Organization of Aerobactin, Hemolysin, and Antibacterial Resistance Genes in Lactose-Negative *Escherichia coli* Strains of Serotype O4 Isolated from Children with Diarrhea

BIANCA COLONNA,[1] LORELLA RANUCCI,[1] PIERA ASSUNTA FRADIANI,[1]
MARIASSUNTA CASALINO,[1] ATTILIO CALCONI,[1] AND MAURO NICOLETTI[2]*

*Dipartimento di Biologia Cellulare e dello Sviluppo, Sezione di Scienze Microbiologiche, Universita'
"La Sapienza," 00185 Rome,[1] and Instituto di Medicina Sperimentale, Cattedra di
Microbiologia, Universita' "G. D'Annunzio," 63100 Chieti,[2] Italy*

Epidemiologically related, non-lactose-fermenting (NLF) *Escherichia coli* strains of serotype O4 have been isolated at a high frequency from children with diarrhea in Somalia (M. Nicoletti, F. Superti, C. Conti, A. Calconi, and C. Zagaglia, J. Clin. Microbiol. 26:524–529, 1988). In order to define the virulence potential of these strains, we characterized the replication properties of their high-molecular-weight plasmids and studied the genetic locations and organization of the aerobactin (*aer*) and hemolysin (*hly*) determinants encoded by 23 NLF O4 *E. coli* strains. Southern blot hybridizations, mobilization assays of nonconjugative plasmids, and incompatibility-exclusion experiments conducted with a conjugative incompatibility group FI (IncFI) plasmid showed that (i) 20 out of the 23 strains examined harbor a 160- to 180-kb IncFI plasmid that shares homology with the basic replicons RepFIA, RepFIB, and (except for the plasmid of one strain) RepFIC, and 22 strains also contain a 40- to 140-kb IncFII plasmid sharing homology with the RepFIIA replicon; (ii) the IncFI plasmid is nonconjugative and carries antibiotic resistance genes; (iii) the *aer* system is located on the IncFI plasmids and/or the chromosomes in the three strains not harboring IncFI, and it is found in an inverted orientation; (iv) the *hly* determinants are located on the chromosome, and their genetic organization is well conserved and closely resembles that of the reference hemolytic plasmid pHly152; and (v) Hly⁻ mutants obtained by transposon insertion mutagenesis are not cytotoxic to HeLa cell monolayers, indicating that hemolysin is responsible for the high cytotoxic activity we have previously reported for these strains. The structural organization of the plasmid-encoded *aer* operon, together with the finding that those plasmids also carry antibiotic resistance genes, indicates that the IncFI plasmid of the NLF O4 *E. coli* strains studied more closely resembles *aer*-encoding virulence IncFI *Salmonella* R plasmids than *E. coli* ColV plasmids. The data presented here cannot rule out whether the strains examined are potentially intestinal or extraintestinal pathogens. Nevertheless, the genetic organization of the virulence genes, together with the epidemiological behavior and the wide spectrum of antibiotic resistance of the NLF O4 *E. coli* strains, indicates that these strains are structured as typical *E. coli* pathogenic isolates of human origin.

Escherichia coli is a normal inhabitant of the human large bowel, but under certain conditions it can also cause a variety of diseases. Upon its acquisition of specific virulence determinants, *E. coli* may be the causative agent of diarrhea, sepsis, or urinary tract infections. Four major categories of *E. coli* are now recognized as causing diarrheal diseases. They include enteropathogenic, enterotoxigenic, enteroinvasive, and enterohemorrhagic strains (21). However, sporadic cases of diarrheal diseases caused by other *E. coli* strains (atypical) have also been described previously (19). With typical and atypical diarrheagenic *E. coli* strains, little attention has been focused on non-lactose-fermenting (NLF) isolates (with the exception of those of enteroinvasive *E. coli*), probably because diarrheagenic *E. coli* strains are usually screened with strains which produce typically lactose-positive colonies when stool specimens are plated on primary enteric media.

During a 2-year study of diarrheal diseases in children in Somalia, we reported the isolation of NLF *E. coli* strains at a rather high frequency (6). Twenty-three out of 64 NLF strains examined represented a very homogeneous and epi-

demiologically interesting group. They (i) had an O4 serotype, (ii) produced mannose-resistant hemagglutination with human, chicken, and monkey erythrocytes, (iii) adhered to HeLa and HEp-2 cells grown in culture, and (iv) were hemolytic and produced hydroxamate-type siderophores. Also, intact bacteria were able to destroy HeLa cell monolayers very rapidly (27). None of them contained heat-labile or heat-stable enterotoxin genes or had enterotoxic effects in rabbit ileal loop assays. Bacterial cell extracts or culture supernatants did not produce high-level cytotoxic effects on HeLa cells. Moreover, these strains were multiply antibiotic resistant, and the identity of the plasmid profile in strains isolated over a period of years led us to postulate the epidemiological importance of these strains and propose a possible clonal origin (27).

E. coli strains of serotype O4 have been included in the family of facultative enteropathogenic *E. coli* strains, i.e., strains that have been associated with sporadic cases of diarrhea as well as extraintestinal infections such as meningitis, urinary tract infections, and bacteremia (11, 13). In order to better understand the role, if any, of NLF O4 *E. coli* strains as the causative agents of diarrheal diseases, we have further characterized the high-molecular-weight plasmids harbored by these strains for replication properties as well as

---

* Corresponding author.

TABLE 1. Antibiotic resistance patterns and relevant characteristics of plasmids harbored by the
NLF O4 *E. coli* strains used in this study

Strain[a]	Resistance pattern	Characteristics of plasmids[b]		
		RepFIA + RepFIB	RepFIIA	Other
HN1, HN2, HN5, HN7, HN32, HN33, HN69, HN73, HN96, HN107, HN162, HN168, HN175	Tc Cm Km Ap Su Sm Hg	160 kb, Aer$^+$	40 kb	
HN18, HN87	Tc Km Ap Su Sm Hg	160 kb, Aer$^+$	40 kb	
HN100	Cm Km Su Hg Tp	160 kb, Aer$^+$	40 kb	
HN117	Tc Cm Km Ap Su Sm Hp Tp Sp	180 kb, Aer$^+$	NP	
HN202	Tc Su	160 kb, Aer$^+$	40 kb	
HN48	Tc	160 kb, Aer$^+$	40 kb	100 kb
HN166	Tc	160 kb, Aer$^+$	40 kb	
HN15	Tc	NP	120 kb	
HN119	Tc Cm Km Ap Su Sm Hg	NP	40 kb	100 kb
HN126		NP	140 kb	

[a] In addition to the plasmids listed, bacterial strains also contained small plasmids ranging from 3 to 8 kb. All strains were hemolytic. The *hly* operon is located on the chromosome.

[b] Molecular sizes were estimated by comparison with known reference plasmids. Rep, basic replicons of plasmids; Aer$^+$, production of aerobactin and ferric aerobactin receptor; NP, not present.

for the presence of antibiotic resistance genes and we have studied the genetic locations and organization of the hemolysin (*hly*) and aerobactin (*aer*) operons. Moreover, to ascertain whether factors other than hemolysin were responsible for the cytotoxic effect that we previously reported for these strains (27), we isolated Hly-negative mutant strains by transposon insertion mutagenesis and tested them for cytotoxicity with HeLa cell monolayers.

MATERIALS AND METHODS

Bacterial strains. A total of 23 NLF *E. coli* strains of serotype O4:K−:H8 isolated from patients with diarrhea in Somalia were studied (Table 1) (6, 27). ZM87, a nalidixic acid-resistant mutant of *E. coli* K-12 strain 803 (7), was used as the recipient in mobilization experiments with nonconjugative plasmids and as the indicator strain in colicin assays. Strain ZM111 (ZM87 carrying the conjugative IncFI plasmid pZM111 [8]) was used as the donor in incompatibility experiments. Strains ZM3 (ZM87 carrying the IncFI plasmid pZM3 [8]), ZM111, and J5R100 (J5 carrying the IncFII plasmid R100 [36]) were used in hybridization experiments. *E. coli* LG1315 (F⁻ *ara entA lac leu mtl proC rpsL supE thi tonA trpE xyl*) (35) carrying the pColV-K30 plasmid and *E. coli* LG1522 (F⁻ *ara fepA lac leu mtl proC rpsL supE thi tonA trpE xyl*) carrying pColV-K30 *iuc* (4) were kindly supplied by P. H. Williams.

Media and chemicals. Enriched and minimal growth media were LB medium (30), M9 minimal salt medium (30), and Mueller-Hinton agar medium (BBL Microbiology Systems, Cockeysville, Md.). The following antibiotics in the following concentrations were used: ampicillin (Ap), 30 μg/ml; chloramphenicol (Cm), 25 μg/ml; gentamicin (Gm), 10 μg/ml; kanamycin (Km), 25 μg/ml; nalidixic acid, 40 μg/ml; spectinomycin (Sp), 10 μg/ml; sulfonamide (Su), 600 μg/ml

in minimal medium; trimethoprim (Tp), 10 μg/ml; and tetracycline (Tc), 5 μg/ml. When required, α,α'-dipyridyl (150 μM) was added in M9 minimal medium to reduce iron availability.

Genetic procedures. The techniques for conjugation, incompatibility testing, mobilization of nonconjugative plasmids, transformation, and P1 *vir* transduction have been described previously (5, 32, 37). Selection of Tc-sensitive mutants of Tc-resistant O4 *E. coli* strains was accomplished by growing bacterial strains in LB broth supplemented with fusaric acid (12 μg/ml), chlortetracycline hydrochloride (50 μg/ml), and ZnCl₂ (0.1 mM) as described previously (23).

Detection of siderophores. Hydroxamates were assayed by the method of Csàky (10). Aerobactin production was monitored by a cross-feeding bioassay with LG1522 as the indicator strain on M9 minimal medium containing 150 μM α,α'-dipyridyl (4). Synthesis of the ferric aerobactin receptor was assayed by measuring the sensitivity to cloacin DF13 as described previously (8).

Hemolysin production and isolation of Hly-negative strains. Hemolysin production was determined on 5% sheep blood agar plates, and hemolytic activity was assayed as described previously (27). Hly-negative mutants were constructed, by transposon insertion mutagenesis, by introducing pCHR84 (31) into representative O4 *E. coli* strains. pCHR84 is a thermosensitive replication mutant of the conjugative plasmid R388 which codes for resistance to Tp, Su, and Gm. The Gm resistance gene is contained within a functional Tn*5* element in which the gene coding for Km resistance has been replaced by the gene coding for Gm resistance (Tn*5*-GM). pCHR84 was introduced by filter mating at 30°C separately into HN1 and HN119 strains. Three independent Gm-, Tp-, and Cm-resistant transconjugants from each mating, all Hly positive, were purified, and independent colonies were

grown overnight in LB broth at 30°C and then plated at 42°C on prewarmed LB agar plates supplemented with Gm. Hly⁻, Gm-resistant, Tp-sensitive clones resulting from the loss of the plasmid pCHR84 and the transposition of Tn5-GM were selected by replica plating on blood agar plates. The hly::Tn5-GM mutations were transduced by P1 vir into isogenic wild-type O4 E. coli HN1 and HN119, which were then used for further studies.

Colicin assay. The capacities of O4 E. coli strains to produce colicin were determined by the agar overlay method (22). ZM87, the indicator strain, was sensitive to colicin V produced by LG1315 (pColV-K30) (35).

Molecular procedures and DNA probes. Plasmid DNA extractions were performed by the method of Kado and Liu (18) or by the method of Birnboim and Doly (30). The molecular lengths of plasmids were estimated by comparing the plasmids' electrophoretic mobilities with those of known reference plasmids. Chromosomal DNA was prepared according to the method of Silhavy et al. (32). Restriction digestions and electrophoresis were performed as previously described (37). Plasmid DNAs and restriction DNA fragments from agarose gels were denatured and transferred to nitrocellulose filters by the method of Southern (30). Restriction DNA fragments used as DNA probes were obtained from low-melting-point agarose gels labelled with [α-^{32}P]dATP by the random-priming method (30), and unincorporated nucleotides were removed by passage through a Sephadex G-50 column. Labelled DNA fragments showing specific activities in the range of 2×10^8 to 5×10^8 cpm/μg of DNA were used in hybridization experiments. Filters were hybridized in a solution buffer containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 8.0]), 0.01 M EDTA, 0.5% sodium dodecyl sulfate (SDS), 0.1% polyvinylpyrrolidone, 0.1% Ficoll, and 0.1% bovine serum albumin at 68°C for 18 h. Filters were washed in 2× SSC–0.5% SDS for 5 min, 2× SSC–0.1% SDS for 15 min, and 0.1× SSC–0.5% SDS for 2 h at 68°C (high stringency). The DNA probes used for the identification of replication origins were a 917-bp EcoRI fragment isolated from plasmid pULB2154 and containing part of the RepFIA replicon of the IncFI F plasmid (RepFIA probe) (9); a 1.2-kb PstI fragment isolated from plasmid pULB2404 and containing part of the RepFIB replicon of the IncFI P307 plasmid (RepFIB probe) (9); and a 543-bp PstI fragment isolated from plasmid pULB2401 and containing an internal sequence of the RepFIIA replicon of the IncFII R1drd-19 plasmid (RepFIIA probe) that also shows homology with the RepFIC replicon of IncFI plasmids (9, 29). The DNA probes specific for antibiotic resistance genes were a 750-bp EcoRI-PstI fragment isolated from plasmid pBR322 that contains part of the TEM-type B β-lactamase gene (Ap probe) (30) and a 1.8-kb SalI fragment isolated from plasmid pZM1033 that contains part of the type I APH(3') gene (Km probe) (7). The DNA probes for hemolysin were a 3-kb HindIII fragment isolated from plasmid pANN215 containing part of the hlyA gene, a 1.2-kb HindIII-SmaI fragment from plasmid pANN202 containing hlyC and part of hlyA, and a 3.2-kb EcoRI fragment from pANN250 containing part of the hlyB gene of plasmid pHly152 (hemolysin probe) (16). The DNA probe for aerobactin was a 2.8-kb AvaI fragment isolated from recombinant plasmid pZM1301 that contains part of the aer operon of the Salmonella wien plasmid pZM61 (aerobactin probe) (8).

Tests for cytotoxicity. The cytotoxicity of O4 E. coli strains and that of their Hly-negative isogenic mutants were evalu-ated by using HeLa cell monolayers as previously described (27).

RESULTS

Aerobactin production. Since all of the 23 NLF O4 E. coli strains examined produce hydroxamate-type siderophores (27), we performed cross-feeding experiments with LG1522. Under iron-deprived conditions of growth, all O4 E. coli strains were able to cross-feed LG1522 and were sensitive to cloacin DF13. This indicated that functional aerobactin molecules as well as the specific ferric aerobactin receptors were synthesized by all of the strains tested.

Characterization of plasmids. The 23 NLF O4 E. coli strains showed a highly uniform plasmid profile which consisted mainly of one large plasmid ranging from about 100 to 180 kb, one plasmid of about 40 kb (Table 1), and small plasmids ranging from 3 to 8 kb.

Since the NLF O4 E. coli strains were all shown to produce aerobactin and all harbored large plasmids (27), features often associated with the presence of plasmids in the FI incompatibility group (IncFI) (8, 34), we analyzed the plasmids for the presence of sequences homologous to RepFIA, RepFIB, and RepFIIA in Southern blot experiments. RepFIA and RepFIB are two basic replicons present in IncFI plasmids, while RepFIIA is the basic replicon in IncFII plasmids (9). Figure 1 shows agarose gel electrophoresis of plasmid DNA contents (Fig. 1A) and the corresponding Southern hybridizations with the RepFIA (Fig. 1B) and RepFIIA (Fig. 1C) probes of 10 out of the 23 NLF O4 E. coli strains examined, which were chosen as representative of each plasmid or antibiotic resistance pattern variant (Table 1). The RepFIA probe hybridized with the high-molecular-weight plasmids, with the exceptions of those present in strains HN15, HN119, and HN126 (Fig. 1B, lanes a, h, and i). The hybridization pattern of the RepFIB probe was identical to that of the RepFIA probe (data not shown), indicating that those high-molecular-weight plasmids belong to the IncFI group (9). On the other hand, the RepFIIA probe hybridized strongly with all of the 40-kb plasmids present in the strains tested, as well as with the 120- and the 140-kb plasmids of strains HN15 and HN126 (Fig. 1C), showing that they belong to the IncFII group (9). The weak hybridization of the putative and reference IncFI plasmids (defined as those which hybridized with both RepFIA and RepFIB probes) with the RepFIIA probe (Fig. 1C, lanes b through f, j, and k) reflected the presence of the RepFIC replicon, which is known to share partial homology with RepFIIA sequences (29). This indicates that these plasmids contain at least parts of the three basic replicons of IncFI plasmids (1, 9). Interestingly, the putative IncFI plasmid harbored by strain HN117 did not hybridize with the Rep-FIIA probe, thus indicating that the RepFIC region is probably deleted in this plasmid. The high-molecular-weight plasmid present in strain HN119 (Fig. 1C, lane h) and that with a similar size (about 100 kb) in strain HN48 (Fig. 1C, lane c) did not hybridize with the RepFIA, RepFIB, or RepFIIA probe. Southern blot experiments with the three Rep probes were also performed with the strains not included in Fig. 1, and they produced identical hybridization profiles among strains grouped together in Table 1 (data not shown).

Incompatibility exclusion experiments introducing the IncFI conjugative plasmid pZM111 (Cm, Km, Ap, Hg, and Gm) (8) into 15 representative IncFI-harboring O4 NLF E.
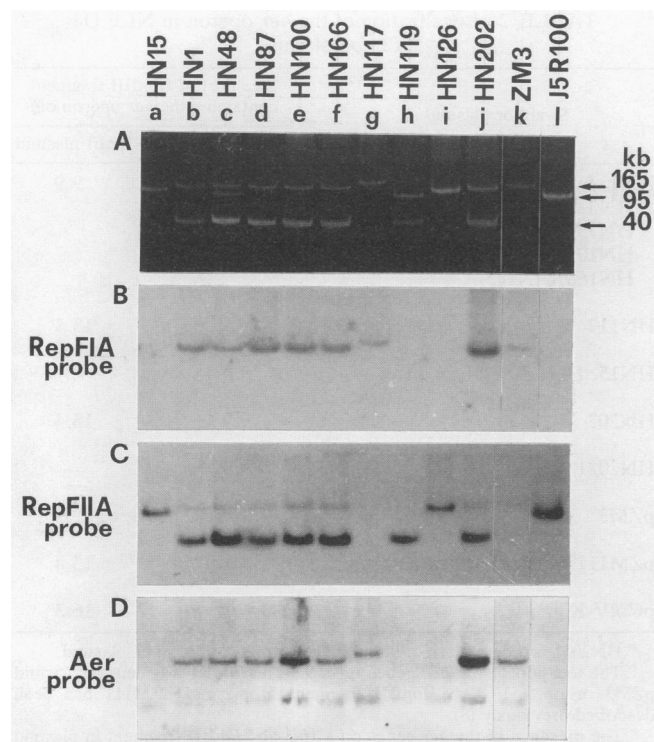
FIG. 1. Characterization of plasmids carrying aerobactin genes. Electrophoretic separation on a 0.8% agarose gel of plasmids prepared from 10 representative NLF O4 *E. coli* strains and 2 control strains (ZM3 and J5R100) (the control strains are described in Materials and Methods, and the electrophoresed plasmids were blotted onto nitrocellulose filters and hybridized with labelled probes as described in Materials and Methods) (A); autoradiograms of hybridization with the 917-bp *Eco*RI fragment of plasmid pULB2154 (RepFIA) (B), the 543-bp *Pst*I fragment of plasmid pULB2401 (RepFIIA) (C), and the 2,800-bp *Ava*I fragment of plasmid pZM1301 (aerobactin) (D). Molecular sizes are on the right.

coli strains confirmed the presence of a functional IncFI system in all of the plasmids (data not shown).

Genetic locations of antibiotic resistance genes. Since the NLF O4 *E. coli* strains studied showed multiple drug resistance, with the exceptions of HN126, which was sensitive to all of the antibiotics used, and HN15, HN48, and HN166, which were resistant only to Tc (Table 1), and since we could not transfer the antibiotic resistance markers to strain ZM87 either in liquid or in filter-mating experiments, we performed mobilization experiments using the conjugative plasmid pCHR84 (31). Plasmid pCHR84 was introduced by conjugation into three representative NLF O4 *E. coli* strains with different and broad antibiotic resistance patterns (HN1, HN100, and HN117; Table 1). Purified transconjugants were then individually filter mated with the *E. coli* K-12 strain ZM87 at 30°C. Since we isolated ZM87 transconjugants with the same antibiotic resistance profile as that of the donor strains, we concluded that the antibiotic resistance genes in strains HN1, HN100, and HN117 formed a linkage group which was located on defective conjugative plasmids. Agarose gel electrophoresis of plasmid DNA from independent ZM87 transconjugants revealed the presence of only two plasmids: a plasmid with a size corresponding to that of pCHR84 and a plasmid with the size of the corresponding IncFI plasmid. To provide further evidence of localization of

the antibiotic resistance genes on IncFI plasmids, we hybridized plasmid DNA from all multiply drug-resistant strains with Km and Ap probes. The two probes hybridized only with the high-molecular-weight IncFI plasmids and the 100-kb uncharacterized plasmid of strain HN119 (data not shown).

Tc-sensitive mutants of strains HN202, HN48, HN166, and HN15 were obtained by plating bacteria in the presence of 12 μg of fusaric acid per ml (23). Agarose gel electrophoresis of plasmid DNA preparations of Tc-resistant and Tc-sensitive derivative strains allowed us to establish that the Tc determinant (in strain HN202, together with Su) is located on the high-molecular-weight IncFI plasmid. Since all of the HN15 Tc-sensitive derivatives isolated from independent experiments showed plasmid contents identical to those of the parental strains, we concluded that in HN15 the Tc-resistant gene might be located on the chromosome. Moreover, with the exception of HN15, which produced a colicin that was active both on ZM87 (ColV sensitive) and LG1315 (ColV resistant), the remaining 22 O4 *E. coli* strains examined did not produce colicins.

Genetic locations and organization of the virulence genes. The location of the aerobactin operon was assessed by comparing the results of Southern blot experiments with plasmid DNA and those of Southern blot experiments with *Hind*III digests of total DNA from the 23 NLF O4 *E. coli* strains. The probe used was a 2.8-kb *Ava*I fragment isolated from plasmid pZM1301 that contains part of the *aer* operon of the IncFI plasmid pZM61 (8). The aerobactin genes were determined to occur on the bacterial chromosome when homology to the probe was detected only in *Hind*III digests of total DNA, with a negative result occurring in plasmid DNA Southern blot hybridizations. Figure 1D shows the hybridization of the *aer* probe with plasmids from the 10 O4 *E. coli* strains chosen as representatives of all of the O4 strains studied. The probe hybridized only with IncFI plasmids (Fig. 1D, lanes b through g, j, and k). In *Hind*III digests of total DNA from the three strains not containing IncFI, HN15, HN119, and HN126 (Fig. 2A), the probe recognized a 9.9-kb fragment (Fig. 2B, lanes a, h, and i) with *Hind*III-digested total DNA, indicating that in strains that do not contain IncFI, the *aer* system is located on the chromosome. Moreover, a 9.9-kb (Fig. 2, lanes b through f) or a 13.4-kb (Fig. 2, lane g) *Hind*III fragment showed up in strains containing IncFI. Interestingly, 13.4- and 9.9-kb *Hind*III fragments are recognized by the *aer* probe in the IncFI-containing strain HN202 (Fig. 2, lane j). Since only the 9.9-kb fragment is present in HN2021 (Fig. 2, lane n), an IncFI-cured derivative of HN202 obtained by selecting for resistance to fusaric acid, we concluded that two copies of the *aer* operon are present in strain HN202, with one located on the 13.4-kb *Hind*III fragment of the IncFI plasmid and the other located on a 9.9-kb *Hind*III fragment of the chromosome. Figure 2 also shows the following *Hind*III fragments of known IncFI plasmids hybridizing with the *aer* probe: a 9.9-kb fragment of pZM3 (lane k), a 13.4-kb fragment of pZM111 (lane 1), and a 16.3-kb fragment of pColV-K30 (2, 8, 34). Since 9.9- and 13.4-kb *Hind*III hybridization bands are obtained from pZM3 and pZM111, two *S. wien* virulence R plasmids which we have previously shown have the 16.7-kb IS*1* composite element containing the *aer* operon present in the opposite orientation (8), we concluded that the aerobactin-encoding DNA region of the O4 *E. coli* strains is well conserved, that it more closely resembles that of *S. wien* IncFI R plasmids than that of ColV plasmids (2, 4, 34), and that it is present in either orientation. Moreover, hybridiza-
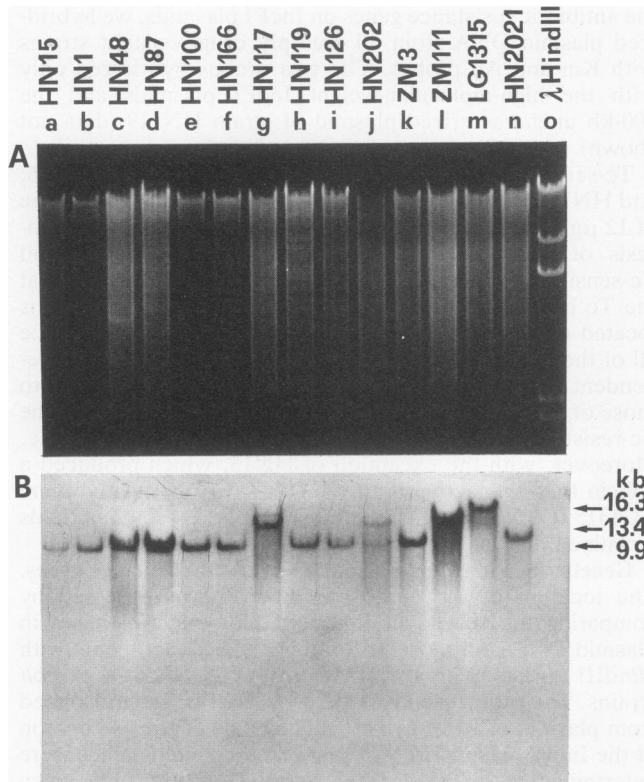
FIG. 2. Locations of aerobactin genes in different *E. coli* strains. (A) Electrophoretic separation on a 0.8% agarose gel of *Hind*III-restricted genomic DNA from 10 representative NLF O4 *E. coli* strains (lanes a to j). Also shown are ZM3 and ZM111 (*S. wien* aerobactin plasmids described in the text), LG1315 (aerobactin plasmid pColV-K30), and HN2021 (a derivative of strain HN202, shown in lane n, which has been cured of its IncFI plasmid). (B) Autoradiogram of hybridization of the genomic blots described above with the 2,800-bp *Ava*I fragment of plasmid pZM1301 (aerobactin). Molecular sizes are on the right.

TABLE 2. Localization of the *aer* operon in NLF O4 *E. coli* strains

Strain or plasmid	Size (kb) of *Hind*III fragment containing the *aer* operon on:	
	Chromosome	IncFI plasmid
HN1, HN2, HN5, HN7, HN18, HN32, HN33, HN48, HN69, HN73, HN87, HN96, HN100, HN107, HN162, HN166, HN168, HN175		9.9
HN117		13.4
HN15, HN119, HN126	9.9	
HN202	9.9	13.4
HN2021[a]	9.9	
pZM3[b]		9.9
pZM111[b]		13.4
pColV-K30[c]		16.3

[a] HN2021 is a derivative of strain HN202 cured of its IncFI plasmid.

[b] The location of the *aer* genes on a 9.9-kb *Hind*III fragment in plasmid pZM3 or on a 13.4-kb *Hind*III fragment in plasmid pZM111 has been described previously (8).

[c] The presence of the *aer* genes on a 16.3-kb *Hind*III fragment in plasmid pColV-K30 has been previously described (34).

tion experiments with the *aer* probe of *Hind*III total DNA digests of the remaining strains not shown in Fig. 2 displayed an identical hybridization pattern among the strains grouped together in Table 1. Table 2 summarize the results of plasmid and/or chromosomal *Hind*III fragments which hybridized with the *aer* probe for each of the 23 NLF O4 *E. coli* strains examined.

In a previous study, we showed that the 23 NLF O4 *E. coli* strains produced hemolysin and that they were highly cytotoxic to HeLa cell monolayers (27). To study the genetic organization of the *hly* operon, we performed Southern blot experiments with plasmid DNA as well as with *Hind*III digests of total DNA preparations from the 23 NLF O4 *E. coli* strains. We used three hemolysin probes spanning the *hlyABC* genes carried by the hemolysin-encoding plasmid pHly152 as probes (16). While the probes did not hybridize to plasmid DNA, in all *Hind*III-digested total DNA preparations examined they recognized fragments with the same sizes as those of the *Hind*III-digested pHly152. This indicated that the *hly* genes are located on the chromosome, that the *hly* DNA region is well conserved, and that the *hly* DNA region closely resembles that of pHly152 (15). Figure 3 shows the results of a typical Southern blot experiment with DNA from four representative NLF O4 *E. coli* strains

probed with the 3-kb *Hind*III fragment from the recombinant plasmid pANN215 (*hly* probe) (16).

Isolation of Hly-negative strains by Tn5-GM insertion mutagenesis. To ascertain whether the production of hemolysin was responsible for the highly cytotoxic effect on HeLa cell monolayers, we isolated Hly⁻ mutants of two Hly⁺ NLF O4 *E. coli* strains (HN1 and HN119) by transposon-induced mutagenesis. Plasmid pCHR84 was introduced into both
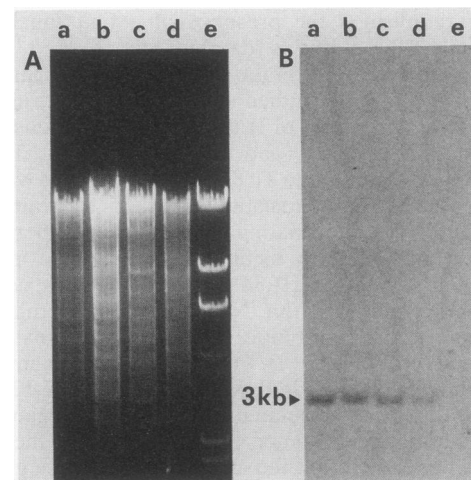

FIG. 3. Locations of hemolysin genes in NLF O4 *E. coli* strains. (A) Electrophoretic separation on a 0.8% agarose gel of *Hind*III-restricted genomic DNA from NLF O4 *E. coli* HN1, HN15, HN119, and HN202 (lanes a to d, respectively). Lane e shows *Hind*III-cut lambda DNA. (B) Autoradiogram of hybridization of the genomic blots described above with the 3,000-bp *Hind*III fragment of plasmid pANN215 (hemolysin).

strains by filter mating at 30°C. Tn5-GM-induced Hly⁻ mutants were isolated by plating at 42°C (nonpermissive temperature for pCHR84 maintenance [31]) on blood agar plates supplemented with Gm, and Tn5-GM-induced Hly⁻ mutations were transferred to isogenic Hly⁺ wild-type strains by P1 *vir* transduction. Independent *hly*::Tn5-GM (Hly⁻) mutants of strains HN1 and HN119 were then tested for cytotoxicity with HeLa cell monolayers. Since all of the Hly⁻ mutants tested were not cytotoxic to HeLa cells, we concluded that the chromosomally encoded hemolysin was responsible for the cytotoxic activity of the NLF O4 *E. coli* strains on HeLa cells.

DISCUSSION

The 23 O4 NLF *E. coli* strains described in this study are an interesting group of epidemiologically related strains that have been isolated from children with diarrhea in Somalia (6). In spite of being isolated in different years (1983 and 1984) and from different children, they have been shown to be very homogeneous in several respects, such as their hemagglutination properties, their hemolysin and hydroxamate-type siderophore production, their wide spectrum of antibiotic resistance, and their plasmid DNA profiles (27). These strains cannot be classified in the classical major diarrheagenic *E. coli* categories, because they do not produce the conventional diarrhea-associated virulence factors (27). Since *E. coli* strains of serotype O4 have been described as the causative agents of sporadic outbreaks of enteritis or meningitis, urinary tract infections, and bacteremia as well as common inhabitants of the human intestine (facultative enteropathogenic *E. coli* [11, 13]), we decided to further investigate those strains with the aim of better defining their virulence potential.

In this paper, we have shown the presence and the expression of the aerobactin iron uptake system in all of the 23 O4 NLF *E. coli* strains examined. The positive cross-feeding bioassays with the *E. coli* K-12 strain LG1522 and its sensitivity to cloacin DF13 indicated production of both aerobactin and specific ferric aerobactin receptors. As was shown from combined hybridization experiments with plasmid and *Hin*dIII-digested total DNA probed with the 2.8-kb *Ava*I fragment (*aer* probe), the aerobactin iron uptake system is located on a large, 160- to 180-kb plasmid present in 20 out of the 23 strains examined (one strain carries the *aer* system also on the chromosome). In the remaining three strains, the *aer* system is found on the chromosome (Table 1). The plasmids carrying the *aer* system were shown to belong to the IncFI incompatibility group by DNA hybridization experiments with the replicon-specific probes Rep-FIA, RepFIB, and RepFIC and by incompatibility-exclusion experiments with the IncFI plasmid pZM111.

The IncFI plasmids of the NLF O4 *E. coli* strains studied more closely resemble IncFI virulence R plasmids from epidemic *Salmonella* strains isolated in North Africa, Europe, and the Middle East (5, 8) than *E. coli* ColV plasmids (34, 35). In fact, they are nonconjugative, they do not encode colicins, and they carry several antibiotic resistance genes. Moreover, the *aer* probe specifically recognized a 9.9- or a 13.4-kb *Hin*dIII fragment, both in IncFI plasmids from NLF O4 *E. coli* strains (Fig. 2, lanes b to g and j) and in the IncFI reference R plasmids pZM3 and pZM111 (Fig. 2, lanes l and m) (8). The same probes hybridized with a 16.3-kb *Hin*dIII fragment in the pColV-K30-harboring strain LG1315 (Fig. 2, lane m) (25, 34). pZM3 and pZM111 are two naturally occurring, aerobactin-encoding IncFI virulence R plasmids

originally isolated from *S. wien* epidemic strains (8). In a previous study, we showed that the *aer* system of these plasmids, as well as that of plasmids isolated from *Salmonella typhimurium* strains, is part of a 16.7-kb DNA region flanked by two inverted copies of IS*1* that is present in an opposite orientation in pZM3 and pZM111 and that, depending on the orientation, is contained entirely in a 9.9- or a 13.4-kb *Hin*dIII fragment (8). This indicates that the genetic organization of the *aer* system among IncFI plasmids of NLF O4 *E. coli* and *Salmonella* strains is highly conserved and that inversion of the IS*1*-bound element may also occur in *E. coli* IncFI plasmids, thus excluding the hypothesis that inversion is mediated by a *Salmonella*-specific factor (34). In ColV plasmids, the IS*1*-bound *aer* system (25, 28) is highly homologous to those of the *Salmonella* R plasmids pZM3 and pZM111, but it has always been found in one orientation, with high conservation of the downstream and divergence of the upstream IS*1*-flanking DNA regions (2, 25, 34). For this reason, the aerobactin-containing *Hin*dIII fragments of the characterized ColV plasmids are of different sizes, ranging from 14.5 to 45 kb in length (34).

Interestingly, those NLF O4 *E. coli* strains containing a chromosomally encoded *aer* system presented the *aer* genes on a 9.9-kb *Hin*dIII fragment identical to that found in some IncFI plasmids (Fig. 2, lanes a, h, and i). Since the 9.9-kb *Hin*dIII fragment contains the *aer* operon, an IS*1* element (6a), and a downstream DNA region outside the IS*1* element (8, 34), an apparently identical 9.9-kb *Hin*dIII fragment in the chromosomally encoded *aer* system indicates that there is also a homologous plasmid and a chromosomal DNA region extending downstream of the IS*1*-bound *aer* system. The IS*1*-flanked aerobactin-encoding DNA region has been proposed as a genetically mobile element (12) to explain the ubiquity of the *aer* genes on both plasmids and chromosomes of *E. coli* and *Shigella* and *Salmonella* species (2, 3, 8, 20, 25). If the IS*1*-bound *aer* system were spread by transposition, one would expect variations in the flanking DNA regions outside the IS*1*-flanked transposon. On the contrary, the results presented here seem to indicate that the diffusion of the IS*1*-bound *aer* system among IncFI plasmids and chromosomes of different bacterial species might have originated from recombinational events not necessarily involving IS*1* sequences.

A well conserved, chromosomally encoded hemolysin genotype has been found for all of the 23 O4 NLF *E. coli* strains examined. The genetic organization of the *hly* operon has been studied by Southern hybridizations with a set of three DNA probes encompassing the *hlyABC* genes. The results confirmed a high degree of relatedness with the previously described *hly* operon in the reference hemolytic plasmid pHly152 (26). Hemolysin production by the O4 NLF *E. coli* strains was found to be responsible for the cytotoxic effect that we have previously reported for those strains (27). In fact, Hly-negative derivative strains obtained by Tn5-GM transposon-induced mutagenesis (31) were found to be completely noncytotoxic when challenged with cultured cells.

In conclusion, the NLF O4 *E. coli* strains examined in this study all presented chromosomally encoded hemolysin determinants and a plasmid- or chromosomally encoded *aer* system. These features have been previously reported for pathogenic extraintestinal *E. coli* isolates (14, 17, 24, 33). The data presented here cannot rule out whether the NLF O4 *E. coli* strains are intestinal pathogens. However, the genetic organization of the virulence genes, which is typical of that of pathogenic *E. coli* isolates of human origin (3, 15, 17, 26, 33), together with their epidemiological behavior and

the wide spectrum of antibiotic resistance (27) of the NLF O4 *E. coli* strains suggests that they can be viewed as potential pathogens which under different circumstances might induce intestinal and/or extraintestinal infections in the colonized host. In this respect, it would be of great importance to assess whether the occurrence of the O4 NLF *E. coli* strains in the intestines of children in Somalia alone would augment the probability of contracting specific intestinal or extraintestinal infections due to this serotype.

ACKNOWLEDGMENTS

REFERENCES

1. Bergquist, P. L., S. Saadi, and W. Maas. 1986. Distribution of basic replicons having homology with RepFIA, RepFIB, and RepFIC among IncF group plasmids. Plasmid 15:19–34.
2. Bindereif, A., and J. B. Neilands. 1985. Aerobactin genes in clinical isolates of *Escherichia coli*. J. Bacteriol. 161:727–735.
3. Carbonetti, N. H., S. Boonchai, S. H. Parry, V. Vaisanen-Rhen, T. K. Korhonen, and P. H. Williams. 1986. Aerobactin-mediated iron uptake by *Escherichia coli* isolates from human extraintestinal infections. Infect. Immun. 51:966–968.
4. Carbonetti, N. H., and P. H. Williams. 1984. A cluster of five genes specifying the aerobactin iron uptake system of plasmid ColV-K30. Infect. Immun. 46:7–12.
5. Casalino, M., A. Comanducci, M. Nicoletti, and F. Maimone. 1984. Stability of plasmid content in *Salmonella wien* in late phases of the epidemic history. Antimicrob. Agents Chemother. 25:499–501.
6. Casalino, M., M. W. Yusuf, M. Nicoletti, P. Bazzicalupo, A. Coppo, B. Colonna, C. Cappelli, C. Bianchini, V. Falbo, H. J. Ahmed, K. H. Omar, K. B. Maxamuud, and F. Maimone. 1988. A two-year study of enteric infections associated with diarrhoeal diseases in children in urban Somalia. Trans. R. Soc. Trop. Med. Hyg. 82:637–641.
6a. Colonna, B. Personal communication.
7. Colonna, B., M. L. Bernardini, G. Micheli, F. Maimone, M. Nicoletti, and M. Casalino. 1988. The *Salmonella wien* virulence plasmid pZM3 carries Tn*1935*, a multiresistance transposon containing a composite IS*1936*-kanamycin resistance element. Plasmid 20:221–231.
8. Colonna, B., M. Nicoletti, P. Visca, M. Casalino, P. Valenti, and F. Maimone. 1985. Composite IS*1* elements encoding hydroxamate-mediated iron uptake in F*Ime* plasmids from epidemic *Salmonella* spp. J. Bacteriol. 162:307–316.
9. Couturier, M., F. Bex, P. Bergquist, and W. K. Maas. 1988. Identification and classification of bacterial plasmids. Microbiol. Rev. 52:375–395.
10. Csàky, T. Z. 1948. On the estimation of bound hydroxylamine in biological materials. Acta Chem. Scand. 2:450–454.
11. Czirók, E., J. Madàr, J. Budai, Z. Stverteczky, A. Kertèsz, I. Dombi, and L. Gyengèsi. 1976. The role in sporadic enteritis of facultatively enteropathogenic *Escherichia coli* serogroups. Acta Microbiol. Acad. Sci. Hung. 23:359–369.
12. de Lorenzo, V., M. Herrero, and J. B. Neilands. 1988. IS*1*-mediated mobility of the aerobactin system of pColV-K30 in *Escherichia coli*. Mol. Gen. Genet. 213:4877–4900.
13. Evans, D. J., Jr., D. G. Evans, L. S. Young, and J. Pitt. 1980. Hemagglutination typing of *Escherichia coli*: definition of seven

hemagglutination types. J. Clin. Microbiol. 12:235–242.
14. Hacker, J., H. Hof, L. Emody, and W. Goebel. 1986. Influence of cloned *Escherichia coli* hemolysin genes, S fimbriae, and serum resistance on pathogenicity in different animal models. Microb. Pathog. 1:533–547.
15. Hacker, J., and C. Hughes. 1985. Genetics of *Escherichia coli* hemolysin. Curr. Top. Microbiol. Immunol. 118:139–162.
16. Hacker, J., S. Knapp, and W. Goebel. 1983. Spontaneous deletions and flanking regions of the chromosomally inherited hemolysin determinant of an *Escherichia coli* O6 strain. J. Bacteriol. 154:1145–1152.
17. Johnson, J. R., S. L. Mosely, P. L. Roberts, and W. E. Stamm. 1988. Aerobactin and other virulence factors among strains of *Escherichia coli* causing urosepsis: association with patient characteristics. Infect. Immun. 56:405–412.
18. Kado, C. I., and S. T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365–1373.
19. Kelly, M. T., D. J. Bremer, and J. J. Farmer III. 1985. *Enterobacteriaceae*, p. 263–277. In E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
20. Lawlor, K. M., and S. M. Payne. 1984. Aerobactin genes in *Shigella* spp. J. Bacteriol. 160:266–272.
21. Levine, M. M. 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. 155:377–389.
22. Lewis, M. I. 1968. Transferable drug resistance and other transferable agents in strains of *Escherichia coli* from two human populations. Lancet i:1389–1393.
23. Maloy, S. R., and W. D. Nunn. 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. 145:1110–1112.
24. Martinez, J. L., A. Delgado-Iribarren, and F. Baquero. 1990. Mechanism of iron acquisition and bacterial virulence. FEMS Microbiol. Rev. 75:45–56.
25. McDougall, S., and J. B. Neilands. 1984. Plasmid- and chromosome-coded aerobactin synthesis in enteric bacteria: insertion sequences flank operon in plasmid-mediated systems. J. Bacteriol. 159:300–305.
26. Muller, D., C. Hughes, and W. Goebel. 1983. Relationship between plasmid and chromosomal hemolysin determinants of *Escherichia coli*. J. Bacteriol. 153:846–851.
27. Nicoletti, M., F. Superti, C. Conti, A. Calconi, and C. Zagaglia. 1988. Virulence factors of lactose-negative *Escherichia coli* strains isolated from children with diarrhea in Somalia. J. Clin. Microbiol. 26:524–529.
28. Perez-Casal, J. F., and J. H. Crosa. 1984. Aerobactin iron uptake sequences in plasmid pColV-K30 are flanked by IS*1*-like elements and replication regions. J. Bacteriol. 160:256–265.
29. Saadi, S., W. K. Maas, D. F. Hill, and P. L. Bergquist. 1987. Nucleotide sequence analysis of RepFIC, a basic replicon present in IncFI plasmids P307 and F, and its relation to the RepA replicon of IncFII plasmids. J. Bacteriol. 169:1836–1846.
30. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. Sasakawa, C., and M. Yoshikawa. 1987. A series of Tn*5* variants with various drug-resistance markers and suicide vector for transposon mutagenesis. Gene 56:283–288.
32. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusion. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. Valvano, M. A., R. P. Silver, and J. H. Crosa. 1986. Occurrence of chromosome- or plasmid-mediated aerobactin iron transport systems and hemolysin production among clonal groups of human invasive strains of *Escherichia coli* K1. Infect. Immun. 52:192–199.
34. Waters, V. L., and J. H. Crosa. 1991. Colicin V virulence plasmids. Microbiol. Rev. 55:437–450.

35. **Williams, P. H.** 1979. Novel iron uptake system specified by ColV plasmids: an important component in the virulence of invasive strains of *Escherichia coli*. Infect. Immun. **26:**925–932.

36. **Womble, D. D., and R. R. Rownd.** 1988. Genetic and physical map of plasmid NR1: comparison with other IncFII antibiotic-resistance plasmids. Microbiol. Rev. **52:**433–451.

37. **Zagaglia, C., M. Casalino, B. Colonna, C. Conti, A. Calconi, and M. Nicoletti.** 1991. Virulence plasmids of enteroinvasive *Escherichia coli* and *Shigella flexneri* integrate into a specific site on the host chromosome: integration greatly reduces expression of plasmid-carried virulence genes. Infect. Immun. **59:**792–799.