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Large, Unstable Inserts in the Chromosome Affect Virulence Properties Of Uropathogenic *Escherichia coli* O6 Strain 536

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The hemolytic, uropathogenic *Escherichia coli* 536 (O6:K15:H31) contains two inserts in its chromosome (insert I and insert II), both of which carried *hly* genes, were rather unstable, and were deleted spontaneously with a frequency of 10^{-3} to 10^{-4}. These inserts were not found in the chromosome of two nonhemolytic *E. coli* strains, whereas the chromosomal sequences adjacent to these inserts appeared to be again homologous in the uropathogenic and two other *E. coli* strains. Insert I was 75 kilobases in size and was flanked at both ends by 16 base pairs (bp) (TTCGACTCCTGTGATC) which were arranged in direct orientation. For insert I it was demonstrated that deletion occurred by recombination between the two 16-bp flanking sequences, since mutants lacking this insert still carried a single copy of the 16-bp sequence in the chromosome. Both inserts contained a functional hemolysin determinant. However, the loss of the inserts not only affected the hemolytic phenotype but led to a considerable reduction in serum resistance and the loss of mannose-resistant hemagglutination, caused by the presence of S-type fimbriae (*sfa*). It is shown that the Sfa-negative phenotype is due to a block in transcription of the *sfa* genes. Mutants of strain 536 which lacked both inserts were entirely avirulent when tested in several animal model systems.

Escherichia coli is one of the main opportunistic bacterial pathogens causing extraintestinal infections, such as urinary tract infections (UTI; e.g., cystitis and pyelonephritis), sepsis, and meningitis (14, 20). Most of these pathogenic *E. coli* strains exhibit certain virulence properties, i.e., specific O and K antigens, serum resistance, special iron uptake systems, synthesis of cytolysins (hemolysins), and different adhesins which exhibit mannose-resistant (MR) or mannose-sensitive (MS) hemagglutinating activity (14). MR adhesins recognize different receptors, such as those containing digalactosides (P adhesins) and sialic acid (S adhesin).

Recently, some of these virulence determinants were cloned and their molecular organization was investigated (22). These studies indicated that the hemolysin (*hly*) determinant comprises four genes which are responsible for hemolytic activity (*hlyA* and *hlyC*) and transport of hemolysin across the two membranes of the gram-negative *E. coli* cell envelope (*hlyB* and *hlyD*) (2, 31, 39, 40). The genetic determinants for MS, P, and S adhesins consist of DNA stretches 6 to 8 kilobases (kb) long, which for the MR adhesins can be divided into two functionally distinct regions. One region is required for fimbriae formation, the other for the specific binding (indicated by the hemagglutinating activity) of the bacteria (10, 36) to the target cells.

While our knowledge of the genetic structure of virulence determinants from extraintestinal *E. coli* strains has grown considerably in recent years, few data are available on the regulation of these genes. Phase variation (i.e., reversible alternation between two unstable phenotypes) was observed for MS, P, and S fimbriae (1, 25, 28), but the molecular base for the switch has been worked out only for the MS adhesin. An invertible element and a gene coding for a negatively regulating protein seem to be involved in the regulation of MS pilus expression (1, 26).

Nothing is known of possible genetic interactions between various virulence determinants. The physical linkage of the genes coding for hemolysin and MR adhesin (P-type fimbriae) on the chromosome of an O4:K6 strain has been described (21), but common regulation of these determinants was not demonstrated. Recently (9, 18) we have shown that the phenotype of nonhemolytic derivatives of the uropathogenic Hly$^+$ *E. coli* 536 was caused by deletions of the hemolysin genes.

Here we present evidence that these *hly* genes are parts of two specific, large DNA inserts in the chromosome of this strain which can be lost by recombination between two 16-base-pair (bp) direct repeats located at the ends of the insert. Deletion of the inserts affects the expression of other virulence determinants, as shown here for the *sfa* determinant.

MATERIALS AND METHODS

Bacterial strains. *E. coli* 536 and the deletion mutants 536-21, 536-111, 536-112, 536-113, 536-225, and 536-14 have been described previously (9, 18). A more detailed description of these strains is given in the text. For cloning experiments, *E. coli* HB101 (H. Boyer) and JM103 were used.

The fecal nonhemolytic *E. coli* 91, isolated from a healthy person, was a gift from T. Chakraborty. *E. coli* K-12 5K was originally isolated by S. Glover.

Media, chemicals, and enzymes. Cultures were grown in enriched nutrient broth, alkaline broth extract (Oxoid, Basingstoke, England), or YT broth. Antibiotics used here were a gift from Bayer (Leverkusen, Federal Republic of Germany [FRG]). Radiochemicals were obtained from Amersham (Braunschweig, FRG). Restriction enzymes and T4 ligase were purchased from Biolabs (Beverly, Mass.). DNA polymerase I and Klenow fragment were obtained from Boehringer (Mannheim, FRG). DNase I (RNase free) was purchased from Cooper Biomedical (Freehold, N.J.). Other chemicals were obtained from Merck (Darmstadt, FRG).

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Isolation of chromosomal and plasmid DNA. Chromosomal DNA was extracted as described previously (18). Small- and large-scale isolation of recombinant plasmid DNA was performed by the cleared lysate procedure (3).

Restriction enzyme cleavage and agarose gel electrophoresis. DNA was digested with appropriate restriction enzymes, and the resulting fragments were separated by agarose gel electrophoresis as described previously (23). Two agarose concentrations, 0.7 and 1.0%, were used. DNA fragments were isolated by electroelution (23).

Nick translation. Plasmids and DNA fragments were labeled by nick translation with a mixture of all four α-^{32}P-labeled deoxynucleotide triphosphates as described elsewhere (29) and purified by ethanol precipitation.

Southern blots. DNA fragments separated by agarose gel electrophoresis were transferred to nitrocellulose filters by the blotting technique of Southern (32). The blots were preincubated in hybridization buffer (50% formamide, 5× SSC [1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate, 1 mM EDTA [pH 7.5], 5× Denhardt solution, denatured salmon sperm DNA [50 μg/ml], 50 mM Tris hydrochloride pH 7.5) for 3 to 6 h at 42°C and then incubated overnight at the same temperature in hybridization buffer containing the nick-translated DNA probe at 3×10^6 to 5×10^6 cpm/ml. Stringent conditions were used for the washing procedure. The filters were exposed to Fuji X-ray film at −20°C for 8 to 16 h.

DNA sequencing. Nucleotide sequencing was carried out by the technique with the M13 mp8/mp9 system described previously (13).

Transformation. *E. coli* K-12 strains were transformed by the $CaCl_2$ method (23).

Cosmid cloning procedure and colony hybridization. Construction of genomic libraries of *E. coli* 536 and mutant 536-21 were performed by partially digesting approximately 500 μg of chromosomal DNA from *E. coli* 536 with 2 U of *Sau*3A (Biolabs) for 30 min at 37°C in a buffer containing 6 mM Tris hydrochloride, pH 7.5, 6 mM $MgCl_2$, and 50 mM NaCl. Following heat inactivation of the enzyme at 65°C for 15 min, the cleaved DNA was fractionated by sedimentation through a 5 to 40% sucrose gradient, and fractions containing 35- to 40-kb fragments were pooled and precipitated with ethanol. Partially digested DNA (3 μg) was mixed with 1.5 μg of *Bam*HI-cleaved and alkaline phosphatase (Boehringer)-treated pHC79 (12). After ligation, the DNA was packaged into phage particles (4). Infection of host bacteria and selection of infected cells on ampicillin (50 μg/ml) LB agar plates was performed as described previously (2). From several thousand Ap^r colonies, 1,500 were picked randomly and transferred to nitrocellulose filters. Amplification of recombinant cosmid DNA, lysis of the cells on the filters, and binding of liberated DNA were done as described (16, 23). Filters were treated for 2 h at 80°C before use in colony hybridizations.

Annealing of single-stranded recombinant M13 DNA. Annealing of M13 recombinant phage DNAs was done by boiling 0.5 μg of single-stranded M13mp8-W4 and M13mp9-M12 DNAs for 5 min in 10 mM Tris hydrochloride, pH 7.5, 6 mM $MgCl_2$, and 50 mM NaCl. The DNA solution was then incubated for 15 min at 70°C and slowly cooled to room temperature, and the DNA was precipitated with ethanol. DNA was treated with 10 U of S1 nuclease (Bethesda Research Laboratories) in 0.2 M NaCl, 0.05 M sodium acetate (pH 4.5), 10 mM $ZnSO_4$, and 5% glycerol at 37°C for 30 min to remove single-stranded DNA.

Isolation of RNA. Total RNA was extracted from cells grown on YT plates by the hot phenol method essentially as described previously (11). The preparations were treated with DNase I (RNase free; Cooper Biomedical), phenol extracted, ethanol precipitated, and suspended in water.

RNA dot-blot procedure. RNA was denatured by glyoxylation and spotted on nitrocellulose paper as described by Thomas (35). The blots were baked for 2 h at 80°C, treated with 20 mM Tris hydrochloride, pH 8.0, for 10 min at 100°C, and prehybridized and hybridized as described above for Southern blots.

Assays for virulence properties of strain 536 and its derivatives. The O6 and K15 antigens were detected with specific antisera as described previously (8). Hemolysin production was tested on blood plates and confirmed in a liquid assay as described previously (39). Expression of S fimbriae adhesin was estimated by agglutination of human, bovine, and guinea pig erythrocytes with and without 1% mannose (6). For detection of S-specific agglutination, erythrocytes were incubated with neuraminidase as described previously (10). The serum resistance was determined by growth of 2×10^5 bacteria in 90% human serum for 3 h (14, 34). Adhesion of bacteria to uroepithelial cells was tested by the method of Svanborg-Eden et al. (33) by incubating bacteria with human uroepithelial cells isolated from fresh morning urine. The in vivo toxicity of the bacterial strains was tested in a mouse peritonitis system (8, 27, 31; J. Hacker, H. Hof, L. Emödy, and W. Goebel, Microb. Pathogenesis, in press). A 100% lethal dose (LD_{100}) of $<4 \times 10^7$ bacteria was considered positive; an LD_{100} of $>2 \times 10^8$ bacteria was considered negative; and an LD_{100} of about 8×10^7 was considered intermediate. In a chicken embryo assay (15, 27; Hacker et al., in press), more than 80% lethality after application of 10^7 bacteria was considered positive, less than 30% lethality was considered negative, and 50% lethality was considered intermediate. The LD_{100} values were calculated by the method of Kärber (17).

RESULTS

Identification and mapping of two unstable DNA regions. As previously reported (18), uropathogenic *E. coli* 536 carries two hemolysin determinants in its chromosome, which are spontaneously deleted at a relatively high frequency (10^{-3} to 10^{-4}). The deletions lead to mutants which lack one or both sets of the *hly* genes. It was further shown that sequences flanking the two *hly* determinants can also be deleted, indicating that the *hly* genes are part of larger regions in the chromosomes of this strain. To characterize these chromosomal segments, we performed walking experiments starting with labeled border fragments of the cloned *hly* determinant I and identified by colony hybridization in a cosmid gene library of strain 536, which carry overlapping fragments 35 to 40 kb in size. These fragments extended more than 50 kb to the right and 50 kb to the left of *hly* I. A detailed restriction map of the covered DNA stretch is given in Fig. 1A. A similar chromosome walk was started with border fragments of *hly* II. These fragments covered a continuous stretch of 30 kb to the right and 80 kb to the left of *hly* II, with one gap in between which we could not close (Fig. 1B).

Characterization of the two DNA regions. The cloned fragments which covered the flanking regions of the two *hly* determinants were used as probes for hybridization of chromosomal DNA from the Hly$^-$ deletion mutant 536-21 and two nonhemolytic *E. coli* strains (*E. coli* K-12 5K and the wild-type fecal strain 91). These experiments were per-

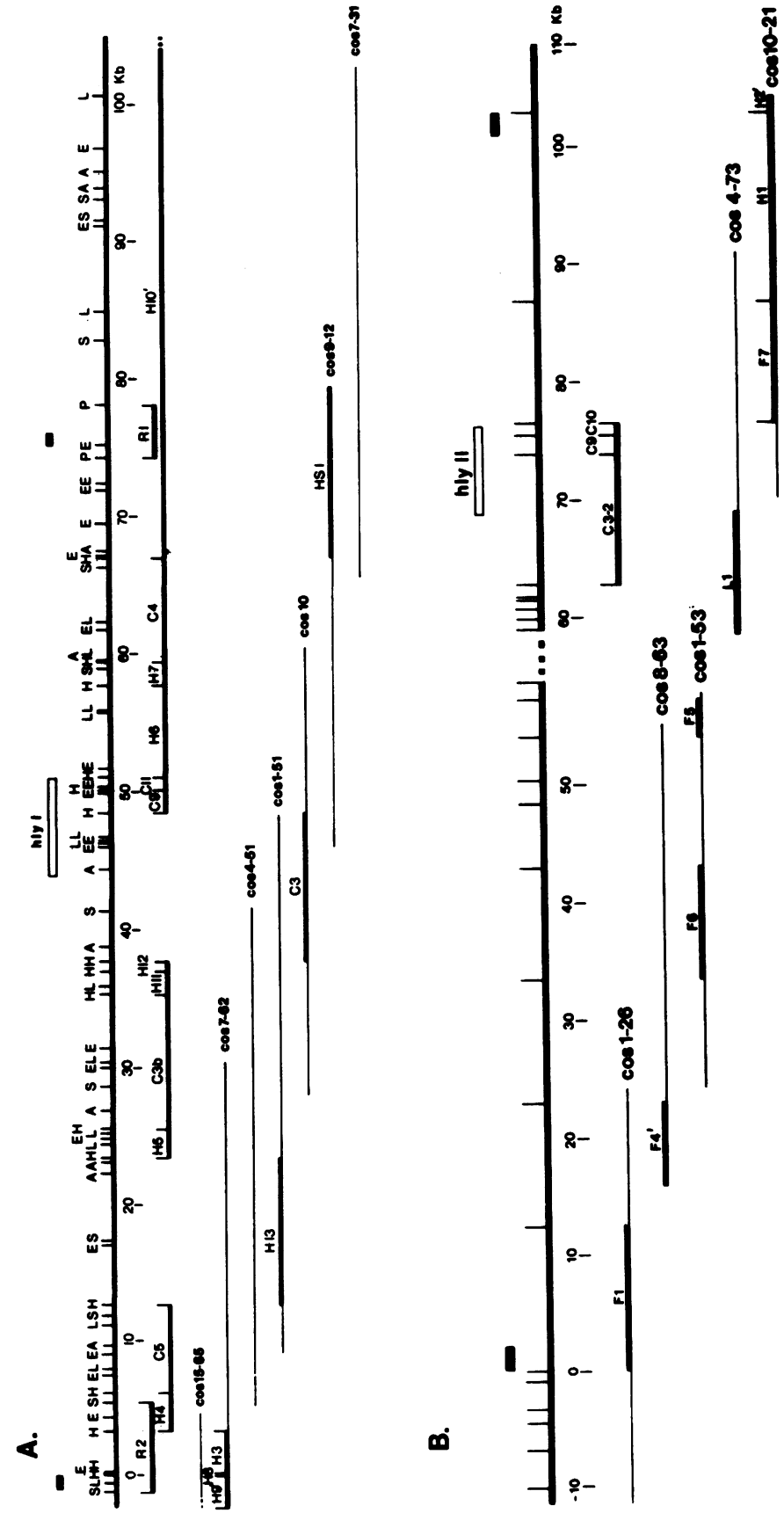

FIG. 1. Restriction maps of iI (A) and iII (B) and their flanking regions in the chromosome of *E. coli* 536. The location of both *hly* determinants is shown in the upper part in both panels. The short black bars indicate the location of the border sequences of the two inserts. The scale underneath both restriction maps marks the distances from the left end point of the inserts. Letters indicate mapped restriction sites: A (*Bam*HI), E (*Eco*RI), H (*Hin*dIII), L (*Bgl*II), P (*Pst*I), S (*Sal*I). Insert II was only mapped by *Hin*dIII. These sites are indicated. A series of representative overlapping recombinant cosmids is shown below both maps. The restriction fragments used in the walk toward the left or right of *hly* I and *hly* II are designated HS1, C3, H13, and H3 for insert I and F7, L1, F5, F6, and F4′ for insert II and are indicated by thicker lines. The restriction fragments used as hybridization probes in Southern blot analysis, H3, H4, H5, H6, H13, C3, C4′, H9′, and R1′, are indicated by the black bars below the map in panel A. H9′ and R1′ (an *Eco*RI-*Sma*I subfragment of R1) are border fragments of insert I which were subcloned into phage M13mp8 and M13mp9 (see map in Fig. 4A).

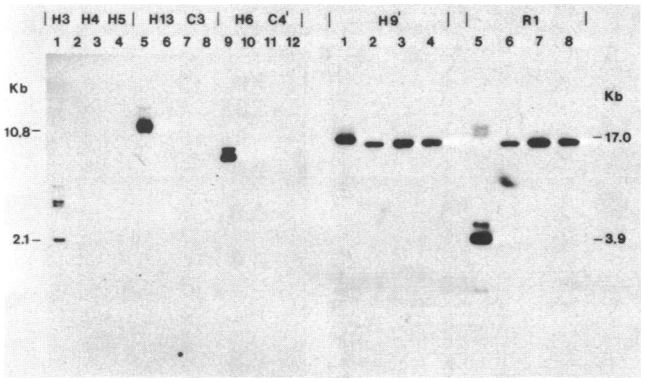

FIG. 2. Southern blot analysis carried out with total, *Hin*dIII-restricted DNA from *E. coli* 536, Hly⁻ mutant 536-21, and two nonhemolytic *E. coli* strains with internal H3, H4, H5, H13, C3, H6, and C4′ (used as probes in hybridizations with *Hin*dIII-restricted DNA) and end point fragments H9′ and R1 (used as probes in hybridizations with *Pst*I-restricted DNA) from iI as hybridization probes. Lanes 1, 5, and 9; *E. coli* 536; 2, 6, and 10; *E. coli* 536-21; 3, 7, and 11; *E. coli* 91; 4, 8, and 12; *E. coli* 5K.

formed to characterize the size and specificity of the chromosomal segments carrying the *hly* genes in *E. coli* 536.

As shown in Fig. 2, all subfragments (H3, H4, H5, H13, C3, H6, and C4) carrying sequences of the *hly* I flanking regions failed to hybridize with the DNA mutant 536-21 and the two nonhemolytic strains 5K and 91, indicating that this *hly* I-flanking DNA is not present in the chromosomes of these strains. The same was found for all other *hly* I-deficient deletion mutants that we tested. Hybridization occurred again with chromosomal DNAs of all strains when the *Hin*dIII fragment H9′ and the *Pst*I fragment R1 (see Fig. 1A), which are approximately 75 kb apart, were used as hybridization probes. As shown in Fig. 2, both probes labeled a 17.0-kb *Pst*I fragment when the DNA of the mutant 536-21 or the two nonhemolytic isolates 5K and 91 were hybridized. Since none of the probes which mapped in the DNA stretch covered by the two fragments H9′ and R1 showed homology to the nonhemolytic strains, we assume that H9′ and R1 contain the border sequences of the unstable DNA region which we designated insert I (iI). Accordingly, the 17.0-kb *Pst*I fragment which seemed to be labeled by both probes should carry the flanking sequences to either side of insert I, indicating that the *hly* I-deficient mutants and the two nonhemolytic *E. coli* strains lacked the whole insert I-specific DNA stretch.

A summary of the data obtained from Southern hybridizations in the region of insert II is given in Fig. 1B. An unstable DNA stretch of approximately 100 kb (this is a minimal size due to the discontinuity of this DNA stretch), designated insert II (iII), was missing in all *hly* II-deficient deletion mutants and both the fecal and K-12 isolates.

To test whether these insert deletions occurred by a site-specific event, we hybridized the iI specific border fragments R1 and R2 (Fig. 1A) against *Pst*I-*Sal*I-digested DNA from the deletion mutants listed in Table 1. As shown in Fig. 3, in all mutants which were deficient for iI, the DNA probe R1 labeled a *Pst*I-*Sal*I fragment slightly larger (4.3 kb) than the 3.9-kb *Pst*I fragment of the wild-type strain *E. coli* 536 and type II mutant 536-225, which has lost iII but retains iI. The same 4.3-kb fragment was also labeled by probe R2 (6.9 kb) in all iI-negative mutants, indicating that the 4.3-kb fragment M1 resulted from the iI deletion.

Detection of the iI deletion junction site in the chromosome of mutant 536-21. The 4.3-kb fragment M1 was isolated from the recombinant cosmid cosM15-4, which was detected in a gene library of mutant 536-21 with fragment R1 as a hybridization probe. As expected, fragment M1, when used as hybridization probe in Southern blot analysis of *Pst*I-*Sal*I-cleaved mutant and wild-type DNA (Fig. 3), labeled strains which contained iI (wild-type strain 536 and mutant 536-225), a 6.9-kb and a 3.9-kb fragment. This indicates that the 4.3-kb fragment M1 from iI-deficient mutants carries the deletion junction site and the sequences immediately flanking the two ends of iI.

DNA sequence analysis of the left and right borders of iI and of the iI deletion junction site of mutant 536-21. To further investigate the specificity of the iI deletion, we determined the two end sequences of insert iI from *E. coli* 536 and the sequence at the iI deletion junction site in mutant 536-21. For this purpose the recombinant plasmid pSK731-1, constructed by inserting the 3.9-kb *Pst*I fragment R1 (right border fragment of iI, see Fig. 1A) into the *Pst*I site of pBR322, and the isolated 4.3-kb *Pst*I-*Sal*I fragment M1 was mapped with several restriction enzymes. Both restriction maps are shown in the upper part of Fig. 4A, where they are lined up by their common restriction sites. Subfragments of M1 and pSK731-1 were cloned in the M13 derivative mp9, yielding the recombinant phages M13mp9-W4, M13mp9-M5, and m13mp9-M12. M13mp9-W4 carries the 1.55-kb *Eco*RI-*Sma*I subfragment (R1′) from plasmid pSK731-1. The recombinant phages M13mp9-M5 and M13mp9-M12 contain the *Sma*I-subfragment of M1 in the opposite orientation. Single-stranded DNAs of both recombinant phages M13mp9-W4 and M13mp9-M12 were annealed to form duplexes in the homologous region. After S1 digestion a hybrid fragment of 1.45 kb was detected on a 0.7% agarose gel (data not shown). The size of this fragment correlates with the distance from the right end sequence of iI to the common *Sma*I site in the inserts of both recombinant M13 DNAs. This fixes the location of the right-end sequence in the immediate vicinity of the second *Sma*I site in M1 and the *Eco*RI site in pSK731-1. To isolate the left end sequence of

TABLE 1. Genetic and virulence properties of *E. coli* 536 and of insert-deletion mutants derived from *E. coli* 536

Strain[a]	*hly* I	*hly* II	Sfa[b]	Sre[c]	Toxicity[d]	Adhesion to human uroepithelial cells[e]
Wild type 536	+	+	R	+	+	+
Mutants						
Type I						
536-21	−	−	−	−	−	−
536-111	−	−	−	−	−	−
536-112	−	−	−	−	−	−
536-113	−	−	−	−	−	−
Type II						
536-225	+	−	−	−	(+)	−
536-14	−	+	R	+	(+)	+

[a] All the strains tested exhibited the O6 antigen and streptomycin resistance as recognition markers.

[b] Presence of the S fimbriae adhesin (Sfa). R, *M*annose-resistant hemagglutination (10).

[c] Serum resistance (Sre), measured by survival of 2×10^5 bacteria after incubation for 3 h in 90% human serum (14, 34).

[d] Toxicity was estimated in a mouse peritonitis system and in a chicken embryo test (Hacker et al., in press).

[e] The test was done by the method of Svanborg-Edén et al. (33).

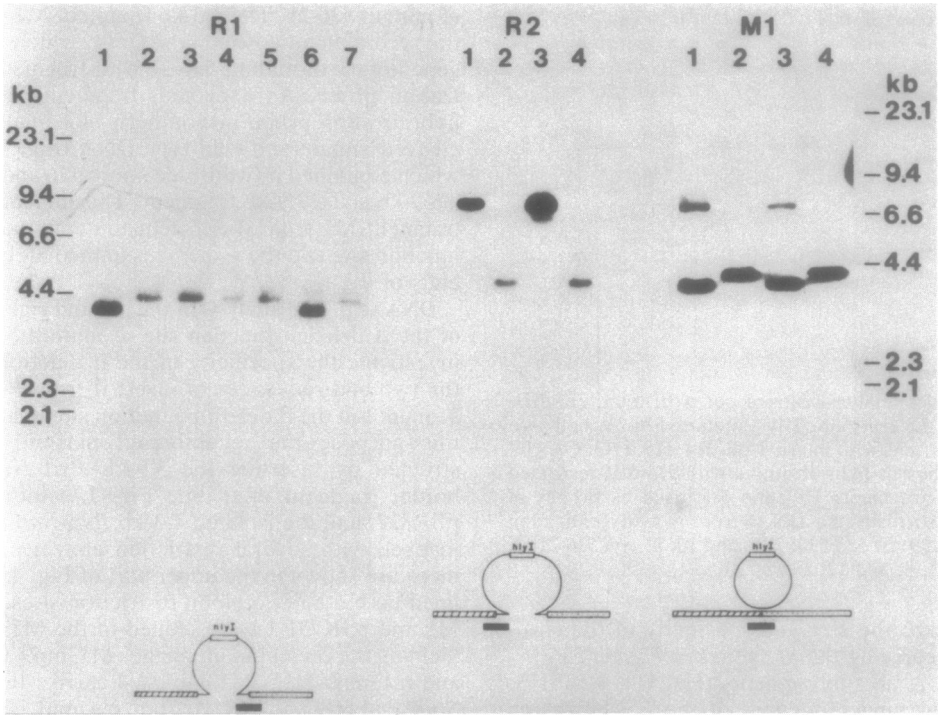

FIG. 3. Southern blot analysis of *Pst*I- and *Sal*I-cleaved chromosomal DNA of *E. coli* 536 and mutants deficient for either iI or iII. The hybridization probes used contained the left (R2) or the right (R1) end sequence of iI or a fragment with the deletion junction site of mutant 536-21 (M1). The precise location of the fragments for these hybridization probes is indicated in the drawings by the black bars. The thin lines represent iI carrying *hly* I (shown as an open box in the circle). The stippled and hatched boxes indicate the left and right flanking regions of iI, respectively. The autoradiograms show hybridizations of *Sal*I- and *Pst*I-cleaved total DNA from the following *E. coli* strains with the probes R1, R2, and M1. Hybridization with R1: lane 1, *E. coli* 536; 2, mutant 536-21; 3, mutant 536-111; 4, mutant 536-112; 5, mutant 536-113; 6, mutant 536-225; 7, mutant 536-14. Hybridizations with R2 and M1: lane 1, *E. coli* 536; 2, mutant 536-21; 3, mutant 536-225; 4, mutant 536-14. The size of *Hin*dIII fragments used as internal probe is indicated.

insert iI, the 2.1-kb *Hin*dIII restriction fragment (H9′) located between map position −2.0 and +0.12 kb (see Fig. 1A) was isolated from recombinant cosmid cos7-62 and subcloned into M13mp8, yielding recombinant phages M13mp8-44 and M13mp8-46, which differ only in the orientation of the insert. The restriction map of this fragment is aligned by the common cleavage sites of fragment M1 in Fig. 4A. The DNA of the recombinant phages M13mp9-W4, M13mp8-M5, and M13mp8-44 was partially sequenced by the Sanger technique.

As shown in Fig. 4B, the left and right ends of iI contain a 16-bp nucleotide sequence arranged as direct repeats. The copy of this sequence to the right of iI mapped 125 bp 3′ to the *Eco*RI site at map position 75.19 (see Fig. 1A). The copy to the left of iI is located 120 bp 5′ to the *Hin*dIII site at map position 0.12 kb (see Fig. 1A). As indicated in Fig. 4B, the mutant strain 536-21 lacked the entire 75-kb DNA stretch between the two 16-bp sequences and retained one copy of this sequence. Complete sequence homology in the chromosomal DNA of wild-type strain 536 and mutant 536-21 was restored in the region immediately flanking the 75-kb iI. This suggests that the deletion of iI occurs by recombination between the two 16-bp end sequences.

Hybridization with an *sfa*-specific DNA probe. As indicated in Table 1, *E. coli* 536 was able to agglutinate bovine erythrocytes in the presence of mannose. The hemagglutination was sensitive to neuraminidase, which is characteristic for S fimbriae adhesin (Sfa). Mutant strains which have lost both inserts (iI and iII) or iII alone were not only affected in the hemolysin phenotype (Hly^- or $Hly^{\pm}$, respectively), but also failed to produce S fimbriae (Sfa^-). To test whether the structural genes determining the S-specific adhesion are located on iII, we hybridized chromosomal DNA from the wild-type strain 536 (iI^+ iII^+) and the mutant 536-225 (iI^+ iII^-) with a radioactively labeled probe (pANN801-1) carrying a *Pst*I fragment which contains the structural genes of the *sfa* adhesin determinant.

As shown in Fig. 5, hybridization with this probe occurred in the DNA of mutant 536-225 and the wild-type 536 with restriction fragments of indistinguishable size, suggesting that the structural genes for the adhesion phenotype were not localized on iII. It further shows that the adhesion genes were still structurally intact but not expressed.

RNA dot-blot analysis of wild-type 536 and Sfa^- mutant 536-21. Total RNA of the wild-type strain 536 and mutant strain 536-21, which had lost the ability to produce the S fimbriae, was isolated. Filter-bound RNA samples were hybridized with radioactively labeled DNA fragments carrying internal regions of the cloned *sfa* determinant (10). As shown in Fig. 6, this RNA dot-blot analysis indicated that the amount of mRNA produced by the Sfa^- mutant strain 536-21 was drastically reduced in comparison to that of the wild-type strain. Thus it seems that the lack of expression of Sfa in these mutants is due to a block in transcription of the *sfa* genes.

Virulence tests with wild-type strain 536 and deletion mutants. The in vivo behavior of the wild-type strain and some mutants was determined in two toxicity assays and an adhesion test. While wild-type strain 536 was toxic to mice and chicken embryos, the mutants lacking iI and iII were

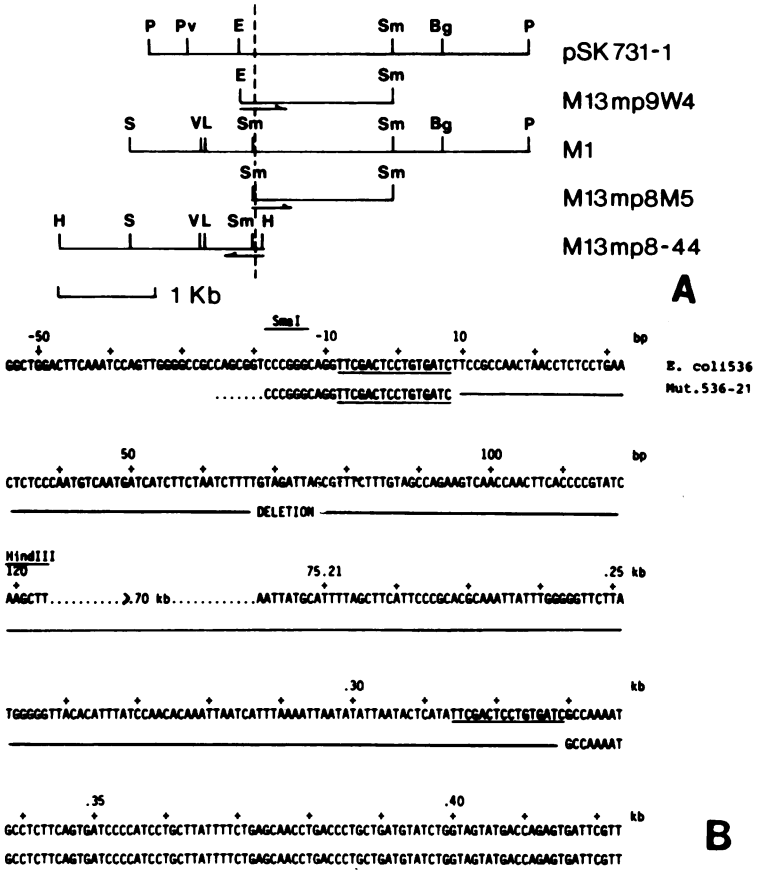

FIG. 4. Restriction maps of cloned DNA fragments carrying the right (pSK731-1, M13mp9-W4) and the left (M13mp8-44) border of iI and the iI deletion junction site of mutant 536-21 (M1, M13mp8-M5). The restriction maps of the border fragments of iI are lined up by their common sites to fragment M1. The cross points of the maps with the vertical dashed line indicate the location of the border sequences and the deletion junction site of iI in mutant 536-21. Letters indicate mapped restriction sites: Bg (*Bgl*I), E (*Eco*RI), H (*Hin*dIII), P (*Pst*I), Pv (*Pvu*I), L (*Bgl*II), V (*Pvu*II), S (*Sal*I), Sm (*Sma*I). Single-stranded DNA of the M13 derivatives M13mp9-W4, M13mp8-M5, and M13mp8-44 were used in the Sanger sequencing reaction. Arrows indicate the direction of the primer elongation. (B) DNA sequence of the left and right borders of iI and of the iI deletion junction site of mutant 536-21. The given sequence of the wild-type strain 536 starts 174 nucleotides upstream of the *Hin*dIII site at map position 121 bp (see Fig. 1) and represents the left border sequence of iI. The right end sequence of iI starts at map position 75.2 kb of iI (see Fig. 1A), about 10 bp downstream of the *Eco*RI site in M13mp9-W4. Both ends of iI contain a single copy of a common 16-bp sequence in direct orientation (underlined). Mutant 536-21 lacks the whole 75-kb sequence of iI but retains one copy of the 16-bp sequence.

completely avirulent. The LD_{100} of these strains increased from 4×10^7 to 6×10^8. The type II mutant strains were of intermediate virulence (LD_{100} about 8×10^7).

Bacteria of the wild-type strain 536 attach to human uroepithelial cells in numbers exceeding 10 per cell. Mutant strain 536-14 (iI^- iII^+), which retained its adhesion property, was able to adhere to uroepithelial cells, while mutants missing iII lost the adhesive ability.

DISCUSSION

The wild-type *E. coli* strain 536 (O6:K15:H31) expresses S-type fimbriae, resistance to human blood serum, and synthesis of hemolysin. Two sets of hemolysin (*hly*) determinants (*hly* I and *hly* II) are carried on the chromosome of this (and other) uropathogenic *E. coli* strains (18). As shown here, the two *hly* determinants are part of specific large chromosomal DNA inserts. This was shown in several ways. (i) Chromosome walking experiments, in which we used labeled, overlapping DNA fragments starting from the left and right ends of the two cloned *hly* determinants (*hly* I and *hly* II). (ii) The recombinant cosmids and suitable subfragments from these cloned DNAs taken as probes for hybridizing chromosomal DNA from a spontaneously generated Hly^- mutant and two nonhemolytic strains (one from the normal human fecal flora and a K-12 strain) revealed one DNA stretch of 75 kb (25 kb to the right and 50 kb to the left of *hly* I) including *hly* I and a second stretch of 100 kb (25 kb to the right and 65 kb to the left of *hly* II; this is a minimal size due to a gap to the left of *hly* II which we have not yet closed) including *hly* II, both of which were completely absent in the three Hly^- strains. (iii) Homology between the chromosomal DNAs from the uropathogenic wild-type strain 536, mutant 536-21, and the two randomly selected strains was restored outside these regions as shown by hybridization of border fragments carrying flanking sequences which are located outside either insert. This indicates that the two *hly* determinants of *E. coli* 536 are located on specific segments which are not present on the chromosome of a fecal or a K-12 *E. coli* strain.

The distance between the two inserts on the chromosome is not yet known. No cosmid clone isolated from the gene library of *E. coli* 536 carried border sequences of both inserts, suggesting that iI and iII are separated by at least 30 kb.

Both inserts were spontaneously deleted in the Hly^- mutants. Since several independently isolated Hly^- mutants and the *hly* II-deficient deletion mutant 536-225 of this strain appear to have the same deletion end point, as revealed by hybridization of the mutant chromosomal DNA with probes containing restriction fragments which include the left or right end sequence of iI, we assume that the deletions occur by a site-specific recombination event. This assumption was confirmed by nucleotide sequence analysis of the left and right border sequences of iI and the deletion junction site of mutant 536-21. Both ends of iI are marked by a 16-bp sequence arranged as direct repeats. No other direct or inverted sequences were identified at the two ends of iI, ruling out a transposon-like structure for this insert. Sequence analysis around the iI deletion junction site of mutant 536-21 yielded a single copy of the 16-bp repeat, indicating that the deletion event removed the entire sequence of iI between the two 16-bp repeats and left only one copy of the 16-bp sequence behind. This suggests that the deletions occur by recombination which involves the two 16-bp direct repeats. The observed fixed deletion end point observed in all iII (*hly* II-specific insert) deletion mutants (data not shown) suggests that a similar mechanism is also responsible for the deletion of this insert. We do not know at the moment whether this insert is flanked by end sequences similar to those of iI. However, our DNA hybridization data indicate that the DNA sequences of iI and iII outside the *hly* determinants are by and large different (both inserts carry, however, at least one copy of a common element which is most likely an insertion element; S. Knapp, unpublished).

Mutants of *E. coli* 536 which have lost both inserts (iI and iII) are not only hemolysin negative but also fail to express the S fimbriae adhesin (Sfa) responsible for adhesion to uroepithelial cells and serum resistance. Loss of S fimbriae was also observed in mutants which suffered a deletion of iII only. Since the hybridization patterns were indistinguishable

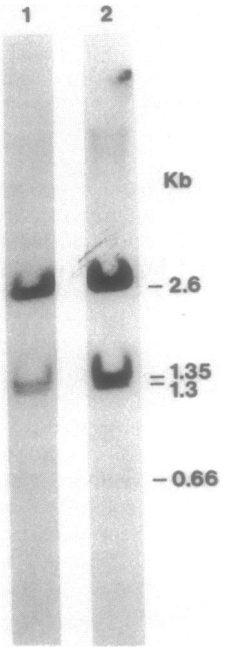

FIG. 5. Southern hybridization of *Pst*I-digested chromosomal DNA of *E. coli* 536 (lane 1) and mutant 536-225 (lane 2) with plasmid pANN801-1, which carries genes of the S fimbriae adhesin (*sfa*) determinant of *E. coli* 536.

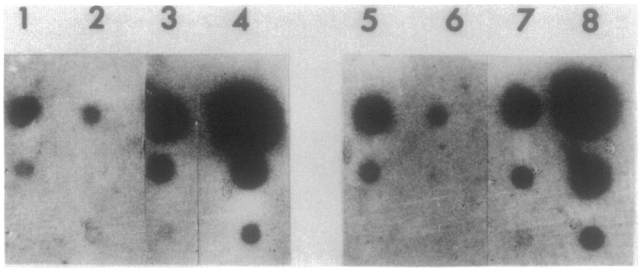

FIG. 6. RNA dot-blot hybridization of total RNA of *E. coli* 536 (Sfa^+; lanes 1 and 5), mutant 536-21 (Sfa^-; lanes 2 and 6), and HB101 (pANN801-13-cloned *sfa* determinant; lanes 3 and 7). Samples in each lane contained 4, 0.4 or 0.04 μg of RNA. As radioactively labeled probes, internal *Cla*I-*Sma*I (lanes 1 through 4) and *Eco*RI-*Cla*I (lanes 5 through 8) DNA fragments of the *sfa* determinant were used. These fragments are specific for the fimbriae and adhesin coding regions of the *sfa* determinant. The precise location of the fragments is indicated in Fig. 5. As a control, unlabeled DNA of the respective restriction fragment was included in lanes 4 and 8.

when chromosomal DNA of the wild-type strain and the Sfa^- mutant 536-225 was hybridized with a DNA probe carrying most of the *sfa* determinants of *E. coli* 536, we conclude that the adhesion property is not impaired by a deletion of the structural *sfa* genes. The *sfa*-specific probes did not hybridize with RNA of the deletion mutant 536-21, indicating that the lack of expression of the adhesion property is due to a block in the transcription of the *sfa* genes. It remains to be seen whether this transcriptional block is a direct or indirect consequence of the deletion of iII. There was always a strong correlation between the loss of iII and the loss of expression of the adhesion property in several independently isolated iII-negative and iI- and iII-negative mutants that we tested. These findings seem to rule out the possibility that the loss of the adhesion property is caused by an independent mutation and is not connected with the deletion of iII. Mutants of *E. coli* 536 that lost both inserts were entirely avirulent in several animal model systems that we tested, indicating that the virulence of this strain is strongly influenced by the two inserts. Furthermore, our preliminary data indicate that the *hly* genes of other uropathogenic *E. coli* strains are likewise located on specific large chromosomal inserts (Knapp et al., unpublished observations). The close functional (9) and structural (21) association of the genetic determinants for hemolysin synthesis and (specific) adherence has been recently demonstrated for several UTI *E. coli* strains. This suggests that the findings described here in detail for one strain may be of general importance for an understanding of the genetic basis of *E. coli* pathogenicity.

It has been demonstrated that most hemolytic uropathogenic *E. coli* isolates carry the *hly* genes on the chromosome (18, 21). In hemolytic *E. coli* strains from animal sources, these genes reside predominantly on transmissible plasmids (5, 7, 30, 38). The data presented here indicate that the chromosomal *hly* genes in uropathogenic *E. coli* strains are part of large specific segments which are not found in the chromosome of nonhemolytic *E. coli* strains. These inserts are easily deleted when the bacteria are grown under laboratory conditions. Are the inserts integrated plasmids or lysogenic phages? There is no evidence in support of this possibility. (i) We have recently shown that most plasmid-encoded *hly* genes are flanked by specific sequences which were absent in all the chromosomal *hly* determinants we

tested (19). (ii) All our experiments designed to demonstrate replication origins on the inserts or transfer of the Hly phenotype or other chromosomal markers to suitable recipients have failed so far. (iii) Treatment of the strains under conditions known to induce lysogenic phages (UV irradiation, mitomycin C) does not cause cell lysis or release of phage particles. Thus, the origin of these inserts, which are essential for virulence, remains unknown.

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