Phylogenetic Analysis of *Escherichia coli* Strains Causing Neonatal Meningitis Suggests Horizontal Gene Transfer from a Predominant Pool of Highly Virulent B2 Group Strains

Edouard Bingen, Bertrand Picard, Naïma Brahimi, Stéphanie Mathy, Patricia Desjardins, Jacques Elion, and Erick Denamur

Laboratoire de Recherche en Bactériologie Pédiatrique (ER 321) and INSERM U458, Hôpital Robert Debré and Université Denis Diderot, Paris, and Laboratoire de Microbiologie, Hôpital Morvan, Brest, France

Phylogenetic relationships of 69 neonatal meningitis *Escherichia coli* strains isolated worldwide were studied. Restriction fragment length polymorphism of *rrn* operons (*rrn* RFLP) in these isolates was compared with that of the 72 strains of the ECOR reference collection. Distributions of K1 antigen, of polymerase chain reaction–detected *ibe10* gene, *pap, afa, sfa/foc, hly,* and *aer* operons, and of a 14.9-kb *rrn*-containing *Hin*dIII fragment previously associated with neonatal meningitis were compared. Oligoclonality was observed for the meningitis strains. Factorial analysis of correspondence on the *rrn* RFLP data showed a frequency gradient of meningitis strains from the phylogenetic B2 group (68%) to the A group (6%), via the D and B1 groups (26%). The distribution of the virulence determinants argues for their horizontal transfer during the evolution of *E. coli.* Analysis of the status of some neonates further suggests that neonatal meningitis results from a balance between bacterial genes of virulence and host factors.

The species *Escherichia coli* has the two characteristics of encompassing both commensal and pathogenic strains that cause a wide range of specific pathologies and of having been [1] and still being the most thoroughly studied bacterial species. The complete nucleotide sequence of the *E. coli* K12 chromosome is now available [2]. These characteristics can be used as an advantage to try to decipher the pathophysiology of human *E. coli* infections by studying the genetic relationships between strains causing a specific type of infection within the genetic diversity of the species as a whole.

Neonatal hematogenous *E. coli* meningitis still remains a significant health problem [3]. Previous studies have shown that this very specific pathology is associated with a limited number of clones, in sharp contrast with the wide genetic diversity of the human commensal *E. coli* isolates. These studies, essentially based on the analysis by multilocus enzyme electrophoresis [4] and on the analysis of restriction fragment length polymorphism in and around *rrn* operons (*rrn* RFLP) (ribotyping) [5], can be considered valid, as horizontal gene transfer observed in *E. coli* does not disrupt the clonal structure of the population [6–9]. Indeed, there is a good correlation between the various phylogenies established with different genetic markers [9]. Although *E. coli* clones causing neonatal meningitis have been directly linked to other collections of extraintestinal *E. coli* causing infection [10, 11], the phylogenetic position of these clones within the ECOR collection [12] is not readily available.

Pathogenic determinants for *E. coli* meningitis identified thus far include essentially surface structures and adhesins. For instance, epidemiologic [13–15] and experimental [16, 17] studies have clearly linked neonatal meningitis with *E. coli* strains expressing the K1 antigen.

S fimbria adhesins *(sfa/foc)* [18] and the product of the *ibe10* gene [19] have been shown to promote, respectively, receptor-mediated adhesion and invasion of brain microvascular endothelial cells. These pathogenic determinants are chromosomally encoded and often involve several genes linked on a specific stretch of the bacterial chromosome [20, 21], the so-called ''pathogenicity islands.'' For example, *E. coli* strains causing pyelonephritis possess two pathogenicity islands that contain two distinct genes related to the associated pili *(pap),* a hemolysin structural gene, and *sfa* transcriptional activators [22]. At this stage, there is no information concerning the mechanism by which bacteria may acquire the chromosomal determinants that make them virulent.

Finally, it is clear that none of the determinants described thus far is sufficient by itself to account for the virulence properties of strains causing neonatal meningitis. It is thus very likely that some other determinants in the genetic background of the bacterium are involved that still remain to be identified. In this context, the description of a highly informative anonymous marker (a 14.9-kb *Hin*dIII *rrn*-containing fragment) linked to meningitis *E. coli* isolates is interesting. This fragment was present in 81.3% of 43 isolates isolated from the cerebrospinal fluid of neonates versus 12.8% of isolates from vaginal

Received 4 April 1997; revised 9 October 1997.

Grant support: Institut National de la Santé et de la Recherche Médicale (grant 920602 to E.B.), Programme Hospitalier de Recherche Clinique (grant AOM96069 to E.B.).

Reprints or correspondence: Dr. E. Bingen, Service de Microbiologie, Hôpital Robert Debré, 48 Bd. Sérurier, 75019 Paris, France (edouard.bingen@rdb.ap-hop-paris.fr).

The Journal of Infectious Diseases 1998; 177:642–50

0022–1899/98/7703–0017$02.00

samples of asymptomatic pregnant women whose neonates remained without infection [5].

In this study, we have used *rrn* RFLP analysis to establish the phylogenetic relationships of 69 *E. coli* strains causing neonatal meningitis isolated on three continents over a 20-year period among a set of 72 reference strains of *E. coli* referred to as the ECOR collection [12]. This collection of strains is believed to be representative of the range of genotypic variation within the *E. coli* species as a whole. The distribution of virulence factors (K1 antigen, *pap, afa,* and *sfa/foc* adhesin-encoding operons, *hly* and *aer* operons, and the *ibe10* gene) and of the 14.9-kb *Hin*dIII fragment has also been studied.

Material and Methods

Bacterial strains and patient characteristics. Sixty-nine strains were recovered from the cerebrospinal fluid of 69 neonates with meningitis in different countries on three continents (table 1). Among them, 44 strains were isolated from 1990 through 1997 in different regions of France: Paris (24, from 4 different hospitals), Pontoise (4), Compiègne (1), Quimper (2), Nancy (4), Lyon (1), Toulouse (1), Aix-en-Provence (3), Saint-Denis (1), Dieppe (1), Beauvais (1), and Senlis (1). Five strains were provided by J. Hacker (Institut für Genetik und Mikrobiologie, Würzburg, Germany): strains IHE 3034 (S17) and IHE 3036 (S14) were isolated in Finland in 1977, strains A21 (S13) and RS176 (S16) in the United States in 1974, and strain A1521 (S15) in Germany in 1974 [24]. Sixteen cerebrospinal fluid strains isolated in North America were provided by R. Bortolussi (Childrens' Hospital, Halifax, Canada) [25]. The last 4 strains were a generous gift of M. Benbachir (Microbiology Department, Casablanca, Morocco) and correspond to isolates from Casablanca. Forty-three of these strains have been previously studied [5]. The set of 72 *E. coli* reference strains (ECOR) [12] was provided by R. Selander (Department of Biology, University of Rochester, Rochester, NY). The ECOR strains have been characterized for the electrophoretically detected allelic variants of 38 enzymes [26, 27] and for *rrn* RFLP and random-amplified polymorphic DNA (RAPD) analysis [9]. Analysis of the genetic distance matrix by the neighbor-joining method has divided the strains into 4 main phylogenetic groups (A, B1, B2, D), plus 1 accessory group containing strains ECOR 31, 37, 42, and 43 [23]. Clinical data were used to classify 23 neonates seen in France as normal-risk or high-risk neonates according to the criteria of Tullus et al. [28].

Capsular typing. K1 antigen determinations were made with an antiserum to *Neisseria meningitidis* group B [29].

rrn RFLP analysis (ribotyping). Total *E. coli* DNA was prepared as previously described [30]. It was digested with *Eco*RI and *Hin*dIII and subjected to Southern blotting analysis with ribosomal 16+23S RNA from *E. coli* as a probe [31]. The probe was labeled by random oligopriming with a mixture of hexanucleotides (Pharmacia, Uppsala, Sweden) and cloned Moloney murine leukemia virus reverse transcriptase (Life Technologies Gibco BRL, Gaithersburg, MD) in the presence of 0.35 m*M* digoxigenin-11-deoxyuridine-5′-triphosphate (Boehringer Mannheim, Mannheim, Germany). Procedure for the chemiluminescence detection was as already reported [31].

Detection of the pap, afa, and sfa/foc adhesin-encoding operons, hly and aer operons, and the ibe10 gene. Polymerase chain reaction (PCR) was used to detect these genes in the DNA of the meningitis and ECOR strains. For the adhesin-encoding operons *(pap, afa, sfa/foc),* PCR was performed by use of the primers and the amplification procedure described by Le Bouguenec et al. [32]. Positive DNA controls from strains carrying the three studied operons (provided by C. Le Bouguenec, Institut Pasteur, Paris) as well as negative controls were used in each set of PCR. PCR detection of the *ibe10* gene was done with the sense (5′-TTACCGCCGTTGATGTTATCA-3′) and the antisense (5′-CATTAGCTCTCGGTTCACGCT-3′) primers at a temperature of 60°C for annealing in a standard amplification protocol that generated a 171-bp fragment. The positive control was DNA from strain S25 (C5), in which the *ibe10* gene has been detected earlier by Southern blotting [19]. Hemolysin *(hly)* and aerobactin *(aer)* operons were detected by PCR in conditions as above. Primers for *hly* operon amplification (sense: 5′-AGGTTCTTGGGCATGTATCCT-3′; antisense: 5′-TTGCTTTGCAGACTGCAGTGT-3′) were positioned to obtain a 556-bp PCR product corresponding to most of the *hlyC* gene and to the 5′ end of the *hlyA* gene. Primers for *aer* operon amplification (sense: 5′-AAACCTGGCTTACGCAACTGT-3′; antisense: 5′-ACCCGTCTGCAAATCATGGAT-3′) drive the amplification of 269 bp of the *iucC* gene. With our protocol (PCR reaction directly performed on crude bacterial lysates), both chromosomal and plasmidic *aer* operon were amplified. Positive control strains for *hly* and *aer* operon amplification were *E. coli* J96 and *E. coli* S13 (A21) [18], respectively.

Statistical analysis. The *rrn* RFLP data were summarized as two-way tables of 72 rows for the 72 ECOR strains [9] and of 69 rows for the 69 *E. coli* strains from meningitis, with the number of columns corresponding to the number of *rrn*-containing DNA fragments detected by *Hin*dIII and *Eco*RI endonucleases. For each column, the *rrn*-containing fragment was coded as a binary code, present = 1 or absent = 0, according to the strains. A factorial analysis of correspondence (FAC) [33–35] was conduced on the ECOR strain data. The data on the 69 meningitis strains were considered supplementary observations and projected on the factorial plane F1, F2 (see figure 1) obtained with the ECOR strains. The computations were obtained by use of Stat-ITCF software (Institut Technique des Céréales et des Fourrages, Paris). Distributions of the K1 antigen, of the adhesin-encoding, *hly,* and *aer* operons, of the *ibe10* gene, and of the 14.9-kb *rrn*-containing *Hin*dIII fragment were analyzed among the phylogenetic groups, and statistical significance between the groups was tested by the χ^2 test.

Results

Genetic diversity of E. coli strains causing neonatal meningitis. For the 69 *E. coli* meningitis strains we studied, depending on the strain, *Eco*RI and *Hin*dIII digestions generated 5–9 and 5–10, respectively, 2.5- to 23-kb *rrn*-containing fragments. Altogether, *Eco*RI produced 22 distinct fragments and *Hin*dIII produced 17 distinct fragments. Twenty-three and 26 *rrn* RFLP patterns were observed with *Eco*RI and *Hin*dIII, respectively. *Eco*RI and *Hin*dIII patterns were combined to define a ribotype.

Table 1. Characteristics of 69 neonatal meningitis *E. coli* strains and their affiliation to phylogenetic groups defined by Herzer et al. [23].

Strain no.*	Location†	K1 antigen	Detection of						14.9-kb *Hin*dIII fragment	Phylogenetic group
			pap operon	*sfa*/*foc* operon	*afa* operon	*ibe10* gene	*aer* operon	*hly* operon		
S1	Paris	+	+	−	−	−	+	−	+	B2
S2	Paris	+	−	+	−	+	+	−	+	B2
S3	Quimper	+	−	+	−	+	+	−	+	B2
S4	Paris	+	−	+	−	+	+	−	+	B2
S5	Paris	+	−	+	−	−	+	−	+	B2
S6	Paris	+	−	+	−	+	+	−	+	B2
S9	Paris	+	−	+	−	−	+	−	+	B2
S10	Lyon	+	+	−	−	−	+	−	+	B2
S11	Paris	+	−	−	−	+	−	−	−	D
S12	North America	+	−	+	−	−	+	−	−	B2
S13	North America	+	+	−	−	−	+	−	+	D
S14	Finland	+	−	+	−	+	−	−	+	B2
S15	Germany	+	−	+	−	+	+	−	+	B2
S16	North America	+	+	−	−	−	−	−	+	D
S17	Finland	+	−	+	−	+	−	−	+	B2
S18	North America	+	+	−	−	−	+	−	+	D
S19	North America	+	+	+	−	−	−	+	−	B2
S20	North America	+	−	+	−	+	−	−	−	B2
S21	North America	+	+	−	−	−	+	−	+	B2
S22	North America	−	−	−	−	+	+	−	−	B2
S23	Paris	+	−	+	−	+	+	−	+	B2
S24	North America	−	−	−	+	−	+	−	−	D
S25	North America	+	+	+	−	+	−	+	+	B2
S26	North America	+	−	+	−	+	+	−	+	B2
S27	North America	−	+	−	−	−	+	−	+	D
S28	North America	+	−	+	−	−	+	−	−	B2
S29	North America	−	+	−	−	−	+	−	+	D
S30	Nancy	+	−	+	−	+	+	−	+	B2
S31	Nancy	+	+	+	−	−	−	+	−	B2
S32	Toulouse	+	+	−	−	−	+	−	+	B2
S33	Paris	−	+	−	−	−	+	−	−	A
S34	Nancy	+	+	−	−	−	+	+	+	B2
S35	Quimper	+	−	+	−	+	+	−	+	B2
S36	North America	+	+	−	−	+	−	+	+	D
S37	Nancy	−	−	+	−	−	−	−	+	B2
S38	Paris	+	+	−	−	−	+	−	+	B2
S39	Paris	+	+	−	−	−	+	−	+	D
S40	Paris	+	−	+	−	+	+	−	+	B2
S41	Paris	+	+	−	−	−	+	−	−	A
S42	Paris	−	−	−	+	−	+	−	−	A
S43	Paris	−	−	+	−	−	+	−	−	B2
S44	Paris	+	+	−	−	+	+	−	+	B2
S45	Paris	+	+	−	−	−	+	−	+	B2
S46	Pontoise	+	−	+	−	+	+	−	+	B2
S48	Paris	+	+	−	−	−	+	−	+	B2
S49	Paris	−	+	+	−	−	+	−	−	B2
S50	Aix-en-Provence	+	+	−	−	−	+	−	+	B2
S51	Paris	+	−	−	−	−	−	−	−	B1
S52	Pontoise	+	+	−	−	−	+	−	+	B2
S53	Compiègne	+	+	−	−	−	+	−	+	B2
S54	Paris	+	+	−	−	−	+	−	+	B2
S55	Aix-en-Provence	+	+	−	−	−	+	−	−	A
S56	Pontoise	+	−	+	−	+	−	−	+	B2
S57	North America	+	−	+	−	−	+	−	+	B2
S58	Paris	+	−	−	−	−	+	−	−	D

Table 1. (*Continued*)

Strain no.*	Location†	K1 antigen	Detection of						14.9-kb *Hin*dIII fragment	Phylogenetic group
			pap operon	*sfa*/*foc* operon	*afa* operon	*ibe10* gene	*aer* operon	*hly* operon		
S59	Morocco	+	+	−	−	−	+	−	+	D
S60	Morocco	+	+	−	−	−	+	−	+	D
S61	Morocco	+	+	−	−	−	+	−	+	D
S62	Morocco	−	−	−	−	−	+	−	−	B1
S63	North America	+	−	−	−	−	−	−	−	B1
S64	North America	+	+	−	−	−	+	−	−	D
S65	Aix-en-Provence	+	+	−	−	−	+	−	+	B2
S68	Paris	+	−	+	−	+	+	−	+	B2
S69	Paris	+	−	+	−	+	+	+	+	B2
S70	Pontoise	+	+	−	−	−	−	−	+	B2
S71	St. Denis	−	+	−	−	−	+	−	−	B1
S72	Dieppe	+	+	−	−	−	+	−	+	B2
S73	Beauvais	+	+	−	−	−	+	−	+	B2
S74	Senlis	−	+	−	−	−	+	−	+	B2

* Strains S13, S14, S15, S16, and S17 correspond to strains A21, IHE 3036, A1521, RS 176, and IHE 3034 [24], respectively, while strain S25 corresponds to strain C5 [19].

† For French isolates, name of city is indicated.

Altogether, 40 ribotypes were observed. Nineteen strains exhibited identical ribotypes. Four other ribotypes were found six, four, three, and two times, while the remaining 35 strains exhibited unique ribotypes. This grouping was not correlated with the geographic origin nor the year of isolation, as the major ribotype was observed for strains isolated in several French hospitals and in Finland (S14 = IHE 3036) between 1977 and 1996. A strain from the United States (S16 = RS176) and 3 strains from Morocco (S59, S60, S61) exhibited identical ribotypes, and 2 strains from North America (S25, S26) were indistinguishable from a French isolate (S30). As a comparison, by use of the same genotyping strategy, 35 ribotypes were found, with no types shared by >2 strains, among 39 *E. coli* K1 strains recovered during the 38th week of pregnancy in cultures of vaginal specimens from 39 asymptomatic women attending a single hospital (Robert Debré Hospital, Paris) from 1989 through 1993 and whose newborns remained healthy [5]. Thus, *E. coli* strains causing meningitis worldwide are less heterogeneous than human commensal strains isolated in one single location. *Eco*RI and *Hin*dIII digests distinguished 64 ribotypes among the 72 ECOR strains [9].

Distribution of the meningitis strains among the E. coli phylogenetic groups. To assess the phylogenetic relationships among the strains, statistical analysis by FAC was done with the *rrn* RFLP data obtained for the meningitis and ECOR [9] strains. FAC is a multidimensional statistical analysis based on the main axes of inertia of a scatter plot associated with strains described by the variables. In most cases, the first few principal axes (F1, F2) account for the majority of the variability in the original data. The technique describes the dispersion and shape of a cloud of *n* objects or *p* variates in a multidimensional space by replacing the original data set by a new set of orthogonal linear coordinates in a space of significantly lower dimension. The explained variances of the elements of the data set are in decreasing order of magnitude with respect to these new coordinates.

A FAC was carried out with the ribotyping data resulting from the combination of the two endonuclease patterns for the 72 ECOR strains [9], and the plane F1, F2 was obtained (figure 1). The 69 *E. coli* meningitis strains, considered as supplementary observations, were projected in the plane F1, F2. This plane, which accounted for 44.9% of the total variance, classified most of the ECOR strains within the 4 phylogenetic groups, A, B1, B2, and D, previously distinguished by multilocus enzyme electrophoresis [23] and by RAPD [9]. Forty-seven *E. coli* meningitis strains were clearly distinguished by the negative values of the first axis and unambiguously classified in the phylogenetic group B2. The unique ribotype exhibited by 19 clinical strains was the ribotype of the strains ECOR 61 and ECOR 62 belonging to the B2 group. Four strains (S33, S41, S42, and S55) were projected on the positive values of the two axes and were clearly classified in the phylogenetic group A. Fourteen strains (S11, S13, S16, S18, S24, S27, S29, S36, S39, S58, S59, S60, S61, and S64) were classified in the phylogenetic group D, whereas the remaining 4 strains (S51, S62, S63, and S71) were identified in the phylogenetic group B1 (table 1).

Thus, a gradient in the number of strains involved in neonatal meningitis is observed from the B2 group to the A group via the D and B1 groups. Interestingly, this gradient is in correlation with the genetic distance between the B2 group and the A group, which corresponds to a marked or ancient divergence during evolution [23].

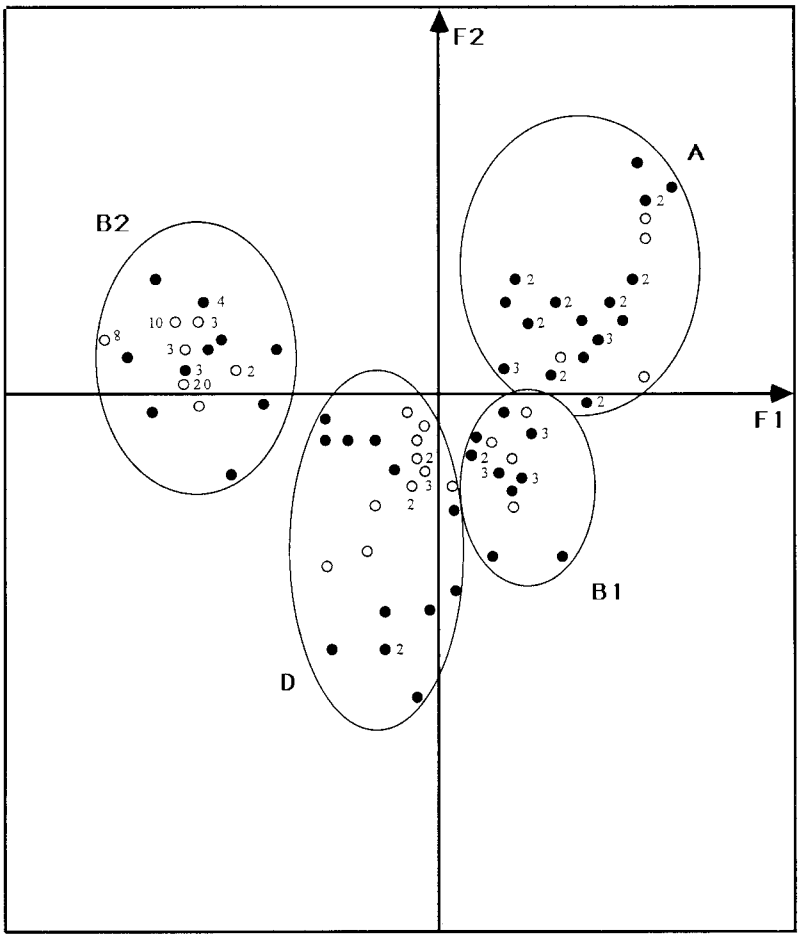

Figure 1. Factorial analysis of correspondence of 72 ECOR strains (●) done on restriction fragment length polymorphism of *rrn* gene data. 69 *E. coli* meningitis strains, considered supplementary observations (○), are projected in plane F1, F2. Phylogenetic groups of ECOR strains A, B1, B2, and D [23] are indicated. No. of strains projected on same point is indicated. Note that decreasing frequency gradient of meningitis strains is observed from phylogenetic B2 group (68%) to A group (6%) via D and B1 groups (26%).

Distribution of virulence genes among the E. coli phylogenetic groups. The presence or absence of the K1 antigen, of the PCR-detected *pap, afa* (a fimbrial adhesin), and *sfa/foc* adhesin-encoding operons, of *hly* and *aer* operons, and of the *ibe10* gene was studied for the meningitis and the ECOR strains, and the distribution of these virulence markers was determined within each phylogenetic group. The presence or absence of the 14.9-kb *rrn*-containing *Hin*dIII fragment was also studied [5].

Our PCR results were in accordance with those obtained from the detection of the *sfa/foc* and *pap, hly,* and *aer* operons with DNA probes and a dot blot procedure for the 5 strains (S13–S17) provided by J. Hacker [24] and with the *ibe10* Southern blotting data for the S25 (C5) strain [19]. A comparison between the meningitis and ECOR strains (tables 2 and 3) shows a global frequency increase for the presence of the *pap* and *sfa/foc* adhesin and *aer* operons and of the *ibe10* gene in the meningitis strains. No difference in the frequency of the *afa* adhesin operon and *hly* operon was observed, but the occurrence of these operons is a rare event in both groups of strains.

Analysis of these data according to the phylogenetic groups revealed some striking features. First, there is a very strong association between the presence of the *sfa/foc* operon and the B2 phylogenetic group for both the meningitis and the ECOR strains. Indeed, all of the *sfa/foc*-positive strains belonged to the *rrn* RFLP-defined B2 group, except for 2 strains (ECOR 58 and ECOR 67), but these 2 strains were positioned within the B2 group by RAPD analysis [9]. In addition, ECOR strain 67 exhibits a B_2-type carboxylesterase B [26]. Second, the *ibe10* gene is also found essentially in meningitis and ECOR strains of the B2 group (34% of the B2 groups and 4% of the *E. coli* of other groups, $\chi^2 = 22.24$, $P < .001$), and the ECOR 67 strain is also *ibe10*-positive. Within the B2 group, *ibe10* is significantly more frequently detected in strains that are positive for the *sfa/foc* operon than in strains that are negative for the *sfa/foc* operon (53% and 9%, respectively, $\chi^2 = 12.6$, $P < .001$). However, 2 *ibe10*-positive meningitis strains belong to the group D (S11 from Paris and S36 from North America). Third, the situation is different for the *pap* operon, because opposite situations are observed for the meningitis and the ECOR strains. For the ECOR strains, its frequency is higher in the B2 group: 60%, versus 42% in group D ($\chi^2 = 0.898$, $P < .01$) and versus 8% in group A ($\chi^2 = 12.715$, $P < .001$). For the meningitis strains, its frequency is lower in the B2

Table 2. Relation between phylogenetic groups of *E. coli* and detection of K1 antigen, *pap, sfa/foc,* and *afa* adhesin-encoding operons, *hly* and *aer* operons, *ibe10* gene, and 14.9-kb *Hin*dIII *rrn*-containing fragment in 69 *E. coli* isolated from neonatal meningitis patients.

Phylogenetic group (no. of strains)	K1 antigen	Detection of						14.9-kb *Hin*dIII fragment
		pap operon	*sfa/foc* operon	*afa* operon	*ibe10* gene	*hly* operon	*aer* operon	
A (4)	2 (50)	3 (75)	0	1 (25)	0	0	4 (100)	0
B1 (4)	2 (50)	1 (25)	0	0	0	0	2 (50)	0
B2 (47)	42 (89)	22 (47)	28 (60)	0	20 (43)	5 (11)	38 (81)	39 (83)
D (14)	11 (79)	11 (79)	0	1 (7)	2 (15)	1 (7)	11 (79)	10 (71)
All groups (69)	57 (83)	37 (54)	28 (41)	2 (3)	22 (32)	6 (9)	55 (80)	49 (71)

NOTE. Data are no. (%).

group (47%) than in the D group (79%) ($\chi^2 = 4.383$, $P < .05$) and in the A group (75%). Within the B2 groups, a significant difference is found between the ECOR and the meningitis strains (60% vs. 47%, $\chi^2 = 0.791$, $P < .01$). Fourth, the 14.9-kb *Hin*dIII fragment is observed at a significantly higher frequency in meningitis strains than in ECOR strains. The same difference is also found in group B2 (83% and 27%, respectively) ($\chi^2 = 16.96$, $P < .001$) and group D (71% and 42%, respectively). Fifth, the *hly* operon is almost always found in the B2 group strains, and within the B2 groups, a clear difference is observed between the ECOR and the meningitis strains, as only 11% of meningitis strains harbored *hly* operon compared with 47% of ECOR strains ($\chi^2 = 9.456$, $P < .01$). Sixth, as observed within the ECOR collection, meningitis strain *aer* determinants belong to all phylogenetic groups.

Thus, virulence markers are present in all of the phylogenetic groups of the *E. coli* species. Those previously reported as being linked to neonatal meningitis are present in the phylogenetic groups B1 and D but are at their highest frequency in group B2. One of them, the *sfa/foc* operon, is restricted to this latter group. The distribution of these markers is different for the ECOR and the meningitis strains; this distribution most notably identifies, among the B2 strains, a subgroup in which some of these virulence markers are grossly enriched and which corresponds to the meningitis strains.

Phylogenetic groups of the meningitis strains and clinical status of the neonates. Clinical data were available for 23 neonates from whom strains were isolated in France. These neonates were classified in high-risk or normal-risk groups as defined by Tullus et al. [28] (table 4). All strains of the phylogenetic A group were isolated from high-risk patients, while the majority of B2 group strains were isolated from normal-risk neonates. One B1 group and 2 D group strains were isolated from normal-risk neonates.

Discussion

Knowledge of the structure of bacterial populations is a prerequisite to the understanding of the epidemiology of infectious diseases. The interpretation of molecular epidemiologic data obtained within a species is largely dependent on the level of horizontal exchanges between strains. Indeed, the level of

Table 3. Relation between phylogenetic groups of 72 ECOR strains and detection of *pap, sfa/foc,* and *afa* adhesin-encoding operons, *hly* and *aer* operons, *ibe10* gene, and 14.9-kb *Hin*dIII *rrn*-containing fragment.

Phylogenetic group (no. of strains)	Detection of						14.9-kb *Hin*dIII fragment
	pap operon	*sfa/foc* operon	*afa* operon	*ibe10* gene	*hly* operon	*aer* operon	
A (25)	2 (8)	0	0	0	1 (4)	5 (20)	0
B1 (16)	1* (6)	2† (12)	0	1* (6)	0	2 (12)	6‡ (37)
B2 (15)	9 (60)	10 (67)	1 (7)	2 (13)	7 (47)	6 (40)	4 (27)
D (12)	5 (42)	0	0	0	0	8 (67)	5 (42)
Accessory group (4)	1 (25)	0	1 (25)	0	0	0	0
All groups (72)	17 (24)	12 (17)	2 (3)	3 (4)	8 (11)	21 (29)	15 (21)

NOTE. Data are no. (%). Strains ECOR 58 and ECOR 67 were positioned within B2 strains by random-amplified polymorphic DNA analysis [9], and ECOR 67 exhibits B_2-type carboxylesterase B [26].

* Strain ECOR 67.

† Strains ECOR 58 and ECOR 67.

‡ Strain ECOR 67 is 1 of 6 strains.

Table 4. Relationship between phylogenetic groups of infecting strains and clinical characteristics of 23 neonates with meningitis.

Phylogenetic group (no. of strains)	High-risk neonates	Normal-risk neonates
A (4)	4	0
B1 (1)	0	1
B2 (15)	3	12
D (3)	1	2

NOTE. Definition of high-risk and normal-risk neonates was as described by Tullus et al. [28].

these exchanges conditions the structure of a bacterial population that may range from highly sexual to almost strictly clonal [7, 8]. It becomes more and more evident that specific bacterial pathogenicities are each linked to a specific clone or group of clones [36]. Continuing new insights into host-parasite interactions at the molecular level will be obtained by combining population genetic analysis of pathogens within their species together with the analysis of virulence and antibiotic resistance genes [36, 37].

In *E. coli,* horizontal exchanges of genetic material do occur, but the overall structure of the population remains clonal [6, 9]. As a success of the approach mentioned above, it has been shown that *E. coli* strains causing hemorrhagic colitis and hemolytic-uremic syndrome belong to a few groups of related organisms, including O157:H7 and O103:H2 clones [36, 38]. Phylogenetic hypotheses have been proposed to explain the emergence of these new diseases, as the O157:H7 clone is closely related to a clone of O55:H7 strains previously shown to be involved in infantile diarrhea worldwide [39], whereas the O103:H2 clone has a common origin with a clone associated with diarrhea in weaned rabbits in France [38]. Likewise, on a rather short series of strains, Selander et al. [4] have shown that the number of electrophoretically defined *E. coli* clones causing neonatal sepsis and meningitis is smaller than that occurring in healthy intestinal floras. These authors suggest that oligoclonality extends to all strains isolated from blood, with or without concomitance of meningitis. However, more data are needed to drive definitive conclusions on the relationships of neonatal meningitis strains, as all of the strains initially studied originated from Finland, only 13 strains were isolated from the cerebrospinal fluid, the genetic relationships of the clinical strains were not analyzed in the context of the species as a whole, and the clinical status of the host was not characterized.

Our data, based on 69 strains causing neonatal meningitis isolated worldwide, confirm the oligoclonality of these strains, mainly due to a high proportion of strains belonging to the phylogenetic group B2. Such an oligoclonality independent of the geographic origin of the strains has been reported in *E. coli* adult extraintestinal strains [10, 11, 40]. However, we find that meningitis strains can belong to all of the phylogenetic groups of the species. These results are in agreement with those of Selander et al. [4], who noted that neonatal pathogenic strains, even though restricted in number, were only slightly less diverse genetically than were commensal strains. It was previously demonstrated that strains belonging to the phylogenetic group B2 of *E. coli* are characterized by a particular electrophoretic pattern (B_2) of carboxylesterase B and correspond to a group of highly pathogenic strains frequently implicated in extraintestinal infections [41, 42]. In our study, strains of the B2 group are significantly more frequent among the strains obtained from meningitis (68%) than among the strains isolated from other *E. coli* extraintestinal infections (40%) [41, 43].

As previously described, 83% of the studied neonatal meningitis strains have the K1 antigen. Here, we show that the presence of the *pap* and *sfa/foc* adhesin-encoding operons, the *aer* operon, and the *ibe10* gene is significantly higher in meningitis strains than in ECOR strains. Interestingly, the *pap* adhesin-encoding operon, which shows a high prevalence among uropathogenic *E. coli* strains (75%–80% of pyelonephritis strains) [32], is observed in 46% of the meningitis strains compared with 24% of the ECOR strains. The low prevalence of *hly* operon within B2 group meningitis strains (11%) has been previously reported [15, 44] and contrasts with its higher prevalence in B2 ECOR strains (47%). The *sfa/foc* operon was observed significantly more often (44%) in the studied meningitis strains than in uropathogenic strains (19%–23%) or in the ECOR strains (17%). The most drastic difference between the meningitis strains and the ECOR strains is observed for the *ibe10* gene (32% vs. 4%), confirming the role of this gene in the pathophysiology of meningitis in newborns [19]. The present data confirm in a larger series our previous observation that the 14.9-kb *Hin*dIII fragment is the most discriminative marker reported to date between commensal and meningitis *E. coli* K1 isolates. Altogether, 64 meningitis strains (93%) harbor at least one of the virulence factors known to be implicated in newborn meningitis (K1, *sfa/foc, ibe10,* 14.9-kb fragment). This fact could be used as a predictive factor in *E. coli* newborn meningitis [5].

When phylogenetic analysis of these virulence factors is done, a striking difference is observed between the *sfa/foc* operon, on the one hand, and the other pathogenic determinants, including the K1 antigen, the *pap* operon, the 14.9-kb *Hin*dIII fragment, the *ibe10* gene, and the *hly* operon, on the other hand. The *sfa/foc* operon is strictly restricted to strains of the phylogenetic B2 group. Similarly, Maslow et al. [11] reported that within adult bloodstream isolates, the presence of this operon was restricted to one cluster. In contrast, the other pathogenic determinants, although being predominant in the B2 group, are also distributed among the other phylogenetic groups. It can be proposed that most of the genes needed for causing neonatal meningitis belonged to the *E. coli* B2 phylogenetic group initially and that horizontal transfer of these genes has occurred toward the more genetically distant groups. The fact that the *sfa/foc* operon was not observed outside of the

phylogenetic B2 group may be because our sample was too small or because it cannot be transferred out from this group of strains. In good agreement with the above considerations, we find a decreasing gradient in the frequency of meningitis strains from the B2 group to the A group, which corresponds to the more divergent *E. coli* lineage from the A group [23] via the D and B1 groups. Thus, the efficiency of genetic exchange probably decreases as divergence between strains increases [45]. The occurrence of pathogenicity islands [46] and the finding that insertional sequences are often located in the vicinity of virulence-associated genes argue for the notion that virulence genes are indeed capable of disseminating among certain *E. coli* strains [21]. Horizontal transfer of *pap* has been previously reported [40, 47]. We sequenced the regions of the *pap* and *sfa/foc* operons chosen for the PCR detection in 12 of the meningitis and ECOR strains selected to represent the distinct phylogenetic groups. Our data yield additional arguments for horizontal transfer, as only two polymorphisms were observed over ~400 nt within the *pap* gene without any correlation with the phylogenetic groups. The same extent of polymorphism was observed in the *sfa/foc* gene, which was detected only in the B2 phylogenetic group (data not shown). Although we propose that the virulence genes have spread from the B2 group, we cannot exclude the possibility that the observed distribution of virulence genes corresponds to their acquisition at different times during the evolution of *E. coli,* with subsequent loss, and that the detection of *sfa/foc* among a single cluster represents a recent acquisition of this operon by *E. coli* [11].

Bacterial infection is a consequence of a disturbed balance between defenses of the host and the colonizing microorganism [28]. In adults, it has been shown that *E. coli* extraintestinal infections in non-immunocompromised hosts involve strains that belong to the carboxylesterase B_2 phenotype and are associated with the presence and expression of multiple chromosomal virulence determinants [48]. In contrast, *E. coli* strains isolated from immunocompromised hosts belong to the B_1 carboxylesterase phenotype and are devoid of virulence factors [48, 49]. Likewise, Maslow et al. [50] have reported a correlation of clinical factors for bacteremia with adhesin carriage by the infecting isolate. Here, we have analyzed the host factors on a sample of the neonates who were classified into high-risk and normal-risk groups on the basis of clinical criteria [28]. Our results generally fit with those reported in the adult, as strains of the A group were observed only in high-risk neonates. However 3 of 15 strains that caused meningitis in normal-risk neonates did not belong to the phylogenetic B2 group. Thus, as in polygenic disease in humans, such as high blood pressure, diabetes, or neuropsychiatric diseases, in which numerous genes and environmental elements are associated to cause a phenotype [51], the phenotype corresponding to *E. coli* neonatal meningitis can be considered as the result of the complex association of the host factors [28] and various *E. coli* virulence genes.

The significantly higher proportion of strains belonging to the phylogenetic B2 group in newborn meningitis strains compared with other extraintestinal *E. coli* strains suggests that neonatal *E. coli* meningitis represents a specific pathophysiologic entity. Coexistence of *pap* and *sfa/foc* adhesin-encoding operons, the *ibe10* gene, and K1 antigen, and the presence of the 14.9-kb *Hin*dIII fragment seem critical in the pathogenesis of neonatal meningitis. However, because some meningitis in normal-risk neonates was due to isolates that lacked these determinants, a role for other, as-yet-undefined virulence factors of the bacteria is predictable. Now that the complete sequence of *E. coli* K12 has been determined [2], sequencing of genomic DNA of prototype strains of the phylogenetic B2 group [52] will be important to undertake. Indeed, *E. coli* K12 belongs to the phylogenetic group A, and comparison of the two sequences can be expected to provide invaluable data concerning virulence in *E. coli* and lead to an understanding of the pathogenesis and pathophysiology associated with *E. coli* neonatal meningitis.

Acknowledgments

We thank H. Dabernat, M. Thibaut, H. Chardon, J. C. Philippe, J. Freney, T. Lambert, J. Raymond, M. Weber, M. Benbachir, K. Kim, J. Hacker, and R. Bortolussi for providing some of the strains used in this study and M. Fageon for typing the manuscript.

References

1. Neidhardt FC. *Escherichia coli* and *Salmonella:* cellular and molecular biology. 2nd ed. Washington DC: American Society for Microbiology, **1996**.
2. Blattner FR, Plunkett G III, Bloch CA, et al. The complete genome sequence of *Escherichia coli* K12. Science **1997**;277:1453–75.
3. Bradley JS. Neonatal infections. Pediatr Infect Dis **1985**;4:315–20.
4. Selander RK, Korhonen TK, Väisanen-Rhen V, Williams PH, Pattison PE, Caugant DA. Genetic relationships and clonal structure of strains of *Escherichia coli* causing neonatal septicemia and meningitis. Infect Immun **1986**;52:213–22.
5. Bingen E, Denamur E, Brahimi N, Elion J. Genotyping may provide rapid identification of *Escherichia coli* K1 organisms that cause neonatal meningitis. Clin Infect Dis **1996**;22:152–6.
6. Selander RK, Lewin BR. Genetic diversity and structure in *Escherichia coli* populations. Science **1980**;210:545–7.
7. Maynard Smith J, Dowson CG, Spratt BG. Localized sex in bacteria. Nature **1991**;349:29–31.
8. Maynard Smith J, Smith NH, O'Rourke M, Spratt BG. How clonal are bacteria? Proc Natl Acad Sci USA **1993**;90:4384–8.
9. Desjardins P, Picard B, Kaltenböck B, Elion J, Denamur E. Sex in *Escherichia coli* does not disrupt the clonal structure of the population: evidence from random amplified polymorphic DNA and restriction fragment length polymorphism. J Mol Evol **1995**;41:440–8.
10. Arthur M, Johnson CE, Rubin RH, et al. Molecular epidemiology of adhesin and hemolysin virulence factors among uropathogenic *Escherichia coli.* Infect Immun **1989**;57:303–13.
11. Maslow JN, Whittam TS, Gilks CF, et al. Clonal relationships among bloodstream isolates of *Escherichia coli.* Infect Immun **1995**;63:2409–27.
12. Ochman H, Selander RK. Standard reference strains of *Escherichia coli* from natural population. J Bacteriol **1984**;157:690–2.
13. Achtman M, Mercer A, Kusecek B, et al. Six widespread bacterial clones among *Escherichia coli* K1 isolates. Infect Immun **1983**;39:315–35.

14. Achtman M, Heuzenroeder M, Kusecek B, et al. Clonal analysis of *Escherichia coli* O2:K1 isolated from diseased humans and animals. Infect Immun **1986**;51:268–76.
15. Korhonen TK, Valtonen MV, Parkkinen J, et al. Serotypes, hemolysin production, and receptor recognition of *Escherichia coli* strains associated with neonatal sepsis and meningitis. Infect Immun **1985**;19: 486–91.
16. Kim KS, Itabashi H, Gemski P, Sadoff J, Warren RL, Cross AS. The K1 capsule is the critical determinant in the development of *Escherichia coli* meningitis in the rat. J Clin Invest **1992**;90:897–905.
17. Saukkonen KMJ, Nowicki B, Leinonen M. Role of type 1 and S fimbriae in the pathogenesis of *Escherichia coli* O18:K1 bacteremia and meningitis in the infant rat. Infect Immun **1988**;56:892–7.
18. Ott M, Hacker J, Schmoll T, Jarchau T, Korhonen TK, Goebel W. Analysis of the genetic determinants coding for the S-fimbrial adhesin *(sfa)* in different *Escherichia coli* strains causing meningitis or urinary tract infections. Infect Immun **1986**;54:646–3.
19. Huang SH, Wass C, Fu Q, Prasadarao NV, Stins M, Kim KS. *Escherichia coli* invasion of brain microvascular endothelial cells in vitro and in vivo: molecular cloning and characterization of invasion gene *ibe10.* Infect Immun **1995**;63:4470–5.
20. Johnson JR. Virulence factors in *Escherichia coli* urinary tract infection. Clin Microbiol Rev **1991**;4:80–128.
21. Mühldorfer I, Hacker J. Genetic aspects of *Escherichia coli* virulence. Microb Pathog **1994**;16:171–81.
22. Morschhauser J, Vetter V, Emody L, Hacker J. Adhesin regulatory genes within large, unstable DNA regions of pathogenic *Escherichia coli:* cross-talk between different adhesin gene clusters. Mol Microbiol **1994**; 11:555–66.
23. Herzer PJ, Inouye S, Inonye M, Whittman TS. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli.* J Bacteriol **1990**;172:6175–81.
24. Ott M, Bender L, Blum G, et al. Virulence patterns and long-range genetic mapping of extraintestinal *Escherichia coli* K1, K5, and K100 isolates: use of pulsed-field gel electrophoresis. Infect Immun **1991**;59: 2664–72.
25. Bortollussi R, Ferrieri P, Björksten B, Quie PG. Capsular K1 polysaccharide of *Escherichia coli:* relationship to virulence in newborn rats and resistance to phagocytosis. Infect Immun **1979**;25:293–8.
26. Goullet P, Picard B. Comparative electrophoretic polymorphism of esterases and other enzymes in *Escherichia coli.* J Gen Microbiol **1989**;135: 135–43.
27. Selander R, Caugant KDA, Ochman H, Musser JM, Gilmour MN, Whittman TS. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl Environ Microbiol **1986**;51: 873–84.
28. Tullus K, Brauner A, Fryklund B, et al. Host factors versus virulence-associated bacterial characteristics in neonatal and infantile bacteraemia and meningitis caused by *Escherichia coli.* J Med Microbiol **1992**;36: 203–8.
29. Cross A, Orskov I, Orskov F, Sadoff J, Gemski P. Identification of K1 *Escherichia coli* antigen. J Clin Microbiol **1984**;20:302–4.
30. Bingen E, Denamur E, Lambert-Zechovsky NY, et al. DNA restriction fragment length polymorphism differentiates crossed from independent infections in nosocomial *Xanthomonas maltophilia* bacteremia. J Clin Microbiol **1991**;29:1348–50.
31. Bingen E, Denamur E, Lambert-Zechovsky N, et al. Analysis of DNA restriction fragment length polymorphism extends the evidence for breast milk transmission in *Streptococcus agalactiae* late-onset neonatal infection. J Infect Dis **1992**;165:569–73.
32. Le Bouguenec C, Archambaud M, Labigne A. Rapid and specific detection of the *pap, afa,* and *sfa* adhesin-encoding operons in uropathogenic *Escherichia coli* strains by polymerase chain reaction. J Clin Microbiol **1992**;30:1189–93.
33. Greenacre MJ. Theory and applications of correspondence analysis. London: Academic Press, **1984**:364.
34. Lebart L, Morineau A, Warwick KM. Multivariate descriptive analysis: correspondence analysis and related technique for large matrices. New York: Wiley-Interscience, **1984**.
35. Tenehaus M, Young FW. An analysis and synthesis of multiple correspondence analysis, optimal scaling, dual scaling, homogeneity analysis, and other methods for quantifying categorical multivariate data. Psychometrika **1985**;50:91–119.
36. Musser JM. Molecular population genetic analysis of emerged bacterial pathogens: selected insights. Emerg Infect Dis **1996**;2:1–17.
37. Doit C, Denamur E, Picard B, Geslin P, Elion J, Bingen E. Mechanisms of the spread of penicillin resistance in *Streptococcus pneumoniae* strains causing meningitis in children in France. J Infect Dis **1996**;174: 520–8.
38. Mariani-Kurkdjian P, Denamur E, Milon A, et al. Identification of a clone of *Escherichia coli* O103:H2 as a potential agent of hemolytic-uremic syndrome in France. J Clin Microbiol **1993**;31:296–301.
39. Whittam TS, Wolfe ML, Wachsmuth IK, Orskov F, Orskov I, Wilson RA. Clonal relationship among *Escherichia coli* strains that cause hemorrhagic colitis and infantile diarrhea. Infect Immun **1993**;61:1619–29.
40. Plos K, Hull SI, Hull RA, et al. Distribution of the p-associated-pilus *(pap)* region among *Escherichia coli* from natural sources: evidence for horizontal gene transfer. Infect Immun **1989**;57:1604–11.
41. Goullet P, Picard B. Highly pathogenic strains of *Escherichia coli* revealed by the electrophoretic patterns of carboxylesterase B. J Gen Microbiol **1986**;132:1853–8.
42. Picard B, Picard-Pasquiet N, Krishnamoorthy R, Goullet P. Characterization of highly virulent *Escherichia coli* strains by ribosomal DNA restriction fragment length polymorphism. FEMS Microbiol Lett **1991**; 82:183–8.
43. Johnson JR, Goullet P, Picard B, Moseley SL, Roberts PL, Stamm WE. Association of carboxylase B electrophoretic pattern with presence and expression of urovirulence factor determinants and antimicrobial resistance among strains of *Escherichia coli* that cause urosepsis. Infect Immun **1991**;59:2311–5.
44. Sitonen AA, Takala A, Ratiner Y, Pere A, Mäkelä P. Invasive *Escherichia coli* infections in children: bacterial characteristics in different age groups and clinical entities. Pediatr Infect Dis J **1993**;12:606–12.
45. Matic I, Taddei F, Radman M. Genetic barriers among bacteria. Trends Microbiol **1996**;4:69–73.
46. Bloch CA, Rode CK. Pathogenicity island evaluation in *Escherichia coli* K1 by crossing with laboratory strain K12. Infect Immun **1996**;64: 3218–23.
47. Arthur M, Arbeit RD, Kim C, et al. Restriction fragment length polymorphisms among uropathogenic *Escherichia coli* isolates: *pap*-related sequences compared with *rrn* operons. Infect Immun **1990**;58:471–9.
48. Picard B, Goullet P. Correlation between electrophoresis types B_1 and B_2 of carboxylesterase B and host-dependent factors in *Escherichia coli* septicaemia. Epidemiol Infect **1988**;100:51–61.
49. Johnson JR, Orskov I, Orskov F, et al. O, K, and H antigens predict virulence factors, carboxylesterase B pattern, antimicrobial resistance, and host compromise among *Escherichia coli* strains causing urosepsis. J Infect Dis **1994**;169:119–26.
50. Maslow JN, Mulligan ME, Adams KS, Justis JC, Arbeit RD. Bacterial adhesins and host factors: role in the development and outcome of *Escherichia coli* bacteremia. Clin Infect Dis **1993**;17:89–97.
51. Weissman SM. Genetic bases for common polygenic diseases. Proc Natl Acad Sci USA **1995**;92:8543–4.
52. Picard P, Journet-Mancy C, Picard-Pasquier N, Goullet P. Genetic structures of the B_2 and B_1 *Escherichia coli* strains responsible for extraintestinal infections. J Gen Microbiol **1993**;139:3079–88.