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Gene Block Encoding Production of Cytotoxic Necrotizing Factor 1 and Hemolysin in *Escherichia coli* Isolates from Extraintestinal Infections

VINCENZO FALBO,* MARIA FAMIGLIETTI, AND ALFREDO CAPRIOLI

Laboratorio di Ultrastrutture, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

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Cytotoxic necrotizing factors (CNFs) are *Escherichia coli* protein toxins causing cell multinucleation and enlargement in tissue cultures and necrosis in rabbit skin. In *E. coli* isolates causing urinary tract infections in humans, the production of CNF1 is closely associated with hemolysin production. In this study, we obtained data suggesting that this phenotypic association is due to the genetic linkage of the determinants of the two toxins on the chromosome of uropathogenic *E. coli*. The genes encoding hemolysin and CNF1 were shown to be closely linked in a 37-kb cloned DNA fragment from an *E. coli* urinary tract isolate of serotype O4:K12:H5 (E-B35). A DNA region encoding CNF1 production but not hemolysin production was further subcloned as a 12-kb *SalI-EcoRI* fragment and used as a CNF1-specific gene probe. DNA hybridization experiments indicated that the CNF1 and hemolysin determinants were closely linked on the chromosomes of isolate E-B35 and six additional extraintestinal isolates belonging to serogroups O2, O4, O6, O22, O75, and O85.

Certain *Escherichia coli* strains are able to elaborate toxic factors causing cell multinucleation and enlargement in tissue cultures and necrosis in rabbit skin. One of these factors was first described in 1983 in our laboratory (7), and we proposed for that toxin the term cytotoxic necrotizing factor (CNF). CNF was subsequently purified and identified as a protein of about 115 kDa by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (6, 13). CNF production was recognized first in *E. coli* strains from humans with diarrhea (3, 7) and subsequently in a large proportion of strains causing urinary tract infections (UTI) and bacteremia in humans (1, 4, 8, 9). CNF-positive strains have also been frequently isolated from enteritic piglets and calves (5, 12, 24, 29).

Recently, De Rycke and coworkers (11) described another type of CNF, produced by *E. coli* isolates causing calf enteritis and formerly designated Vir isolates (33). This toxin induced cell multinucleation slightly different from that elicited by the original CNF, showed an immunological cross-reaction with CNF (11), and was purified as a protein of about 110 kDa (32). In light of these results, De Rycke and coworkers (11) proposed to call this new factor, mostly produced by animal isolates, CNF2 and the CNF originally described by our group CNF1. Blanco et al. (4) proposed to call the *E. coli* strains producing CNF1 or CNF2 necrotoxigenic *E. coli*.

The role of CNFs in the pathogenesis of *E. coli* infections is still uncertain, but the necrotic and lethal properties in vivo (7, 11, 13) and the ability to alter the cytoskeleton of cultured cells in vitro (17) suggest that CNFs may be true virulence factors.

Unlike CNF2-producing isolates, the large majority of CNF1-producing isolates were also shown to produce hemolysin (Hly) (1, 3, 4, 7, 9, 11), another well-known *E. coli* toxin whose role in pathogenesis is still under debate (10, 20, 26, 27, 36). Despite their close association, CNF1 and Hly activities are carried by different molecular species, as shown by the facts that they did not copurify (7, 13) and that Hly-negative mutants retained the CNF1 activity of the parental strain (13). To study the possible genetic basis of the association existing between Hly and CNF1 production, we decided to use recombinant DNA techniques. We present here data on the linkage of the genes encoding the production of CNF1 and Hly on the chromosomes of extraintestinal *E. coli* isolates belonging to different serotypes.

MATERIALS AND METHODS

Bacterial strains, cosmids, and plasmids. The wild-type *E. coli* strains used were part of our laboratory collection and are listed in Table 1. *E. coli* BHB2688 and BHB2690 (28) were used for the preparation of a bacteriophage lambda in vitro packaging extract. *E. coli* LE392 (28) was used as a host strain for preparing the recombinant cosmid library. Cosmid vector pHC79 (23) was used for cosmid cloning. Recombinant plasmids pANN202, pANN215, and pANN250, containing Hly-specific probes (31), were kindly provided by J. Hacker, Würzburg University, Würzburg, Germany.

Media and reagents. *E. coli* strains were grown in L broth (28) supplemented with selective agents when required. Antibiotics and other chemicals used were purchased from Sigma Chemical Co. (St. Louis, Mo.). All restriction endonucleases, T4 ligase, and other enzymes were obtained from Boehringer (Mannheim, Germany).

Detection of Hly, CNF1, and adhesin production. Recombinant cosmid clones were screened for CNF1 and Hly production as previously described (3, 7). In brief, cultures were grown in Trypticase soy broth containing ampicillin at 25 μg/ml. After overnight growth with aeration at 37°C, cultures were spotted onto blood agar plates for the hemolysis test and then were sonicated in an ice bath. The cell debris was removed by centrifugation, and supernatants were tested for CNF activity on HEp-2 cell monolayers grown in 96-well microtiter plates. Titration of toxic activity

* Corresponding author.

TABLE 1. Hly- and CNF1-producing wild-type *E. coli* strains used in this study[a]

Strain	Serotype	Origin
E-B35	O4:K12:H5	UTI
E-B28	O4:K12:H1	UTI
E-B54	O2:K−:H−	UTI
E-B29	O6:K13:H1	UTI
E-B34	O22:K?:H5	UTI
E-B85	O75:K5:H−	Bacteremia
E-B39	O85:K−:H1	UTI

[a] Information is from reference 9.

and seroneutralization assays were carried out as described by De Rycke et al. (11). CNF1-neutralizing antiserum was produced in rabbits against cell extracts from *E. coli* E-B28 as described by De Rycke and coworkers (12). Antiserum to CNF2 was a kind gift from J. De Rycke, Nouzilly, France. The rabbit skin test was performed as previously described (7). The presence of P fimbriae was assessed by mannose-resistant hemagglutination (MRHA) of human P1 erythrocytes and by the PF latex agglutination test (Orion Diagnostica, Espoo, Finland). P-related fimbrial adhesins (18, 19) were recognized by MRHA of sheep erythrocytes.

Preparation of DNA. Chromosomal DNA from wild-type *E. coli* strains was isolated by the modified Marmur procedure described by Ferragut and Leclerc (16). Cosmid pHC79 and the recombinant plasmids were isolated by the cleared-lysate procedure (2) and further purified, when necessary, by CsCl-ethidium bromide gradient centrifugation. Restriction endonuclease maps were prepared by the method described by Maniatis et al. (28). Electrophoresis of restriction endonuclease-cleaved DNA was performed in horizontal agarose slab gels with a low-salt buffer system composed of 40 mM Tris-acetate and 2 mM sodium EDTA (pH 8.0). The desired fragments were extracted from low-melting-point agarose (28).

Recombinant DNA library construction. Chromosomal DNA from *E. coli* E-B35 was partially digested with restriction enzyme *Sau*3A, and the digest yielding the largest number of DNA fragments in the 20- to 48-kb range was selected by agarose gel electrophoretic analysis. The restricted DNA was ligated to *Bam*HI-cleaved and alkaline phosphatase-treated cosmid vector pHC79. Preparation of the packaging extract, the in vitro packaging reaction, and infection of the recipient strain were carried out as described by Maniatis et al. (28). Recombinant cosmid libraries prepared in strain LE392 were stored frozen at −70°C in L broth containing 25 μg of ampicillin per ml and 5% glycerol. The sizes of intact recombinant cosmids were estimated by comparing their relative electrophoretic mobilities with those of covalently closed circular DNAs of reference plasmids.

Southern blotting and DNA hybridization. Restriction endonuclease-cleaved DNA was transferred to nitrocellulose filters as described by Southern (34). Hly- and CNF1-specific gene probes were labeled with ^{32}P-deoxynucleoside triphosphates by nick translation, and hybridization was carried out under stringent conditions as described by Maniatis et al. (28). In brief, filters were prehybridized in 5× Denhardt's solution at 63°C for 3 h. Hybridization was carried out overnight at 63°C in the same buffer. Filters were washed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS, 2× SSC–0.1% SDS, and 1× SSC–0.1% SDS two times each for 20 min each time at 63°C, air dried, and then autoradiographed with Kodak X-ray film.

Immunoblotting analysis of cell sonic extracts from *E. coli* strains. Bacteria were grown for 18 h at 37°C with shaking in 5-liter Erlenmeyer flasks containing 1 liter of Trypticase soy broth with 25 μg of ampicillin per ml. Cells were collected by centrifugation, resuspended in 10 ml of 0.025 M Tris hydrochloride buffer (pH 7.2), and sonicated. The lysate was centrifuged at 30,000 × *g* for 30 min, and the supernatant was centrifuged again at 200,000 × *g* for 2 h. One milliliter of the resulting supernatant was concentrated about 10-fold with a 100-kDa molecular-mass-cutoff ultrafiltration membrane (Ultrafree-MC; Millipore Corp., Bedford, Mass.). Three milliliters of buffer was added during the concentration procedure. Concentrated samples were analyzed by SDS-PAGE performed with 7.5% polyacrylamide gels by the method of Laemmli (25). Gels were stained with Coomassie blue. For immunoblotting, proteins were transferred to nitrocellulose sheets by use of Tris-glycine buffer with 20% methanol at 100 V for 1 h. Blots were probed with a rabbit CNF1-neutralizing antiserum partially absorbed with sonic extracts of K-12 strain LE392. Following incubation with a peroxidase-conjugated anti-rabbit immunoglobulin G goat antibody (Sigma), labeled proteins were detected by use of an enhanced-chemiluminescence system (ECL Western blotting; Amersham International, Amersham, England).

RESULTS

Cloning of the genes encoding CNF1 and Hly from *E. coli* E-B35. *E. coli* E-B35 was isolated from a case of UTI and belongs to serotype O4:K12:H5. It expresses both Hly and CNF1 activities but does not produce P fimbriae. The presence of P-related fimbrial adhesins was suggested by its ability to agglutinate sheep erythrocytes in the presence of mannose.

A cosmid library was prepared with vector pHC79 and DNA from strain E-B35 and plated on strain LE392. Ampicillin-resistant colonies were obtained at a frequency of 10^4/μg of ligated *E. coli* DNA. Endonuclease cleavage and gel electrophoresis of the cosmids present in these transductants revealed that the average random insert of *E. coli* DNA was 40 kb in size. Four hundred cosmid-containing strains were screened for CNF and Hly production. One cosmid clone was positive for both Hly and CNF activities. The recombinant plasmid acquired by this strain was designated pISS385. No cosmid clone which produced only one of the two toxins was obtained, suggesting that in strain E-B35 the genes encoding the production of CNF1 and Hly were closely linked.

Preliminary characterization of pISS385 and subcloning of the CNF determinant. A partial physical map of pISS385 DNA (Fig. 1) was obtained by a series of double and triple enzyme digestions and revealed a 37.5-kb molecular size for the chromosomal DNA insert. This fragment did not confer on the host strain the ability to agglutinate sheep erythrocytes. The genes encoding Hly production were localized by Southern hybridization with the following Hly-specific probes, derived from the Hly plasmid pHly152 (31): pANN202 (which carries the *hlyC* gene), pANN215 (which carries part of the structural *hlyA* gene), and pANN250 (which carries the C-terminal half of *hlyA* and the functional *hlyB* gene).

Subcloning of the CNF determinant was performed by complete digestion of pISS385 DNA with the restriction enzymes *Sal*I and *Eco*RI. The largest fragment obtained (12

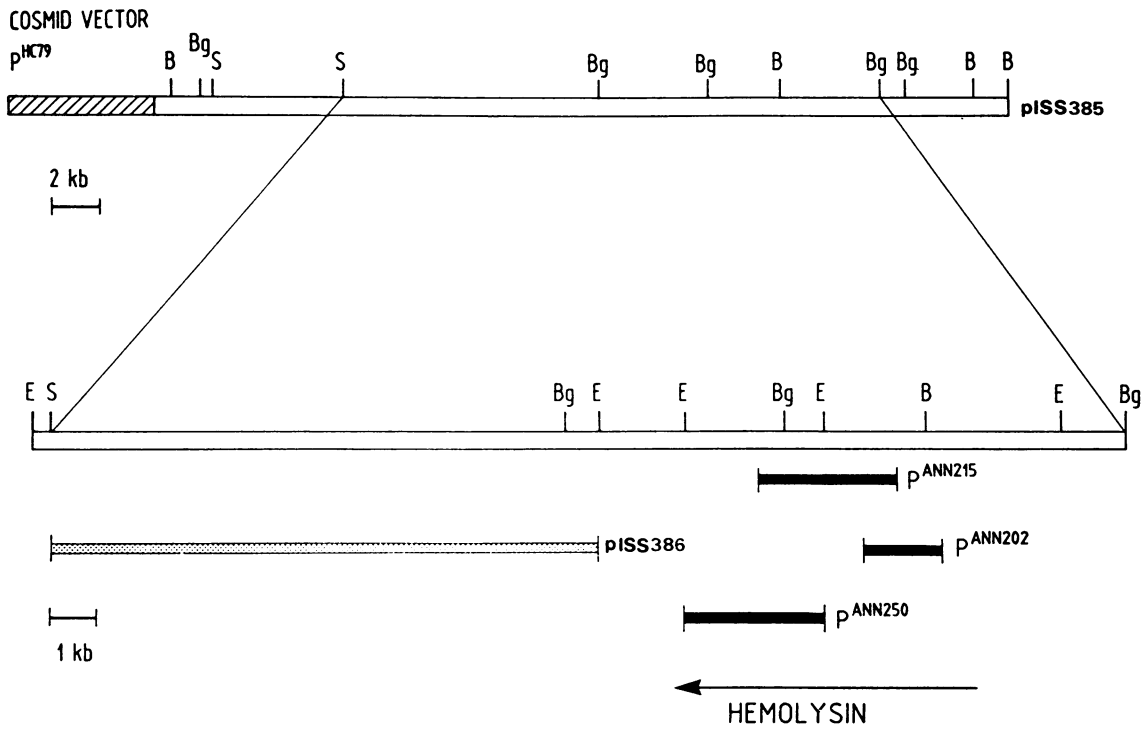

FIG. 1. Physical map of recombinant plasmid pISS385 carrying Hly and CNF1 gene clusters and of subclone derivative pISS386 carrying the CNF1 gene cluster only. The positions of the Hly-specific DNA probes used for Southern blot hybridizations are indicated below. Restriction target sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; S, *Sal*I.

kb) was extracted from the gel, religated into cosmid pHC79, and packaged in vitro. The resulting particles were transduced into LE392, and transductants were selected on medium containing ampicillin. The clones that acquired the recombinant plasmid carrying the 12-kb insert, designated pISS386, still maintained the ability to produce CNF, but they neither produced Hly nor hybridized under stringent conditions with Hly probe pANN250 (Fig. 2C, lane 1). The position of the 12-kb fragment encoding CNF production in the restriction map is shown in Fig. 1.

To rule out the possibility that our 12-kb fragment included any sequence from the Hly operon, we tested whether an entire Hly determinant would hybridize under stringent conditions with the pISS386 probe. The Hly determinant was obtained by digesting Hly plasmid pSU212 (30) with *Sal*I, which does not cut within the Hly operon (15, 20, 21, 27). As shown in Fig. 2B, lane 2, the pISS386 probe did not hybridize with the Hly determinant contained in the 15-kb *Sal*I fragment from pSU212; in contrast, the Hly determinant was recognized by Hly probe pANN250 (Fig. 2C, lane 2). Both probes gave weak hybridization signals with the largest *Sal*I fragment from plasmid pUS212, which probably contains sequences related to vectors pACY184 (31) and pHC79.

Partial characterization of CNF encoded by pISS386. Cell sonic extracts from recombinant strains LE392(pISS385) and LE392(pISS386) contained about fourfold more toxic activity than wild-type strain E-B35; the titers of the multinucleating cytotoxic effect were 1,024 for the recombinant strains and 256 for the wild-type strain. As expected, all toxic extracts induced necrosis in rabbit skin, were neutralized at a high titer (1,024) by CNF1 antiserum, and showed a degree of cross-neutralization with CNF2 antiserum (titer, 32) (titers represent the highest dilution of serum that neutralized the cytotoxicity of four 50% cytotoxic doses). Cell sonic extracts from LE392 carrying cosmid pHC79 or recombinant plasmid pISS386 were concentrated as de-

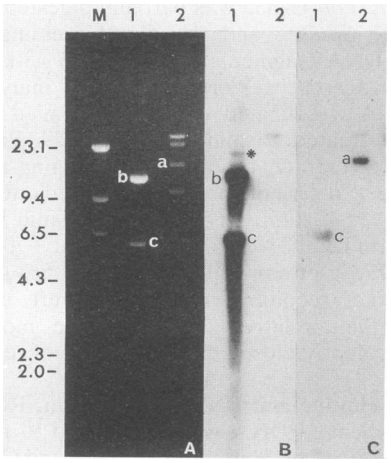

FIG. 2. Southern hybridization of the pSU212 Hly determinant with pISS386 as a probe. (B) *Sal*I-cleaved DNA from plasmid pSU212 (Hly$^+$ CNF$^-$; lane 2) and *Sal*I-*Eco*RI-cleaved cosmid DNA from pISS386 (Hly$^-$ CNF$^+$; lane 1) were hybridized with pISS386 DNA. (C) Hybridization with Hly-specific probe pANN250 was performed as a control. (A) The cleavage products were separated through 0.7% agarose; M, *Hin*dIII-cleaved lambda DNA; sizes are in kilobases. a, Hly determinant; b, CNF determinant; c, pHC79 vector. The asterisk indicates a partial digest fragment.

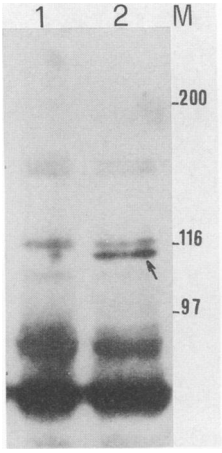

FIG. 3. Comparison by immunoblotting of concentrated cell sonic extracts from *E. coli* LE392 carrying cosmid pHC79 (Hly^- CNF^-; lane 1) and LE392 carrying recombinant plasmid pISS386 (Hly^- CNF^+; lane 2). Proteins were separated by SDS-PAGE in a 7.5% polyacrylamide gel and probed with rabbit CNF1 antiserum. M, molecular mass markers (in kilodaltons). The arrow indicates the 112-kDa protein band, which was absent from the CNF^- strain.

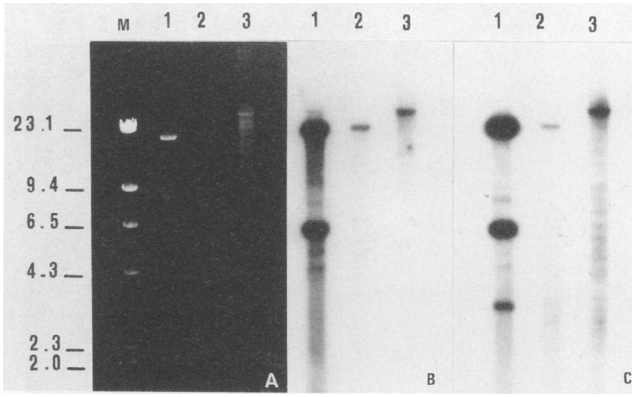

FIG. 4. Linkage of Hly and CNF1 determinants in *E. coli* E-B35. CNF1 (pISS386; B)- and Hly (pANN215; C)-specific gene probes were hybridized to *Bam*HI-*Sal*I-cleaved cosmid pISS385 (lane 1) and chromosomal DNA of strain E-B35 cleaved with either *Bam*HI-*Sal*I (lane 2) or *Bam*HI alone (lane 3). (A) The cleavage products were separated by electrophoresis through 0.5% agarose. M, DNA size markers (in kilobases).

scribed in Materials and Methods and compared by SDS-PAGE. LE392 carrying the CNF1-encoding 12-kb DNA fragment exhibited a faint additional protein band of about 112 kDa (data not shown), a molecular mass very close to those previously estimated for CNF1 (6, 13). To achieve better resolution, we transferred the proteins separated by SDS-PAGE to a nitrocellulose filter and probed them with CNF1 antiserum partially absorbed with sonic extracts of host strain LE392. As shown in Fig. 3, CNF1 antiserum clearly recognized a band of about 112 kDa in the sonic extract from the CNF-producing recombinant strain. The band was absent from the protein profile of host strain LE392.

Linkage of Hly and CNF1 determinants on the chromosomes of strain E-B35 and other *E. coli* strains belonging to different serogroups. To confirm the genetic linkage of the CNF1 and Hly determinants of strain E-B35, we digested total chromosomal DNA from this strain and cosmid pISS385 with *Bam*HI-*Sal*I, cleaving the cosmid DNA to yield a 19.8-kb fragment containing both Hly and CNF1 sequences (Fig. 4A). DNA was hybridized with CNF1 (pISS386; Fig. 4B)- and Hly (pANN215; Fig. 4C)-specific gene probes. Both probes recognized a *Bam*HI-*Sal*I fragment from strain E-B35 (Fig. 4, lane 2) which comigrated with the 19.8-kb *Bam*HI-*Sal*I fragment from pISS385 (Fig. 4, lane 1). This result was confirmed by a different digestion of the E-B35 chromosome with *Bam*HI alone (Fig. 4, lane 3). Again, both probes recognized the same unique fragment, the size of which was about 27 kb, as expected on the basis of the pISS385 restriction map. The identical patterns after hybridization of the chromosomal DNA with the Hly and CNF1 probes confirm the linkage of the two genes on the chromosome of wild-type strain E-B35.

To determine whether the CNF1 and Hly genes in other *E. coli* strains were linked in a manner similar to that in strain E-B35, we performed Southern blotting analysis of Hly^+ CNF^+ *E. coli* strains belonging to some of the O serogroups mainly involved in UTI (Table 1). Total chromosomal DNAs from E-B35 and six additional wild-type strains were digested with *Sal*I, cleaving pISS385 to yield a 29.5-kb fragment containing both Hly and CNF1 sequences. DNA was hybridized with CNF1- and Hly-specific gene probes (Fig. 5). The CNF1 probe hybridized to a *Sal*I fragment of about 30 kb in cosmid pISS385 as well as in the chromosomal DNA digests of strain E-B35 and strains belonging to serogroups O2, O4, O6, O22, and O85 (Fig. 5B, lanes 3 to 6 and 8). The genomic DNA of O75 strain E-B85 showed a hybridization signal for a smaller fragment of about 24 kb (Fig. 5B, lane 7). In all the wild-type strains, the Hly probe (Fig. 5C) hybridized to *Sal*I fragments with electrophoretic mobilities similar to those of the CNF1-specific ones. These results were confirmed by *Bam*HI-*Sal*I digestion of chromosomal DNA (data not shown) and indicated that the Hly and CNF1 genes were linked in all the wild-type strains examined. Strains E-B28 (O4) and E-B85 (O75) had additional Hly-specific *Sal*I fragments of more than 30 kb and about 14 kb, respectively (Fig. 5C, lanes 4 and 7) which were not recognized by the CNF1 probe. This result suggests that two distinct copies of the Hly determinant were present in those strains and that only one copy was linked to the CNF1 genes.

DISCUSSION

It is well known that several factors may contribute to the virulence of *E. coli* strains causing UTI, including the synthesis of specific O and K antigens (9, 14), adherence to urinary tract epithelial cells (18, 35), and elaboration of toxic products, such as Hly and CNF1 (1, 8, 10, 14, 36). None of these factors absolutely correlates with virulence; rather, it appears that the pathogenicity of a strain is determined by a combination of a number of contributing factors.

In studying the nature of these consortia of virulence characteristics, Low and coworkers (26) showed that the genes encoding Hly production and MRHA are closely linked in *E. coli* urinary tract isolates of serogroups O6 and O4. These authors also suggested that the linkage of these two factors occurred through a transposition event and conferred a selective advantage to the isolates involved. Following this first observation, other authors described the existence of DNA segments carrying more than one pathogenicity gene cluster in close association on the chromosome of uropathogenic isolates of *E. coli* (18, 22). These DNA

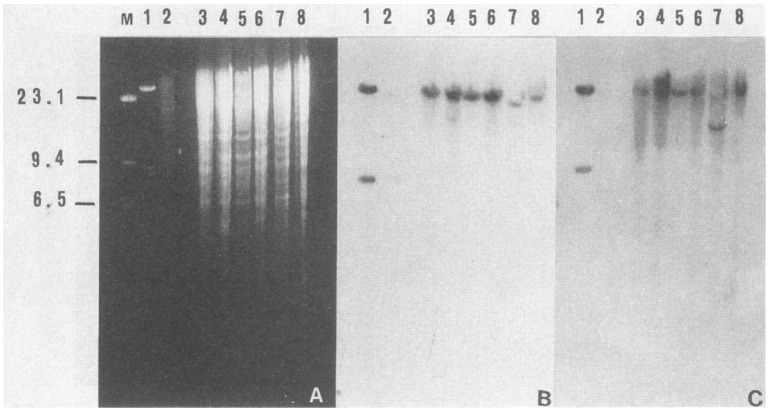

FIG. 5. Linkage of Hly and CNF1 determinants in other *E. coli* strains belonging to different O serogroups. CNF1 (pISS386; B)- and Hly (pANN215; C)-specific gene probes were hybridized to *Sal*I cleavage products (separated by electrophoresis through 0.5% agarose; A) of cosmid pISS385 (lane 1) and chromosomal DNAs of the following *E. coli* strains: lane 2, E-B35 (O4); lane 3, E-B34 (O22); lane 4, E-B28 (O4); lane 5, E-B29 (O6); lane 6, E-B54 (O2); lane 7, E-B85 (O75); and lane 8, E-B39 (O85). M, DNA size markers (in kilobases).

segments were termed virulence gene blocks. High and coworkers (22) described the association of genes encoding Hly and different types of fimbrial antigens in an isolate of *E. coli* O4:K12:H− showing both human and sheep MRHA. Hacker and colleagues (18, 19) presented data on the genetic linkage of an Hly determinant and the genes encoding P-related fimbrial adhesins in isolates belonging to serogroups O6 and O4. They also showed that virulence gene blocks may be part of larger DNA regions which may be deleted from the chromosome and proposed for these regions the term "pathogenicity DNA islands" (19). Our study provides further support for these observations by the description of another virulence gene block constituted by the linkage of an Hly determinant and the genes encoding CNF1 production on the chromosome of *E. coli* O4 strain E-B35. Southern blotting analysis indicated that such a gene block was present in all of the other six strains examined which belonged to the classical uropathogenic serogroups O2, O4, O6, O22, O75, and O85. Two of the strains studied had a second copy of the Hly determinant, which was not linked to the CNF1 genes. The existence of multiple Hly determinants on the chromosomes of uropathogenic *E. coli* strains has been reported by Hacker and coworkers (19). These authors also demonstrated that, in the same strain, each determinant may be linked to different fimbrial adhesin genes (19). Our results seem to confirm the idea that the chromosomal regions containing the Hly determinants may have different DNA arrangements, but further studies are obviously needed to clarify the organization and distribution of these pathogenicity islands.

In our strains, the Hly and CNF1 gene clusters were shown to be very close to each other, indicating that the strong phenotypic association between Hly and CNF production repeatedly observed among different uropathogenic *E. coli* clones (1, 4, 8, 9) has a genetic basis. The role of CNF in the pathogenesis of human and animal *E. coli* infections is still uncertain, and the linkage of its determinant to the Hly determinant in urinary tract isolates may only be a fortuitous genetic event. However, if such an event occurred independently in several different uropathogenic clones (9), we could speculate that there is a selective advantage conferred by this linkage. Such a selective advantage could occur if these two factors acted in a synergistic or complementary way, as previously hypothesized for Hly itself and MRHA adhesins (26). In this respect, the cloning of the DNA region encoding CNF1 production is considered a first step toward (i) a more detailed analysis of the structural organization of the CNF1 determinant and (ii) a comprehensive study of the distribution of the Hly-CNF gene block among uropathogenic *E. coli* clones.

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