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Stéphane Pierre Paul Bonacorsi, Olivier Clermont, Colin R. Tinsley, Isabelle Le Gall, Jean-Christophe Beaudoin, et al.. Identification of Regions of the *Escherichia coli* Chromosome Specific for Neonatal Meningitis-Associated Strains. *Infection and Immunity*, 2000, 68 (4), pp.2096-2101. 10.1128/iai.68.4.2096-2101.2000 . hal-03665348

**HAL Id: hal-03665348**

**<https://agroparistech.hal.science/hal-03665348>**

Submitted on 11 May 2022

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## Identification of Regions of the *Escherichia coli* Chromosome Specific for Neonatal Meningitis-Associated Strains

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Received 23 August 1999/Returned for modification 4 November 1999/Accepted 19 January 2000

Specific virulence factors associated with the pathogenesis of *Escherichia coli* strains causing neonatal meningitis (ECNM), such as the K1 capsular polysaccharide, the S fimbriae, and the Ibe10 protein, have been previously identified. However, some other yet unidentified factors are likely to be involved in the pathogenesis of ECNM. To identify specialized unique DNA regions associated with ECNM virulence, we used the representational difference analysis technique. The genomes of two strains belonging to nonpathogenic phylogenetic group A of the ECOR reference collection were subtracted from *E. coli* strain C5, isolated from a case of neonatal meningitis. Strain C5 belongs to the phylogenetic group B2 as do the majority of ECNM. We have isolated and mapped 64 DNA fragments which are specific for strain C5 and not found in nonpathogenic strains. Of these clones, 44 were clustered in six distinct regions on the chromosome. The *sfa* and *ibe10* genes were located in regions 2 and 6, respectively. A group of genes (*cnf1*, *hly*, *hly*, and *prf*) known to be present in a pathogenicity island of the uropathogenic strain *E. coli* J96 colocalized with region 6. The occurrence of these DNA regions was tested in a set of meningitis-associated strains and in a control group composed of non-meningitis-associated strains belonging to the same B2 group. Regions 1, 3, and 4 were present in 91, 82, and 81%, respectively, of the meningitis strains and in 40, 13, and 47% of the control strains. Together, these data suggest that regions 1, 3, and 4 code for factors associated with the ability of *E. coli* to invade the meninges of neonates.

*Escherichia coli* is responsible for a third of the cases of neonatal meningitis (NM), with an incidence of 0.1 per 1,000 live births (8). Case fatality rates are still very high and range from 25 to 40%. Furthermore, the occurrence of long-term neurologic sequelae in nonfatal cases is 33 to 50% of neonates with *E. coli* meningitis (8, 9, 32). Understanding the pathogenesis of this disease and characterizing these pathogenic strains are prerequisites to the development of new treatments.

Few specific pathogenic determinants have been described for *E. coli* strains causing NM (ECNM). Both expression of the K1 capsular polysaccharide (17) and production of aerobactin (21) are believed to be important for bloodstream dissemination. On the other hand, S fimbrial adhesin (*sfa*) (11, 17, 23) and Ibe10 protein (13), involved in the adhesion and invasion of brain microvascular endothelial cells, likely promote the crossing of the blood-brain barrier.

Phylogenetic approaches have helped to characterize the pathogenic strains. The *E. coli* species has been divided into four main phylogenetic groups designated A, B1, B2, and D (12, 28). Previous studies have shown that ECNM has a clonal structure (3, 27) and that strains mostly belong to the B2 group (5). Considering that only 38% of ECNM have both *sfa* and *ibe10*, it is likely that other determinants remain to be identified (5). In favor of this hypothesis is the fact that 10 pathogen-specific chromosomal segments have recently been detected by comparative macrorestriction mapping of the chromosomes of neonatal meningitis-associated *E. coli* RS218 and the laboratory strain *E. coli* K-12 (26). Two of these segments colocalized

with the *sfa* and *ibe10* genes. However, no functional or epidemiological studies have confirmed the role of these 10 DNA segments in the pathogenesis of ECNM.

In this work, we compared the chromosome of the C5 strain, which belongs to the B2 group, with those of two nonpathogenic *E. coli* strains by using representational difference analysis (18, 30). We obtained a library of sequences which are present only in the virulent strain. These sequences clustered onto the chromosome in six distinct regions. Comparing the presence of these regions among meningitis and non-meningitis-associated strains of the B2 group allowed us to identify three regions as being specific for the ECNM.

### MATERIALS AND METHODS

**Bacterial strains.** Strains used for subtractive hybridization were *E. coli* C5 (serotype O18:K1:H7), isolated from the cerebrospinal fluid (CSF) of a newborn (15) and obtained from R. Bertolussi (Dhalousie University, Halifax, Nova Scotia, Canada), and two nonpathogenic *E. coli* strains, ECOR4 and ECOR15, belonging to the ECOR collection (22). *E. coli* C5 harbors several virulence factors, such as the capsular antigen K1, an *sfa* operon, the *ibe10* gene, Pap pili, and the hemolysin gene (*hly*) (5, 13, 16). This strain belongs to the phylogenetic B2 group (5). On the other hand, the ECOR4 and ECOR15 strains, which belong to the phylogenetic A group, do not express any identified virulence factors, a property of almost all strains of this group (5, 7, 10, 24). Other *E. coli* strains were RS218 (serotype O18:K1:H7), an isolate from the CSF of a newborn, kindly provided by K. Kim (Childrens Hospital, Los Angeles, Calif.) (13), which harbored the same virulence factors as the C5 strain, and the *E. coli* laboratory strain K-12 MG1655, the genome of which has recently been sequenced (6).

In addition we used a set of 54 ECNM obtained from the CSF of newborns with meningitis (age range, 1 to 28 days) and belonging to the phylogenetic B2 group (5). This population was compared to the 15 non-meningitis-associated *E. coli* strains from phylogenetic group B2 of the 72 strains of the ECOR collection (22). This collection was obtained from R. Selander (Department of Biology, University of Rochester, Rochester, N.Y.). These reference strains, isolated from a variety of hosts and geographical locations, are representative of the range of genotypic variation in the species and are divided in four main phylogenetic

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groups (A, B1, B2, and D) (12, 28). Bacteria were grown at 37°C in Luria-Bertani broth or on Luria-Bertani agar. When necessary, ampicillin was used at a concentration of 100 µg per ml.

**Southern blotting.** Southern blotting was performed by capillary transfer onto positively charged nylon membranes. Hybridizations were performed at 65°C in 1% sodium dodecyl sulfate–1 M NaCl–50 mM Tris HCl (pH 7.5)–1% blocking reagent (Boehringer Mannheim, Mannheim, Germany). The membranes were washed first in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min at room temperature, then in 2× SSC–0.1 sodium dodecyl sulfate for 30 min at 65°C, and finally in 0.1× SSC for 5 min at room temperature. Detection by chemiluminescence was performed with the DIG luminescence detection kit for nucleic acid (Boehringer Mannheim) according to the manufacturer's instructions. The *sfa* and *ibe10* probes were produced by PCR, using primers and an amplification procedure previously described (4).

**Representational difference analysis.** The procedure was that of Tinsley and Nassif (30). Chromosomal DNA from the ECOR strains was sheared by repeated passage through a hypodermic needle to obtain fragments ranging between 3 and 10 kb long. This digested DNA was purified by phenol extraction. Chromosomal DNA from *E. coli* C5 was cleaved with the restriction endonuclease *Sau*3AI or *Tsp*509I. This DNA (20 µg) was ligated with 10 nmol of the annealed oligonucleotides RBam12 (5'-GATCCTCGGTGA-3') and RBam24 (5'-AGCACTCTCCAGCCTCTACCGAG-3') or REco12 (5'-AATTCTCGGTGA-3') and REco24 (5'-AGCACTCTCCAGCCTCTACCGAG-3') when the restriction was done by *Sau*AI or *Tsp*509I, respectively. DNA was separated from the excess primers by electrophoresis through a low-melting-point 2% agarose gel. The portion of the gel containing fragments of more than 200 bp was excised and digested with β-agarase. This DNA was purified by phenol extraction.

For the subtractive hybridization (first round), 0.2 µg of *E. coli* C5 DNA, ligated to the oligonucleotides, was mixed with 40 µg of sheared of ECOR4 or ECOR15 DNA in a total volume of 8 µl of 3× EE buffer [1× EE buffer is 10 mM *N*-(2-hydroxyethyl) piperazine-*N'*-(3-propanesulfonic acid)–1 mM EDTA (pH 8.0)]. The solution was overlaid with mineral oil, and the DNA was denatured by heating at 100°C for 2 min; 2 µl of 5 M NaCl was added, and the mixture was allowed to hybridize at 55°C for 48 h. The reaction mixture was diluted 10-fold with preheated 3× EE buffer–1 M NaCl and immediately placed on ice. A portion of the dilution (10 µl) was added to 400 µl of PCR mixture (10 mM Tris HCl [pH 9.0], 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, a 0.25 mM concentration of each deoxynucleoside triphosphate, 50 U of *Taq* polymerase per ml) and incubated for 3 min at 70°C to fill in the ends of the reannealed *E. coli* C5 fragments. After denaturation at 94°C for 5 min and addition of the oligonucleotide RBam24 or REco24 (0.1 nmol per 100 µl), the hybridizations were amplified by PCR (30 cycles of 1 min at 94°C, 1 min at 70°C, and 3 min at 72°C, followed by 1 min at 94°C and 10 min at 72°C in a GeneAmp 9600 thermal cycler [Perkin-Elmer]). The PCR products were gel purified to separate amplified *E. coli* C5 fragments from the primer and high-molecular-weight subtracting ECOR DNA. A second round of subtractive hybridization was performed, using 40 µg of sheared *E. coli* ECOR4 or ECOR15 and 25 ng of RBam24- or REco24-ligated DNA obtained from the first round. These second-round difference products were radiolabeled en masse and used as the probe in Southern hybridization experiments to ensure that the amplified fragments were unique to the pathogenic DNA and not present in the nonpathogenic strains. Thus, four subtractive libraries were obtained.

**Analysis of clones from the subtractive libraries.** DNA from the subtractive libraries was cloned into the *Bam*HI (*Sau*3AI libraries) or *Eco*RI (*Tsp*509I libraries) site of pUC19 (New England Biolabs, Beverly, Mass.) and then transformed into Epicurian coli XL2-blue ultracompetent cells (Stratagene, La Jolla, Calif.). The inserts were amplified by PCRs performed on transformant colonies, using the following primers: P1 (5'-CATGCTGCAGGTGCACTCT-3') and P2 (5'-CGTTGTAAACGACGGCCAG-3'). Clones were designated by the following (in order): the restriction enzyme used ("Tsp" or "Sau"), the strain used for the subtraction (E4 or E15), and an alphanumeric designation.

**(i) DNA sequencing.** After purification of PCR products by solid-phase reversible immobilization, purified PCR fragments were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS (Perkin-Elmer) on an automated ABI PRISM 377 XL DNA Sequencer (Perkin-Elmer) by following the manufacturer's instructions. When problems in obtaining a good-quality sequence were encountered with a particular primer, a sequencing reaction was performed with the dGTP BigDye Terminator Ready Reaction kit (Perkin-Elmer). Sequences were screened for homologies with previously published sequences using the computer programs BLASTN and BLASTX at the National Center for Biotechnology Information (2).

**(ii) Southern blot hybridization.** To check for their specificity, the PCR products obtained by using primers P1 and P2 from the colonies of transformants were labeled by incorporation of digoxigenin-11-dUTP (Boehringer Mannheim) and used as probes for Southern blot analysis of *Dra*I-digested chromosomal DNA from strains *E. coli* C5, ECOR4, and ECOR15 and *E. coli* K-12 MG1655.

**(iii) Pulsed-field gel electrophoresis and mapping of the clones on the chromosomes of strains RS218 and C5.** The position of the DNA sequences corresponding to the cloned difference products was determined relative to the map of *E. coli* RS218 (26) by probing Southern blots of pulsed-field agarose gels. DNA of strain RS218 was digested with *Bln*I, *Nor*I, and *Xba*I and subjected to

pulsed-field gel electrophoresis, as was DNA of strain C5 that had been digested by *Bln*I and *Nor*I. Gels were 1% agarose in 0.5× Tris-borate-EDTA buffer, and were subjected to electrophoresis at 6 V/cm for 27 h, with pulse times varying linearly between 2 and 49 s. The positions on the RS218 chromosome of sequences reactive with each of the clones were determined by comparison of the recognized *Bln*I and *Nor*I restriction fragments with the published macrorestriction map (26).

**Nucleotide sequence accession numbers.** The sequences of the subtractive DNA fragments have been assigned GenBank accession numbers AF222070 to AF222222 for sequences showing no homology and numbers AF222223 to AF222307 for sequences with significant homology.

## RESULTS

### Production of libraries of DNA fragments of pathogenic strain C5 not found in the genome of nonpathogenic *E. coli*.

Using the technique of representational difference analysis, we subtracted the chromosomes of the two nonpathogenic (ECOR4 and ECOR15) strains from the chromosome of the C5 pathogenic strain. Four libraries were produced and designated SauE4, SauE15, TspE4, and TspE15, according to the enzyme used to digest the chromosome of the C5 strain and the strain used for subtraction. In each case, the amplified difference product from the second round of subtraction was labeled and used as a probe against *Dra*I-digested DNA from C5, RS218, ECOR4, and ECOR15. A strong reactivity with the chromosome of pathogenic strains was observed. On the other hand, little or no signal was present in the lanes corresponding to the subtractive nonpathogenic strains (data not shown). Altogether, 494 clones were subsequently isolated and sequenced. Of these, 140 had significant homology with sequences of *E. coli* K-12 and were subsequently discarded. Among the 354 remaining fragments, 259 sequences were unique. Table 1 shows all the clones which had a significant homology with previously described genes (except bacteriophages). Some of these clones corresponded to genes already known to be present in the C5 strain, such as *pap*, *hly*, and *kps*. None of these clones were found to be homologous with *sfa* or *ibe10*. On the other hand, sequences corresponding to virulence factors found in strains not responsible for NM were present, as follows: (i) *prs*, *cnf*, and *hra*, all part of a pathogenicity island (PAI) in the uropathogenic strain *E. coli* J96 (29); (ii) *chuA*, a gene involved in an iron transport system and found in enterohemorrhagic *E. coli* O157:H7 (31); and (iii) *senB*, a gene encoding an enterotoxin on the virulence plasmid of *Shigella* and enteroinvasive *E. coli* (20). Finally, 153 fragments showed no significant homology with any published sequence.

**Mapping of the ECNM-specific sequences on the *E. coli* chromosome.** The availability of a physical map of the chromosome of *E. coli* RS218 (26) made possible the investigation of the distribution of some of the above-mentioned sequences. Of 64 clones that were chosen, 7 showed homology to known virulence factors (*kps*, *hly*, *prs*, *hra*, *cnf*1, *chuA*, and *senB*) and 57 showed no known homology. These latter clones were chosen at random from the TspE4 and SauE15 libraries. These two libraries were chosen because they contained most of the expected pathogen-associated genes (e.g., *pap*, *hly*, and *kps*) and thus were considered the most comprehensive. All the clones showed homology by Southern hybridization to the chromosome of strain RS218. PCR products from these clones were labeled and used to probe Southern blots of DNA from RS218 digested with the infrequently cutting enzymes *Bln*I, *Nor*I, and *Xba*I. The location of both *sfa* and *ibe10* was also determined. To confirm that each one of these clones was specific for ECNM, they were all used to probe *Dra*I-digested DNA from strains ECOR4, ECOR15, and MG1655 and shown to be nonreactive against these strains.

TABLE 1. Summary of BLAST search of clones that were specific for *E. coli* strain C5 and had significant homologies<sup>a</sup>

Clone <sup>b</sup>	Insert size (bp)	Sequence(s) with similarity <sup>c</sup>	Score	Probability	GenBank accession no.
SauE15.A1	163	IS629 (N), plasmid pO157, <i>E. coli</i> O157:H7	315	$e^{-84}$	AB011549
SauE15.A7	190	<i>iroC</i> (N), ATP cassette transporter (iron-regulated locus), <i>Salmonella enterica</i> serovar Typhi	157	$e^{-37}$	U6129
SauE15.B2	294	<i>repB</i> (N); replication protein, plasmid pCD1; <i>Yersinia pestis</i>	123	$e^{-26}$	AF053946
SauE15.B6	157	<i>kps</i> (N), promoter region of polysialic acid gene cluster region 3, <i>E. coli</i>	242	$e^{-62}$	U05251
SauE15.B9	107	<i>traD</i> (N), F sex factor plasmid, <i>E. coli</i>	198	$e^{-50}$	M29254
SauE15.B10	240	ORF 34 and 35 (P), 102-kb unstable region, <i>Y. pestis</i>	69	$e^{-12}$	CAA21357
SauE15.B12	479	unknown protein (P), <i>E. coli</i> ec11	102	$e^{-21}$	AF044503
SauE15.C1	100	<i>r6</i> (N), transposase, pathogenicity island of <i>E. coli</i> CFT073	198	$e^{-49}$	AF081285
SauE15.C6	119	IS100 (N), <i>Y. pestis</i>	228	$e^{-58}$	L19030
SauE15.C7	155	TonB-dependent receptor HI1217 precursor (P), <i>Haemophilus influenzae</i>	52	$e^{-7}$	P45114
SauE15.C9	273	<i>rhuM</i> (N), pathogenicity island of <i>Salmonella enterica</i> serovar Typhimurium (SPI3)	311	$e^{-83}$	AF106566
SauE15.C11	77	<i>orfE</i> (N); promoter-distal region of the <i>tra</i> operon, plasmid R100; <i>S. flexneri</i>	129	$e^{-29}$	X55815
SauE15.D4	153	IS100 (N), <i>Y. pestis</i>	287	$e^{-76}$	L19030
SauE15.D8	347	<i>r3</i> (N), beta-cystathionase, pathogenicity island of <i>E. coli</i> CFT073	615	$e^{-174}$	AF081286
SauE15.E4	281	<i>senB</i> (N), enterotoxin, <i>E. coli</i>	541	$e^{-152}$	Z54195
SauE15.E11	314	<i>traJ</i> , <i>Y</i> (N), plasmid R1-19, <i>E. coli</i>	523	$e^{-147}$	M19710
SauE15.F3	422	<i>chuA</i> (P), heme utilization gene, <i>E. coli</i> O157:H7	98	$e^{-20}$	U67920
SauE15.F9	137	Thioesterase (P), <i>Bacillus</i> sp.	48	$e^{-6}$	AB016427
SauE15.F10	210	<i>r3</i> (N), beta-cystathionase, pathogenicity island of <i>E. coli</i> CFT073	408	$e^{-112}$	AF081286
SauE15.G3	206	<i>traG</i> (N), plasmid R100, <i>S. flexneri</i>	165	$e^{-39}$	U01159
SauE15.G6	328	IS100 (N), <i>Y. pestis</i>	480	$e^{-134}$	L19030
SauE15.H5	200	HMWP1 protein (P), <i>Yersinia enterocolitica</i>	80	$e^{-15}$	CAA73127
SauE15.H7	150	Oxydoreductase (P), <i>Thermotoga maritima</i>	160	$e^{-11}$	AE001762
SauE15.H10	141	<i>traT</i> (N), plasmid R100, <i>E. coli</i>	280	$e^{-74}$	J01769
SauE15.H11	160	Hemoglobin protease (P), <i>E. coli</i> EB1	50	$e^{-6}$	CAA11507
SauE15.I3	176	<i>asst</i> (N), arylsulfate sulfotransferase, <i>Klebsiella</i> sp.	341	$e^{-92}$	U32616
SauE15.I11	162	<i>chuA</i> (N), heme utilization gene, <i>E. coli</i> O157:H7	305	$e^{-82}$	U67920
SauE15.J7	118	<i>iroB</i> (N), glucosyl transferase homolog, <i>Salmonella</i> serovar Typhi	74	$e^{-12}$	U62129
SauE15.J9	96	IS100 (N), <i>Y. pestis</i>	174	$e^{-42}$	L19030
SauE15.M4	193	<i>r3</i> and <i>malX</i> (N), pathogenicity island of <i>E. coli</i> CFT073	383	$e^{-104}$	AF081286
SauE15.M8	149	Delta-(L- $\alpha$ -aminoadipyl)-L-cysteine-D-valine synthetase (P), <i>Penicillium</i> sp.	65	$e^{-11}$	P26046
SauE15.M12	119	<i>senB</i> (N), enterotoxin of enteroinvasive <i>E. coli</i>	228	$e^{-58}$	Z54195
SauE15.N7	188	Plasmid pColBM-C1139 (N), <i>E. coli</i>	208	$e^{-52}$	M35683
SauE4.A2	321	ORF 36 (N), 102-kb unstable region, <i>Y. pestis</i>	135	$e^{-30}$	AL031866
SauE4.A5	249	<i>r3</i> (N), beta-cystathionase, pathogenicity island of <i>E. coli</i> CTF073	355	$e^{-96}$	AF081286
SauE4.B4	360	IS200 (N), <i>E. coli</i>	523	$e^{-147}$	L25845
SauE4.C7	275	Hippurate hydrolase (P), <i>Campylobacter jejuni</i>	54	$e^{-7}$	P45493
SauE4.C11	255	Pristinamycin I synthase (P), <i>Streptomyces</i> spp.	51	$e^{-6}$	CAA67248
SauE4.D3	239	<i>hlyB</i> (N), hemolysin, <i>E. coli</i>	474	$e^{-132}$	M81823
SauE4.E3	263	<i>shuX</i> genes (N), heme utilization genes, <i>Shigella dysenteriae</i>	387	$e^{-106}$	U64516
SauE4.E11	242	IS66 (N), <i>E. coli</i>	329	$e^{-88}$	AF119170
SauE4.F8	188	<i>sorC</i> genes (N), <i>sor</i> operon for L-sorbose utilization, <i>Klebsiella pneumoniae</i>	139	$e^{-31}$	X66059
SauE4.F9	439	<i>YfkN</i> (P), <i>Bacillus subtilis</i>	57	$e^{-8}$	BAA23404
SauE4.F12	324	<i>kpsM</i> (N), polysialic acid gene cluster region 3, <i>E. coli</i> K1	642	0	M57382
SauE4.H2	85	<i>sorM</i> (N), <i>sor</i> operon for L-sorbose utilization, <i>K. pneumoniae</i>	105	$e^{-22}$	X66059
SauE4.I2	431	<i>yihA</i> (N), plasmid R100, <i>S. flexneri</i>	829	0	AP000342
TspE4.A5	271	<i>pap</i> and <i>prkK</i> (N), P-pili protein, <i>E. coli</i>	498	$e^{-139}$	X61239
TspE4.A8	216	ORF 17 kD of <i>prx</i> pili operon (N), cytoplasmic protein, <i>E. coli</i>	387	$e^{-106}$	X61238
TspE4.A9	179	<i>kpsT</i> (N), polysialic acid gene cluster region 3, <i>E. coli</i> K1	347	$e^{-94}$	M57381
TspE4.A10	212	HecB (P), putative hemolysin activator transporter, <i>Erwinia chrysanthemi</i>	73	$e^{-13}$	AAC31980
TspE4.B1	229	<i>r1</i> (N), pathogenicity island of <i>E. coli</i> CFT073	430	$e^{-119}$	AF081286
TspE4.B5	215	Sensory transduction histidine kinase (P), <i>Synechocystis</i> sp.	52	$e^{-7}$	BAA18223
TspE4.B9	319	<i>senB</i> (N), enterotoxin of enteroinvasive <i>E. coli</i>	617	$e^{-175}$	Z54195
TspE4.B12	430	IS100 (N), <i>Y. pestis</i>	698	0	L19030
TspE4.C10	267	Intergenic K42 capsule cluster (N), <i>E. coli</i>	466	$e^{-129}$	AF118251
TspE4.D2	232	<i>waaL</i> (N), lipid A core-to-surface polymer ligase, <i>E. coli</i>	404	$e^{-111}$	AF019746
TspE4.D4	245	ORF 169 (N), plasmid F, <i>E. coli</i>	456	$e^{-126}$	X17539
TspE4.D10	222	<i>cnf1</i> (N), cytotoxic necrotizing factor, <i>E. coli</i>	440	$e^{-122}$	X70670
TspE4.D11	217	<i>hlyB</i> (N), hemolysin, <i>E. coli</i>	422	$e^{-117}$	M81823
TspE4.E3	298	<i>hlyD</i> (N), hemolysin, <i>E. coli</i>	553	$e^{-156}$	M10133
TspE4.E4	267	ORF 95 (N), plasmid F, <i>E. coli</i>	482	$e^{-134}$	X17539
TspE4.E6	190	L-sorbose P reductase (P), <i>K. pneumoniae</i>	112	$e^{-25}$	P37084
TspE4.E8	285	<i>hlyB</i> (N), hemolysin, <i>E. coli</i>	541	$e^{-152}$	M81823
TspE4.G7	238	<i>tra</i> (N), plasmid F, <i>E. coli</i>	448	$e^{-124}$	X61575
TspE4.G8	323	Transmembrane protein (P), <i>E. coli</i>	82	$e^{-15}$	AAA92620

Continued on following page



TABLE 1—Continued

Clone <sup>b</sup>	Insert size (bp)	Sequence(s) with similarity <sup>c</sup>	Score	Probability	GenBank accession no.
TspE4.H1	283	Arginine deiminase (P), <i>Pseudomonas aeruginosa</i>	63	$e^{-10}$	P13981
TspE4.H9	179	<i>traT</i> (N), plasmid R100, <i>E. coli</i>	353	$e^{-96}$	J01769
TspE4.H10	223	<i>prf</i> and <i>papI</i> (N), adhesin regulatory gene, <i>E. coli</i>	418	$e^{-115}$	X76613
TspE4.H11	279	ORF 9 (N), plasmid F, <i>E. coli</i>	456	$e^{-127}$	X17539
TspE4.I10	269	<i>neuC</i> (N), capsule gene cluster, <i>E. coli</i>	492	$e^{-137}$	M84026
TspE4.J1	327	<i>yhtA</i> (N), plasmid R100, <i>E. coli</i>	521	$e^{-146}$	AP000342
TspE4.J6	221	<i>chuA</i> (N), heme utilization gene, <i>E. coli</i> O157:H7	375	$e^{-102}$	U67920
TspE4.K3	180	<i>iss</i> (N), serum survival, <i>E. coli</i>	270	$e^{-70}$	AF042279
TspE4.K8	184	IS100 (N), <i>E. coli</i>	190	$e^{-47}$	L19030
		<i>prf</i> and <i>papB</i> (N), <i>E. coli</i>	143	$e^{-32}$	X76613
TspE15.A1	332	Na <sup>+</sup> H <sup>+</sup> antiporter (P), <i>H. influenzae</i>	96	$e^{-20}$	Q57007
TspE15.C1	299	<i>hrrA</i> (N), heat-resistant agglutinin, <i>E. coli</i> 99	537	$e^{-151}$	U07174
TspE15.C3	386	<i>hcp</i> (N), <i>E. coli</i>	81	$e^{-14}$	AF044503
TspE15.D7	239	Protein STBA (P), plasmid NR1, <i>E. coli</i>	87	$e^{-17}$	P11904
TspE15.D9	230	<i>chuA</i> (P), heme utilization gene, <i>E. coli</i> O157:H7	89	$e^{-18}$	AAC44857
TspE15.E7	360	<i>kpsS</i> (N), capsule gene cluster region 1, <i>E. coli</i> K5	531	$e^{-149}$	X74567
TspE15.G12	287	Putative amino transferase (P), <i>B. subtilis</i>	72	$e^{-12}$	Q08432
TspE15.H2	258	Pyruvate formate lyase-activating enzyme (P), <i>Streptococcus mutans</i>	51	$e^{-6}$	AAB89799
TspE15.H5	310	<i>cnf1</i> (N), cytotoxic necrotizing factor, <i>E. coli</i>	601	$e^{-170}$	U42629
TspE15.H9	273	Major fimbrial subunit of F17-like fimbriae (P), <i>E. coli</i>	48	$e^{-5}$	I41206
TspE15.I2	112	<i>prf</i> and <i>papE</i> (N), P-pili protein, <i>E. coli</i>	222	$e^{-57}$	X62158

<sup>a</sup> Only homologies with at least probability of  $e^{-5}$  were retained. Homologies to bacteriophages ( $n = 21$ ) are not shown.

<sup>b</sup> Clones are designated by the name of the enzyme (Sau or Tsp), followed by the name of the strain used for the subtraction (E4 or E15) and a code composed of a letter and a number.

<sup>c</sup> The name of the gene sequence is given (with similarity type in parentheses), followed by the product or function that is encoded by the gene and/or the gene's location, as well as the organism name. N, similarity at the nucleotide level; P, similarity at the protein level; ORF, open reading frame.

Mapping of these clones revealed a nonrandom distribution of the ECNM-specific sequences (Fig. 1). Forty-four of the clones clustered in six distinct groups on the chromosome. One clone encoding part of the *chuA* gene found in enterohemorrhagic *E. coli* was not associated with any of these clusters and remained isolated. Region 1 is contained within the *BlnI* fragment m (85 kb). The clones of region 2 mapped on *NotI* fragments p and n (~240 kb). Region 3 is contained within *NotI* fragment e (~310 kb), and region 4 is contained within *BlnI* fragment h (~210 kb). Region 5 maps to *BlnI* fragment j (~135 kb). Region 6, which overlaps the *NotI* fragment b and *BlnI* fragment b (~550 kb), has been divided into two subregions by the enzyme *XbaI* (data not shown), regions 6a and 6b. The latter subregion contained clones with homologies to *cnf1*, *hly*, *prf*, and *hrrA*. The *sfa* and *ibe10* probes hybridized in regions 2 and 6a, respectively. The capsule-encoding genes were not linked to any of these regions. Six clones presenting no homology with known sequences and the *senB* gene all mapped on a large plasmid present in the RS218 strain (1).

**Distribution of *E. coli* C5-specific genomic regions among two collections of *E. coli* strains.** To assess the relevance of these regions in terms of pathogenesis, we determined the frequency of occurrence of clones located on regions 1, 3, 4, 5, and 6b as well as of the clone containing part of the *chuA* gene in our collection of 54 ECNM belonging to the phylogenetic group B2. We excluded from this study regions 2 and 6a because they contained the *sfa* and *ibe10* genes, the distribution of which among group B2 *E. coli* strains is well established (5). For each region, two to four clones were used independently to probe Southern blots of genomic DNA of ECNM isolates. The control group corresponded to the 15 B2 strains of the ECOR collection which contains no meningitis strains. Results are shown in Table 2. All the above-mentioned regions are widely present among ECNM except for region 6b which is underrepresented in meningitis-associated strains. In addition, regions 1, 3, and 4 appeared with a significantly higher frequency ( $P <$

0.05) among ECNM than the other B2 strains, thus suggesting that these regions may encode factors specific to ECNM.

## DISCUSSION

The pathogenesis of ECNM is incompletely understood. Until now, only four specific virulence factors have been described: the K1 capsular polysaccharide, the aerobactin, the S fimbrial adhesin, and the Ibe10 protein (11, 13, 17, 21, 23). However, *E. coli* isolates that were obtained from the CSF of neonates with meningitis and which do not express these virulence factors have been described, thus suggesting that some other yet unidentified bacterial attributes may be involved (5). Very recently, a new gene (*ibeB*) required for penetration of brain microvascular endothelial cells was reported (14). In this work, we performed subtractive hybridization to identify regions of the chromosome likely to encode attributes responsible for specific aspects of the pathogenesis of ECNM. We performed two rounds of subtractive hybridization according to a previously described method (30) and obtained libraries of clones specific for ECNM containing inserts with sizes ranging from 100 to 500 bp. The specificity of the subtractive libraries was assessed (i) by Southern blotting with the nonpathogenic strains and (ii) by sequence analysis which showed that 72% of the clones had no homology with the published sequence of *E. coli* K-12. Some clones corresponded to expected virulence-associated genes (*kps*, *pap*, and *hly*). On the other hand, we did not isolate clones corresponding to *sfa* and *ibe10* genes. However, clones derived from regions containing both of these genes were obtained. Together these data confirm the comprehensiveness of these libraries.

Very recently, Rode et al. carried out comparative macrorestriction mapping using rare-restriction-site alleles between the chromosomes of the laboratory strain K-12 MG1655 and *E. coli* RS218 (26). They identified 10 large chromosomal additions in the RS218 strain but not the MG1655 strain (Fig. 1).

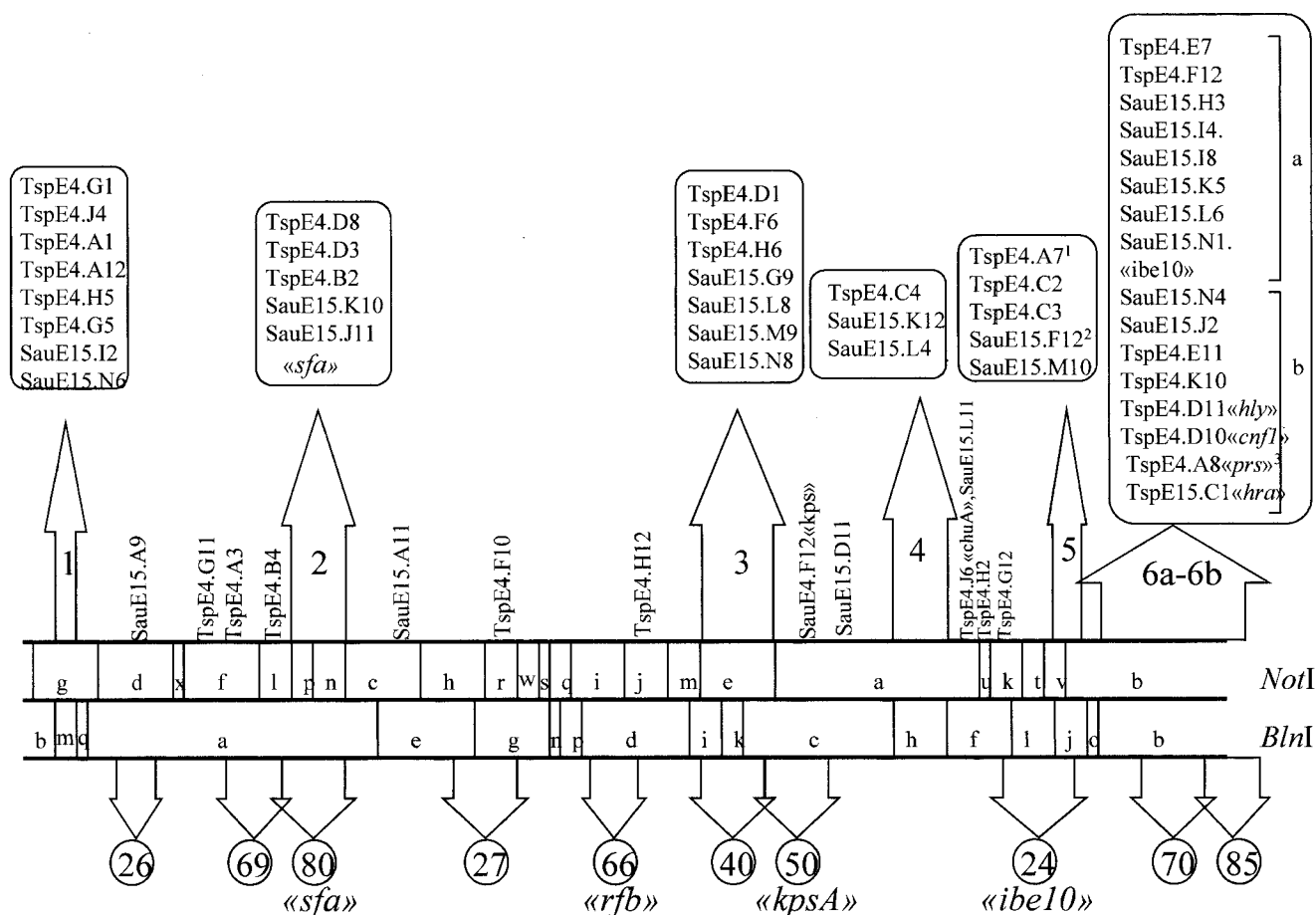


FIG. 1. Distribution of specific sequences on the chromosome of *E. coli* strain RS218. *E. coli* strain C5-specific clones were used as probes on Southern blots of DNA from strain RS218 digested with the infrequently cutting enzymes *BlnI* and *NotI* and were positioned on the linearly represented restriction map (26). The upper arrows indicate the six regions found in this study and delineated by *NotI* and *BlnI* fragments with a high density of C5-specific clones. Clones with known homologies are indicated, as are the positions of the probes *sfa* and *ibe10*. Region 6 was divided into two subregions according to the mapping of the clones on different *XbaI* fragments. The lower arrows indicate the 10 segments of the RS218 chromosome in which Rode et al. found additions relative to the *E. coli* K-12 chromosome. The length (in kilobases) of each addition is indicated in the respective circle. The positions of various virulence factors are also indicated (26). Superscripts to clone names designate the following: 1, TspE4.A7 was positioned by overlapping SauE15.F12; 2, SauE15.F12 also shows reactivity on the plasmid; and 3, TspE4.A8 also shows a weak reactivity on the *NotI* fragment p.

Most of these segments are in perfect accordance with our results. However, four segments undetected by our approach were reported by Rode et al. On the other hand, our regions 1 and 4 were not detected in this previous work. The most likely explanation for not having isolated clones in some of the regions reported by Rode et al. is, first, that these may correspond to sequences deleted during the course of laboratory isolation of *E. coli* K-12 and, second, that these deletions are unlikely to have occurred in the group A ECOR strains. In

favor of this hypothesis is the fact that segments 66 and 69 (Fig. 1), which we did not obtain, correspond to *rfb* genes and the  $\lambda$  bacteriophage, respectively, which are deleted in strain MG1655 (19, 25).

An epidemiological approach was undertaken to shed light on the role of these regions in the infective process of ECNM. Considering that most ECNM belong to the phylogenetic group B2, we determined the prevalence of each region as well as of *chuA* among ECNM of group B2 and among non-men-

TABLE 2. Strains isolated from cases of NM and from the ECOR collection that hybridized with subtractive clones used as probes

Strain source <sup>a</sup>	No. of strains	% of isolates positive by Southern blot hybridization <sup>d</sup>											TspE4J6
		Region 1		Region 3		Region 4			Region 5		Region 6b		
		TspE4.J4	TspE4.H5	SauE15M9	TspE4H6	SauE15.L4	SauE15K12	TspE4.C4	SauE15.M10	TspE4C2	SauE15.N4	PAI V <sup>a</sup>	
NM	54	91 <sup>b</sup>	91 <sup>b</sup>	80 <sup>b</sup>	84 <sup>b</sup>	81 <sup>b</sup>	81 <sup>b</sup>	81 <sup>b</sup>	100	98 <sup>c</sup>	17 <sup>b</sup>	17 <sup>b</sup>	100
ECOR collection	15	40	40	13	13	47	47	47	100	87	47	47	100

<sup>a</sup> The prevalence of PAI V was assessed using the clones TspE4.D11, TspE4.D10, and TspE15.C1, homologous to the *hly*, *cnf1*, and *hra* genes, respectively.

<sup>b</sup>  $P < 0.05$ , compared with strains of the ECOR collection (existence of a difference in the distribution of the studied clones was tested by the  $\chi^2$  test).

<sup>c</sup> Not significant, compared with strains of the ECOR collection.

<sup>d</sup> TspE4.J6 is homologous to *chuA*. The other clones are representative of their respective regions.

<sup>e</sup> All strains were in the phylogenetic B2 group.

ingitis-associated group B2 strains from the ECOR collection. Although small, this control group was chosen because it is composed of reference strains that belong to the ECOR collection, which is considered representative of the range of genotypic variations of the species. We used two to four clones from each region and the clone TspE4.J6, homologous to *chuA*, as probes against Southern blots of genomic DNA prepared from isolates belonging to both groups. The occurrence of these clones among the meningitis isolates indicates that all these regions, except the region 6b, are widely represented among ECNM, thus suggesting the involvement of genes encoded by these regions in the pathogenesis of these strains. On the other hand and surprisingly, region 6b which resembles PAI V, has a low prevalence in ECNM (17%) but was widely represented in the B2 group strains from the ECOR collection (47%). Region 5 had a high but a similar prevalence in both collections and thus may correspond to segments characteristic of phylogenetic group B2. The same distribution was observed for *chuA*, but interestingly, this gene was present in all B2 group strains tested without exception. Considering that regions 1, 3, and 4, which are specific for ECNM, do not contain known virulence factors, one may speculate that these regions correspond to islands of DNA associated with the invasion of meninges by *E. coli*. Further work is in progress to characterize the contents of these regions.

#### ACKNOWLEDGMENTS

This work was supported in part by the Programme de Recherche Fondamentale en Microbiologie, Maladies Infectieuses et Parasitaires (Appel d'offre 1998) (Recherche de déterminants génétiques de pathogénicité chez *E. coli* K1 responsable de méningite néonatale) and by the Programme Hospitalier de Recherche Clinique (grant AOM 96069). The laboratory of X.N. is supported by the INSERM, the Université Paris 5 and the Fondation pour la Recherche Médicale. S.P.P.B. was the recipient of a fellowship obtained from Hoescht Marion Roussel and from the Société de Pathologies Infectieuses de Langue Française.

We thank P. Berche for his help, A. Perrin for her constant support, and H. Dabernat, M. Thibaut, H. Chardon, J. C. Philippe, J. Freney, T. Lambert, J. Raymond, M. Weber, M. Benbachir, K. Kim, J. Hacker, R. Bertolussi, H. Vu Thien, and R. Selander for providing some of the strains used in this study.

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