Cloning of the Chromosomal Determinants Encoding Hemolysin Production and Mannose-Resistant Hemagglutination in *Escherichia coli*

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We have cloned the chromosomal hemolysin determinants from *Escherichia coli* strains belonging to the four O-serotypes O4, O6, O18, and O75. The hemolysin-producing clones were isolated from gene banks of these strains which were constructed by inserting partial *Sau*3A fragments of chromosomal DNA into the cosmid pJC74. The hemolytic cosmid clones were relatively stable. The inserts were further subcloned either as *Sal*I fragments in pACYC184 or as *Bam*HI-*Sal*I fragments in a recombinant plasmid (pANN202) containing cistron C (*hlyC*) of the plasmid-encoded hemolysin determinant. Detailed restriction maps of each of these determinants were constructed, and it was found that, despite sharing overall homology, the determinants exhibited minor specific differences in their structure. These appeared to be restricted to cistron A (*hlyA*), which is the structural gene for hemolysin. In the gene banks of two of these hemolytic strains, we could also identify clones which carried the genetic determinants for the mannose-resistant hemagglutination antigens Vb and VIc. Both of these fimbrial antigens were expressed in the *E. coli* K-12 clones to an extent similar to that observed in the wild-type strains. These recombinant cosmids were rather unstable, and, in the absence of selection, segregated at a high frequency.

Escherichia coli strains very frequently cause extraintestinal infections, particularly those of the urinary tract and bloodstream, and it appears that a variety of factors, e.g., hemolysin production, possession of ColV plasmids, and carriage of specific hemagglutination (HA) O, and K antigens, may influence their virulence (8, 15, 16). With recombinant DNA techniques, these "virulence" factors can now be isolated, and their specific contribution to the complex picture of extraintestinal pathogenesis can be more readily determined.

Hemolysin synthesis (Hly) is encoded either by transmissible plasmids, particularly in hemolytic *E. coli* strains isolated from animals (20), or by the chromosome, especially in hemolytic *E. coli* strains isolated from humans (15, 16). The plasmid-borne genes essential for hemolysin production have been previously cloned and their functions partially characterized (11, 17). Hybridization experiments have subsequently shown that plasmid-mediated hemolysin determinants (*hly*) are generally homologous with one another (7), and similar results have been obtained from hybridizations carried out between plasmid *hly* cistrons and several chromosomal *hly* determinants (D. Müller, C. Hughes, and W. Goebel, submitted for publication). The high degree of homology between these various hemolysin genes suggests a common origin. However, recent studies on the contribution to virulence of isolated hemolysin determinants in vivo have revealed differences in toxicity, despite their high sequence homology (23; J. Hacker, unpublished results).

We have therefore decided to study these hemolysin sequences in more detail and have cloned the genes essential for hemolysin synthesis in *E. coli* strains belonging to the four O-serotypes O4, O6, O18, and O75, which are most often associated with hemolysin production among urinary tract pathogens (5, 6, 18, 19). The cosmid cloning method (3, 4) allows the rapid construction of gene banks from which the required clones can be easily obtained if appropriate screening procedures are available (1, 8, 9, 21). In this paper, we report on the isolation and characterization of the *hly* determinants from four *E. coli* strains belonging to serotypes O4, O6, O18, and O75 and, in addition, the mannose-resistant hemagglutination (MRHA) antigens Vb and VIc from the O6 and O18 isolates. Of particular interest was the finding that, despite sharing extensive homology, the *hly* determinants display minor specific differences which appear to be limited to the structural gene (*cisA*).

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MATERIALS AND METHODS

Bacterial strains. The *E. coli* urinary tract infection (UTI) strains were obtained from the Institut für Hygiene und Mikrobiologie, Würzburg, West Germany (strain 536 [O6]), and from Charing Cross Hospital, London, England (strains 367 [O4] and 341 [O75]). *E. coli* strain 764 (O18), obtained from the Institut für Hygiene und Mikrobiologie, Würzburg, was isolated from the stool of a healthy person. The characters of these strains are indicated in Table 1. *E. coli* HB101 (*hsdR hsdM, leu pro recA*) was obtained from H. W. Boyer; *E. coli* 5K (Sm^r *hsdR hsdM*) was obtained from S. Glover; and *E. coli* K-12 strain N205 (λ *imm*434 *c*Its *b*2 *red*3 *Eam*4 *Sam*7), strain N205 (λ *imm*434 *c*Its *b*2 *red*3 *Dam*15 *Sam*7), and strain N205 (λ) were obtained from B. Hohn.

Media and enzymes. Cultures were grown in Luria broth: 10 g of peptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), 5 g of NaCl per liter of water (pH 7.2). NZ medium, for growth of the strains used to prepare the cell-free packaging mixture, contained in 1 liter: 10 g of NZ-amine (Adlag, Hamburg, West Germany), 5 g of NaCl, and 2 g of MgCl$_2$. The restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, and *Sau*3A and the T4 DNA ligase were purchased from Seakem, Rockland, Maine.

Cosmid cloning system. Recombinant DNA was packaged in vitro as described previously (3, 4), except that NZ was used as the culture medium.

Screening for clones. Hemolysis was determined on blood plates (10 g of meat extract [Difco], 10 g of peptone [Oxoid Ltd., London, England], 5 g of NaCl, 60 ml of washed human erythrocytes in 1 liter). Synthesis of hemolysin was confirmed in a liquid assay (21). Hemagglutination, resistant to 1% mannose, was assayed in phosphate-buffered saline as described previously (9). Erythrocytes were either prepared (human, bovine, guinea pig) or purchased from Flow Laboratories, Bonn, West Germany (chicken, African green monkey).

Isolation of plasmid DNA. Plasmid DNA from purified clones carrying recombinant DNA was screened by the cleared lysate procedure (1). Preparative DNA isolation was achieved as described previously (12).

Restriction and ligation. Cleavage of the DNA by

TABLE 1. Bacterial strains[a]

Strain	Hemolysin production	O type	K type	MRHA fimbriae type	Origin
536[b]	+	O6		Vb	UTI[c]
764[b]	+	O18		VIc	Fecal[d]
367[e]	+	O4	K5	Vc	UTI
341[e]	+	O75	K5		UTI

[a] Wild-type *E. coli* strains from which clones were derived. All were identified by standard biochemical tests (19).

[b] Obtained from the Institute of Hygiene and Microbiology, University of Würzburg, Würzburg, West Germany.

[c] Symptomatic urinary tract infection.

[d] Isolated from the stool of a healthy person.

[e] Obtained from the Charing Cross Hospital, London, England.

restriction enzymes was performed at 37°C in 25 mM Tris-hydrochloride (pH 7.5)–20 mM MgCl$_2$–10 mM NaCl. Gel electrophoresis was carried out in 1% agarose.

Ligation was achieved after heat inactivation of the restriction endonucleases at 65°C for 6 min in 66 mM Tris-hydrochloride (pH 7.6)–6.6 mM MgCl$_2$–2 mM dithiothreitol–1 mM ATP–0.1 mg of bovine serum albumin per ml with 5×10^{-2} U of T4 DNA ligase. The concentration of DNA was between 200 and 300 µg/ml for packaging and about 20 µg/ml for transformation. Before addition of ligase, the samples were mixed, heated to 65°C for 6 min, and cooled slowly. The ligation reaction was continued for about 20 h at 8°C.

Transformation. *E. coli* K-12 strains were transformed with ligated DNA by the CaCl$_2$ method (2).

Electron microscopy. Cells were grown and washed in saline before application of carbon-coated grids. After 2 min, they were washed again in saline and suspended in acetone before drying in liquid CO$_2$. After shadowing with platinum-iridium, the grids were examined under a Zeiss-10A transmission electron microscope.

RESULTS

Construction of gene banks from hemolytic *E. coli* strains and selection of hemolytic clones. The four hemolytic *E. coli* strains chosen were isolated from UTI or normal fecal flora and belong to four serotypes; i.e., *E. coli* 367 belongs to O4, *E. coli* 536 belongs to O6, *E. coli* 764 belongs to O18, and *E. coli* 341 belongs to O75 (Table 1). It has been found that the genes determining synthesis and secretion of hemolysin in such strains are generally located on the chromosome (13, 15; C. Hughes, unpublished data). Total DNAs from these strains were therefore isolated and partially digested with *Sau*3A, and the fragments were cloned into the cosmid pJC74, which was completely digested with *Bam*HI. This cosmid (16 kilobases [kb]) contains a single *Bam*HI site and allows the incorporation of additional DNA with an average size of about 30 kb. Such recombinant DNA molecules can be efficiently packed into phage λ heads, and a collection of a few hundred clones therefore represents the total genome of *E. coli*. In each cloning experiment, a few thousand transformant colonies were obtained, and 10 randomly selected clones from each experiment were screened for plasmid DNA by using the quick miniscreen procedure described by Birnboim and Doly (1). At least 9 of 10 clones contained recombinant DNAs of similar size (about 45 kb) and, on average, 1 hemolytic clone was found per 1,000 clones tested. None of these hemolytic clones could agglutinate erythrocytes. This suggests that each hemolytic strain carries only one hemolysin determinant per chromosome and that the determinants for hemolysin production and mannose-resistant hemagglutination are not carried on the same recombinant cosmids. DNA of five hemo-

lytic clones obtained from the cloning experiments with chromosomal DNA of strain 536 (O6) was isolated and cleaved with restriction enzymes. As shown in Fig. 1, the recombinant DNAs of individual clones, when cleaved with *Eco*RI and *Hin*dIII, yielded mainly different DNA bands. This indicates that, in addition to the common hemolysin determinant (see below), these clones have different adjacent chromosomal sequences.

Thus, all hemolytic clones tested were independently generated and do not represent subclones of one hemolytic transformant. Similar results were obtained with the hemolytic clones carrying recombinant cosmids from strains 367 (O4), 341 (O75), and 764 (O18). All hemolytic clones carrying recombinant cosmids were stable only under selective pressure, i.e., when the cultures were grown in the presence of ampicillin; without the antibiotic, nonhemolytic segregants were observed with a high frequency. In general, these segregants no longer exhibited ampicillin resistance, which indicates that the whole recombinant DNA and not only sequences involved in hemolysin synthesis were lost.

Subcloning of the hemolysin determinants. We have shown previously (14, 17) for the plasmid-borne hemolysin determinant that the genes essential for hemolysin synthesis (three cistrons, termed *hlyC*, *hlyA*, and *hlyB*) are located on a continuous DNA segment of about 7.5 kb, and Southern hybridization of the plasmid *hly* sequences with chromosomal DNAs of the four *E. coli* strains examined here have indicated strong homology between the various *hly* determinants (D. Müller, C. Hughes, and W. Goebel, submitted for publication). From restriction analysis of the plasmid *hly* determinant, it was known that no *Sal*I site was located within the *hly* genes. We therefore chose *Sal*I fragments to subclone the chromosomal *hly* determinants from the recombinant cosmid DNA which should contain more than 20 kb of inserted DNA not associated with hemolysin determination. Cloning of *Sal*I-digested recombinant cosmid DNA carrying the *hly* determinant from strain 536 (O6) into pBR322 or pACY184 yielded hemolysin-producing clones that were tetracycline sensitive, indicating that a *Sal*I fragment was inserted into the *Sal*I site of the vectors used (both of which carry single *Sal*I sites in a gene essential for tetracycline resistance [17]). This was further verified by *Sal*I cleavage of the isolated recombinant DNA. Separation of the *Sal*I digest on an agarose gel resulted in two fragments, the larger of which (~15 kb) seems to carry the hemolysin genes, since the other one is indistinguishable in size from linearized vector DNA. The inserted *Sal*I fragment carries a single *Bam*HI site that,
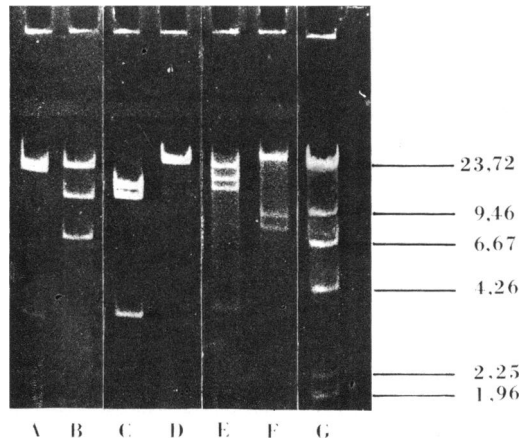

FIG. 1. Restriction endonuclease patterns obtained after digestion of the cosmids pANN521 (lanes A and B), pANN522 (lanes C and D), and pANN523 (lanes E and F) (derived from *E. coli* 536 [O6]) with *Eco*RI (lanes A, C, and E) and *Hin*dIII (lanes B, D, and F). Control (lane G) is *Hin*dIII-cleaved lambda DNA. Sizes are in kb.

as judged from the size of the *Bam*HI-*Eco*RI fragments (see Fig. 3), has a location within the hemolysin determinant similar to that of the *Bam*HI site in the plasmid determinants. As previously shown, the *Bam*HI site is located between *hlyC* and *hlyA*, separating the two cistrons into the functional units (17). We therefore tried to subclone *Bam*HI-*Sal*I fragments from a cosmid clone carrying the *hly* determinant of strain 536 into a recombinant plasmid (pANN202) carrying *hlyC*, thus allowing the insertion of *Bam*HI-*Sal*I fragments. One *Bam*HI-*Sal*I fragment of this recombinant cosmid should carry *hlyA* and *hlyB* of the chromosomal *hly* determinant and therefore yield Hly⁺ clones when inserted into *Bam*HI-*Sal*I-cleaved pANN202, provided that the plasmid determinant *hlyC* can substitute for the chromosomal *hlyC*. Hly⁺ colonies were indeed obtained when the *Bam*HI-*Sal*I-digested recombinant cosmid DNA carrying the *hly* determinant from strain 536 (O6) was ligated with *Bam*HI-*Sal*I-cleaved pANN202 and the ligation mixture was transformed into *E. coli* 5K. Cleavage of the isolated plasmid (pANN5211) from five of these Hly⁺ clones with *Bam*HI and *Sal*I gave identical restriction patterns, which indicated that a single *Bam*HI-*Sal*I fragment (~9 kb) was inserted into pANN202 at its *Sal*I sites.

Similar cloning experiments with pANN202 as the vector were subsequently carried out with the recombinant cosmids carrying the *hly* determinants of *E. coli* strains 367 (O4), 764 (O18), and 341 (O75). Cosmids and subcloned DNAs are listed in Table 2. Hly⁺ subclones with single *Bam*HI-*Sal*I inserts in pANN202 were obtained

TABLE 2. Recombinant cosmids and subcloned DNAs

Recombinant plasmid/cosmid	Cloned *hly* genes	Comments
pANN511	*hly A, B, C* from *E. coli* 367 (O4)	Cosmid clone
pANN521	*hly A, B, C* from *E. coli* 536 (O6)	Cosmid clone
pANN522	*hly A, B, C* from *E. coli* 536 (O6)	Cosmid clone
pANN523	*hly A, B, C* from *E. coli* 536 (O6)	Cosmid clone
pANN531	*hly A, B, C* from *E. coli* 764 (O18)	Cosmid clone
pANN541	*hly A, B, C* from *E. coli* 341 (O75)	Cosmid clone
pANN5211	*hly A, B* from *E. coli* 536 (O6)	Subcloned DNA from pANN521 into pANN202 (*hlyC*)
pANN5311	*hly A, B* from *E. coli* 764 (O18)	Subcloned DNA from pANN521 into pANN202 (*hlyC*)
pANN5411	*hly A, B* from *E. coli* 341 (O75)	Subcloned DNA from pANN541 into pANN202 (*hlyC*)
pANN801	Genes encoding MRHA fimbriae type Vb from *E. coli* 536 (O6)	Cosmid clone
pANN802	Genes encoding MRHA fimbriae type VIc from *E. coli* 764 (O18)	Cosmid clone

from the recombinant cosmids carrying the *hly* determinants of all three strains. These recombinant plasmids, which carry functional *hlyA* and *hlyB* of chromosomal *hly* determinants covalently joined to plasmid *hlyC*, allowed the more precise mapping of the *hlyA* and *hlyB* parts of chromosomal *hly* determinants, which was carried out with several restriction enzymes (see Fig. 3). Figure 2 shows as an example the *Bam*HI cleavage patterns of the recombinant cosmids and plasmids carrying the described *hly* determinants. The smaller size of the recombinant Hly plasmids relative to the recombinant Hly cosmids is evident from the smaller number or size (or both) of the restriction fragments
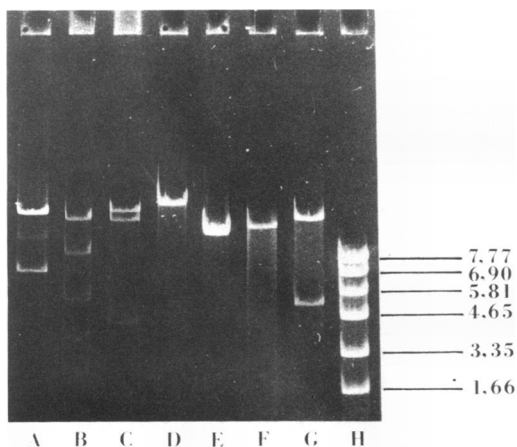

FIG. 2. Restriction endonuclease patterns obtained after *Bam*HI digestion of cosmid and subcloned DNAs. Lane A, pANN521; lane B, pANN531; lane C, pANN541; lane D, pANN510 (all cosmid DNAs); lane E, pANN5211; lane F, pANN5311; lane G, pANN5411 (respective subcloned DNAs in pANN202). Control (lane H) is *Eco*RI-cleaved SPP1 DNA. Sizes are in kb.

obtained from the recombinant Hly plasmids. Figure 3 summarizes the data obtained from these restriction analyses. These maps suggest that (i) the size and order of the hemolysin cistrons are the same in all the *hly* determinants studied; (ii) changes in these determinants occur predominantly within *hlyA*, as indicated by the presence of new restriction sites or the absence of sites when compared with the well-characterized plasmid *hlyA* cistron; and (iii) the *hlyB* cistron of the *hly* determinants seems to be more conserved, since no obvious changes have been observed in this region.

Isolation of recombinant cosmids encoding MRHA VIc and MRHA Vb. The O18 strain 764, like many *E. coli* strains causing UTI, possesses the hemagglutination VIc antigen, i.e., pili causing MRHA. *E. coli* strain 536 (O6), on the other hand, carries the less common mannose-resistant-type MRHA, Vb (8, 9). MRHA VIc causes hemagglutination of human erythrocytes, whereas MRHA Vc agglutinates bovine erythrocytes. Since the recipient strain, *E. coli* K-12, does not agglutinate erythrocytes in the presence of mannose, transductants with recombinant cosmids carrying the MRHA genes could be recognized in the gene banks of these strains by testing individual clones for MRHA of appropriate erythrocytes. Of 600 clones with inserted chromosomal *Sau*3A fragments of strain 764, we found one which had inherited the MRHA VIc determinant. Similarly, we isolated one clone with the MRHA Vb$^+$ phenotype among 200 clones tested from the gene bank of strain 536. These were not hemolytic. Electron microscopy of both clones was performed to visualize the MRHA pili (Fig. 4).

Both clones possess recombinant cosmid DNAs of around 40 kb. Cleavage of these DNAs showed, as expected, different restriction pat-
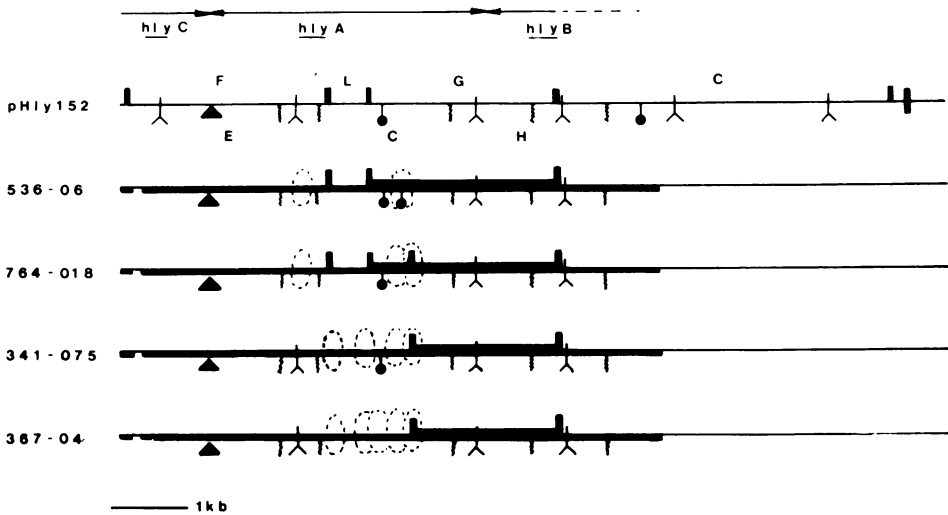
FIG. 3. Physical maps of the *hly* determinants cloned from *E. coli* strains belonging to serotypes O4 (367), O6 (536), O18 (764), and O75 (341) and the well-characterized plasmid *hly* determinant from pHly152 (22). Restriction endonuclease recognition sites shown are λ, HindIII; $\bullet$, BglII; $\{$, PstI; $\blacktriangle$, BamHI; $\mathbf{\bot}$, EcoRI; $\mathbf{+}$, SalI. The dashed circles indicate loss or addition of restriction sites with reference to the plasmid *hly* determinant.

terns, and retransformation of the isolated recombinant cosmids into a second nonpiliated host (*E. coli* 5K) gave Ap^r transformants, all of which exhibited the expected MRHA antigen (Table 3). These clones are rather unstable (segregation rate up to 95% within 20 generations) and can only be maintained in the presence of ampicillin. Further analysis of the inserted DNA will be needed to identify the genes essential for the expression of these pili.

DISCUSSION

Evidence suggests that several factors contribute to the virulence of *E. coli* strains causing extraintestinal infections, including synthesis of specific O, K, and MRHA antigens and hemolysin (6, 8, 13). The high incidence of hemolysin production among *E. coli* UTI isolates is well documented, but only recently has it been confirmed that the hemolysin determinant plays an important role in the pathogenesis of infections (10, 22, 23). The reported data and those gathered in our laboratory indicate differences with regard to the extent of toxicity exerted by cloned hemolysin determinants (J. Hacker, unpublished results), despite their close genetic homology. However, the known data do not allow one to ascertain whether these differences are based on different molecular structures of the secreted hemolysins or on differences in other gene products required for the hemolytic phenotype. As already shown, each hemolysin determinant in *E. coli* consists of three cistrons, *hlyC*, *hlyA*, and *hlyB*, of which only *hlyA* seems to determine the

polypeptide that is ultimately converted into active hemolysin (17); the other two cistrons seem to be involved in the processing and transport of the primary *hlyA* gene product. Thus, differences in the hemolysin proteins encoded by different hemolysin determinants should be reflected by nucleotide changes in *hlyA*. The cloning of four different hemolysin determinants described here is considered a first step toward a detailed analysis of the *hlyA* sequences.

The cloning of partial *Sau*3A fragments of the chromosomal DNA from these strains on a cosmid vector yielded the number of hemolysin-positive clones expected for a genetic determinant which is present only once per chromosome. A convenient way to subclone the hemolysin determinants from the recombinant cosmids onto a multicopy plasmid vector proved to be the cloning of *Bam*HI-*Sal*I fragments into pANN202, a recombinant DNA which carries the plasmid *hlyC* in pACYC184. Hemolytic subclones were obtained by this cloning strategy from recombinant cosmids carrying the *hly* determinants of the O6, O18, O75, and O4 strains. This indicates that the *Bam*HI site located between *hlyC* and *hlyA* is conserved in all these determinants, and covalent linkage between the plasmid *hlyC* part and the chromosomal *hlyA* and *hlyB* sequences via the *Bam*HI site results in functional hybrid hemolysin determinants. As already observed with the cloned plasmid determinant (11), recombinant DNAs carrying the whole determinant, i.e., *hlyC*, *hlyA*, and *hlyB*, on a multicopy vector plasmid can be tolerated
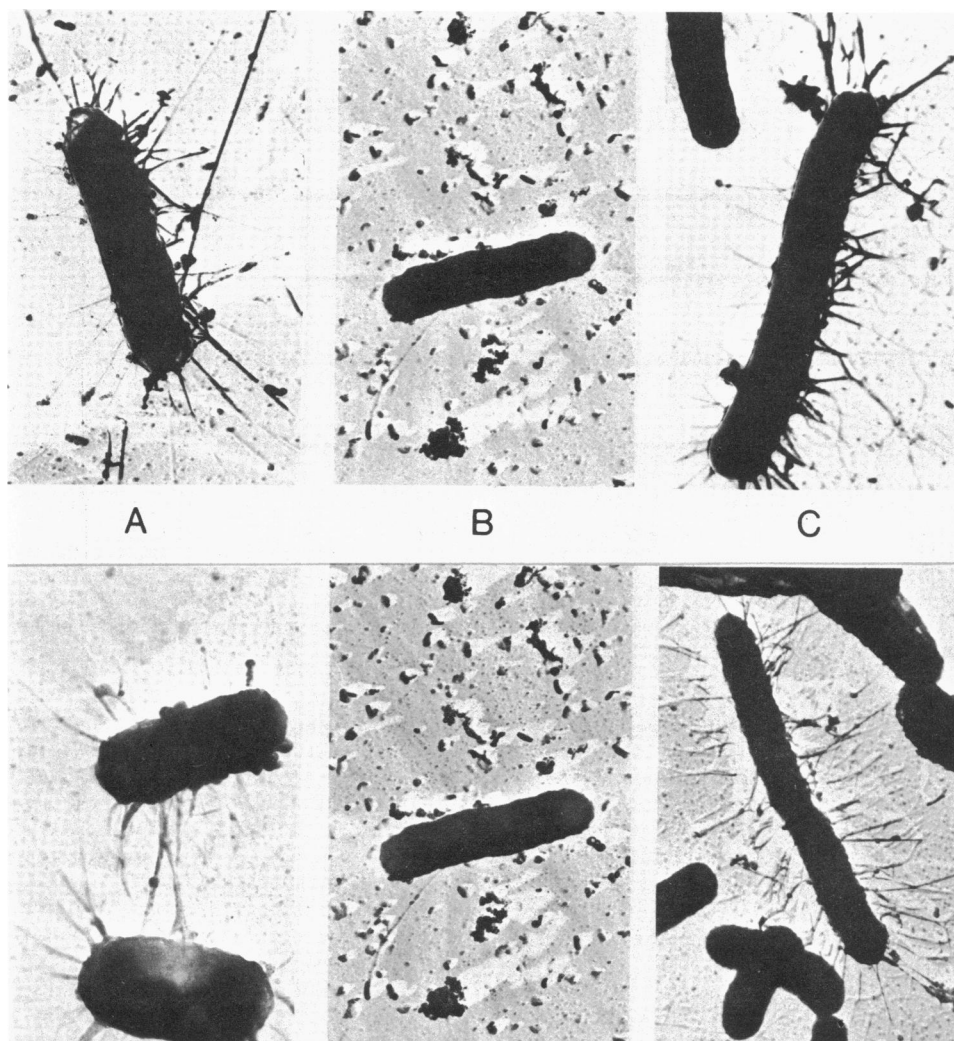
FIG. 4. Electron micrographs of the piliated and nonpiliated *E. coli* strains. (Top) *E. coli* 536 (O6) with MRHA pili type Vb (A), *E. coli* K-12 without pili (B), and *E. coli* K-12 (pANN801) with MRHA pili type Vb (C). (Bottom) *E. coli* 764 (O18) with MRHA pili type VIc (A), *E. coli* K-12 without pili (B), and *E. coli* K-12 (pANN802) with MRHA pili type VIc (C).

TABLE 3. MRHA of *E. coli* isolates and *E. coli* K-12 strains carrying recombinant DNA

E. coli strain	MRHA pattern[a]			MRHA type[b]
	Hu	Bv	Mk	
HB101(pJC74)				
764 (O18)	R		R	VIc
HB101(pANN802)	R		R	VIc
5K				
5K(pANN802)	R		R	VIc
536 (O6)		R		Vb
HB101(pANN801)		R		Vb
5K(pANN801)		R		Vb

[a] Erythrocytes tested: Hu, human; Bv, bovine; Mk, African green monkey. R, MRHA. None of the chicken or guinea pig erythrocytes tested showed MRHA.

[b] Types according to Evans et al. (9).

by the host, but the production of hemolysin is not significantly increased, despite the high copy number of the recombinant plasmid. The high frequency of hemolysin-negative strains which are observed when clones carrying the hemolysin-positive recombinant cosmids or plasmids are grown without selection pressure is always due to the loss of the entire plasmid or cosmid and not to a partial deletion of the hemolysin determinant. The frequent loss of these recombinant DNAs, which is even more dramatic in the MRHA Vb and MRHA VIc clones, suggests that the carriage of these factors is disadvantageous to *E. coli* K-12 strains under normal growth conditions.

We consider that the most interesting observation made with the cloned chromosomal he-

molysin determinants is that, despite their close structural and sequential relationship, defined differences are evident. These seem to be clustered in the *hlyA* cistron; *hlyB*, which is of similar size to *hlyA*, does not show recognizable changes. Preliminary data on the considerably smaller *hlyC* also indicate a very conserved sequence in this part of the hemolysin determinant. These results support our previous observations, based on hybridization data between the plasmid-encoded and several chromosome-mediated hemolysin determinants (D. Müller, C. Hughes, and W. Goebel, submitted for publication). Work is currently under way to determine whether the observed changes in *hlyA* can be correlated with the observed differences in virulence exerted by various isolated hemolysin determinants.

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