td>
</tr>
</tbody>
</table>

\textsuperscript{a} UTI, Urinary tract infection.
\textsuperscript{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\textsubscript{2} method. Wild-type strains were transformed by a modified CaCl\textsubscript{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 SalI, 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 hly\textsubscript{C}), pANN215 (which includes most of hly\textsubscript{A}), and pANN250 (which carries the C-terminal half of hly\textsubscript{A} and the functional hly\textsubscript{B\textsubscript{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 SalI 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 SalI 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 Sau3A fragments (average size, 40 kb) into the cosmid vector pHC79. Among several thousand colonies, we identified 50 which were Hly\textsuperscript{+}. From these Hly\textsuperscript{+} 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 HindIII fragments measuring 1.0 and 1.1 kb, respectively. In addition, hybridization occurred in two larger HindIII fragments (10.3 and 1.6 kb) when HindIII-cleaved DNA of these recombinant cosmids and HindIII-cleaved chromosomal DNA from the wild-type strains were hybridized with a hly-specific probe (pANN260). This probe spans the region from the EcoRI site at 3.9 kb to the PstI site at 8.1 kb (Fig. 3) of the hly determinant (25) and thus includes part of the structural hly\textsubscript{A} gene, hly\textsubscript{B\textsubscript{a}} and hly\textsubscript{B\textsubscript{b}}. HindIII-cleaved chromosomal DNA from strain 536 hybridized with the same probe in both small HindIII fragments (Fig. 2, lane 3) in addition to the two larger HindIII fragments.

As previously reported (9), strain 536 yields nonhemolytic mutants at a relatively high rate (about 10\textsuperscript{-3} to 10\textsuperscript{-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**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>hly cistrons and resistance markers encoded\textsuperscript{a}</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pANN104</td>
<td>hly\textsubscript{C}, part of hly\textsubscript{A} (pHly152), Cm\textsuperscript{r}</td>
<td>19</td>
</tr>
<tr>
<td>pANN202</td>
<td>hly\textsubscript{C}, part of hly\textsubscript{A} (pHly152), Cm\textsuperscript{r}</td>
<td>19</td>
</tr>
<tr>
<td>pANN215</td>
<td>Part of hly\textsubscript{A} (pHly152), Cm\textsuperscript{r}</td>
<td>19</td>
</tr>
<tr>
<td>pANN250</td>
<td>Part of hly\textsubscript{A}, hly\textsubscript{B\textsubscript{a}} (pHly152), Tc\textsuperscript{r}</td>
<td>19</td>
</tr>
<tr>
<td>pANN260</td>
<td>Part of hly\textsubscript{A}, hly\textsubscript{B\textsubscript{a}}, hly\textsubscript{B\textsubscript{b}}, Tc\textsuperscript{r}</td>
<td>Unpublished</td>
</tr>
<tr>
<td>pANN522</td>
<td>hly\textsubscript{C}, hly\textsubscript{A}, hly\textsubscript{B\textsubscript{a}}, hly\textsubscript{B\textsubscript{b}}, of 536 (O6), Ap\textsuperscript{r} (cosmid)</td>
<td>Unpublished</td>
</tr>
<tr>
<td>pANN523</td>
<td>hly\textsubscript{C}, hly\textsubscript{A}, hly\textsubscript{B\textsubscript{a}}, hly\textsubscript{B\textsubscript{b}}, of 536 (O6), Cm\textsuperscript{r}</td>
<td>1</td>
</tr>
<tr>
<td>pANN205-222</td>
<td>hly\textsubscript{B\textsubscript{b}}, hly\textsubscript{B\textsubscript{b}} (pHly152), Ap\textsuperscript{r} (cosmid)</td>
<td>12</td>
</tr>
<tr>
<td>pANN601</td>
<td>Part of hly\textsubscript{C} (pHly152), Ap\textsuperscript{r} (cosmid)</td>
<td>Unpublished</td>
</tr>
<tr>
<td>pANN603</td>
<td>Sequence proximal to hly\textsubscript{B\textsubscript{b}}</td>
<td>Unpublished</td>
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</tbody>
</table>

\textsuperscript{a} Resistance markers: Ap\textsuperscript{r}, ampicillin; Cm\textsuperscript{r}, chloramphenicol; Tc\textsuperscript{r}, tetracycline.

**FIG. 1.** Hybridization patterns of SalI-cleaved chromosomal DNA from E. coli strain 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, 32P-labeled DNA of pANN202 (lanes 1 through 4), pANN215 (lanes 5 through 8), and pANN250 (lanes 9 through 12). As size markers, BglII fragments of a DNA were used (lane 13).
of these mutants lacked either of the two hly determinants. This was demonstrated by hybridization of hly-specific probes with SalI-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 SalI-fragments of strain 536 (Fig. 1, lanes 2, 6, and 10), whereas mutant 536-14 hybridized only in the smaller SalI fragment (Fig. 1, lanes 3, 7, and 11). When the chromosomal DNA from these two mutants was cleaved with HindIII and hybridized as described above with pANN260, hybridization was observed between chromosomal DNA from mutant 536-225 and the probe with the small HindIII 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 HindIII fragments (10.3 and 1.6 kb). These data indicate that the small 1.0-kb HindIII 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 HindIII 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. 2. Hybridization and restriction patterns of HindIII-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 HindIII-cleaved cosmid clones pANN522 (lanes 1 and 7) and pANN523 (lanes 2 and 8). As the hybridization probe, nick-translated, 32P-labeled pANN260 (see text) was used. As size markers, HindIII fragments of λ DNA were used (lane 6).

FIG. 3. Physical map of the hlyC and hlyBb proximal regions of the hemolysin determinant of pHyl52. 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: \( \text{BamHI} \), \( \text{BglII} \), \( \text{EcoRI} \), \( \text{HaeIII} \), \( \text{HindIII} \), \( \text{PvuI} \), \( \text{Sau3A} \).
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 pH152-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* or *hlyB* 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 *SalI* and hybridized with the *hlyC*-specific probe pANN202 gave positive signals from several *SalI* fragments (Fig. 4). The hybridization patterns were rather similar in all three independently isolated *E. coli* strains. The only visible difference was a small *SalI* fragment of 2.6 kb which hybridized in the DNA of strain 768 but not in that of strains 251 and 764. The *SalI* 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 *SalI* fragments which specifically hybridize with the *hlyC* probe represent complete *hly*-determinants or part of it, we hybridized *SalI*-cut DNA of the three O18 strains with the *hlyA* probe pANN215 and the *hlyB* probe pANN250. Only *SalI* fragment 5 hybridized with these two probes in all three strains (Fig. 4), indicating that only this *SalI* 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) *SalI* fragment 5 was absent, but all other *SalI* 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.

To test whether the hybridization in the other six *SalI* fragments is caused by homology with the structural *hlyC* gene or the adjacent sequence approximately 1 kb upstream from *hlyC* also located on the *HindIII*-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 *HaeIII*-BamHI fragment (Fig. 3) contains a 280-bp sequence located upstream of the BamHI 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 BamHI 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 *SalI*-cleaved chromosomal DNA of the three strains, hybridization was observed only with *SalI* fragment 5 (Fig. 5) and not with the other *SalI* fragments hybridizing with pANN202. This suggests that the homology between the other six (7) *SalI* fragments and pANN202 is not caused by homology with the structural *hlyC* gene.

When the 575-bp *HaeIII* fragment located farther upstream from the 280-bp *HaeIII*-BamHI 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 *SalI* fragments and pANN202 is located on this *HaeIII* fragment. The next *HaeIII*-HindIII fragment upstream (750 bp; Fig. 3) adjacent to the 575-bp *HaeIII* fragment of pANN202 yielded strong hybridization with all chromosomal *SalI* fragments except *SalI* 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 *SalI*-cleaved chromosomal DNA with the adjacent 1,060-bp *HindIII*-EcoRI fragment (Fig. 3) from pH1y152 (19). This fragment showed again strong hybridization in the same *SalI* fragments as the 750-bp *HaeIII*-HindIII probe, but an *EcoRI*-Sau3A fragment which originates 250 bp downstream from the *EcoRI* site no longer hybridized with these chromosomal *SalI* fragments (data not shown). This indicates that the sequence ends at least 250 bp before the *EcoRI* site. The described data suggest that a sequence outside of the structural *hlyC* gene but close to *hlyC* on plasmid pH1y152, and hence at one end of the *hly* determinant of plasmid pH1y152, is causing the hybridization of multiple chromosomal fragments from the hemolytic O18 *E. coli* strains. The failure of *SalI* fragment 5, which carries the *hly* determinant (see above), to hybridize with the sequence-specific 750-bp *HindIII*-HaeIII 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 pH1y152 *hly* determinant. Since the 575-bp *HaeIII* fragment hybridizes weakly with *SalI* fragment 5 and the other pANN202-homologous *SalI* fragments, one can conclude that this fragment carries,

### 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hemolysin activity (U)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>internal</td>
</tr>
<tr>
<td><em>E. coli</em> 536</td>
<td>60</td>
</tr>
<tr>
<td>Mutant 536-225</td>
<td>60</td>
</tr>
<tr>
<td>Mutant 536-14</td>
<td>12</td>
</tr>
<tr>
<td><em>E. coli</em> K-12 (pANN522)</td>
<td>56</td>
</tr>
<tr>
<td><em>E. coli</em> K-12 (pANN523)</td>
<td>11</td>
</tr>
<tr>
<td><em>E. coli</em> K-12 (pANN5211)</td>
<td>61</td>
</tr>
<tr>
<td><em>E. coli</em> (pANN5211)</td>
<td>61</td>
</tr>
</tbody>
</table>

*The determination of the hemolytic activity and the definition of hemolytic units were as described (25).*
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 SalI 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 (hlyBb-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 hlyBb-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 hlyBb-proximal sequence for strain 536 (9). Chromosomal DNAs of these strains were cut with PstI (the two sequences do not contain internal PstI 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 hlyBb-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 hlyBb-proximal sequence. These copy numbers are based on the assumption that the sequences in E. coli K-12 also do not contain PstI sites. (iii) In contrast to the deletion of the hly determinants in E. coli 536, in which the involvement of the hlyBb-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 PstI-cut chromosomal DNA was used (lane 22).

FIG. 4. Hybridization patterns of SalI-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, 32P-labeled DNA of pANN202 (lanes 1 through 6), HindIII fragment C from pANN215 (lanes 7 through 12), and EcoRI fragment G from pANN250 (lanes 13 through 18) and vector pACYC184 (lanes 19, 20, 21). As size markers, HindIII fragments of λ DNA were used (lane 22).

FIG. 5. Hybridization patterns of SalI-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, 32P-labeled DNA of the 280-bp BamHI-HaeIII fragment of pANN202 (lanes 1 through 3), the adjacent 575-bp HaeIII fragment (lanes 4 through 6), and the next 750-bp HaeIII fragment (lanes 7 through 9). As size markers, HindIII fragments of λ DNA were used (lane 10). Explanations for the hybridization probes are given in the text.
DNA from both mutants with the probe specific for the hlyBb-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 SalI-cut chromosomal DNA since previous results indicated that there is no SalI 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 HindIII 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 hlyBb, 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 cosmids 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 pHCT9 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 SalI fragments to probes which carry fragments adjacent to the two proximal hly genes, i.e., hlyC and hlyBb (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 (hlyBb-proximal) end of the plasmid pHly152-encoded hly determinant. Both sequences occur in multiple copies in the chromosomome 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 hlyBb. This is indicated by the observation that hybridization with probes specific for these sequences does not occur in those chromosomal SalI 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 IS91 described by Zabala et al. (26). This sequence was identified on several hemolytic plasmids, including pH1Y152, and was shown to be transposable to pACYC184.

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ADDITIONAL MATERIAL

Subsequent DNA sequence analysis of the hlyC flanking sequence revealed its identity with IS2.

LITERATURE CITED


