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Characterization of an Anonymous Molecular Marker Strongly Linked to *Escherichia coli* Strains Causing Neonatal Meningitis

Olivier Clermont, Stéphane Bonacorsi, and Edouard Bingen*

Laboratoire d'Études de Génétique Bactérienne dans les Infections de l'Enfant (EA3105), Université Denis Diderot-Paris 7, Service de Microbiologie, Hôpital Robert-Debré (AP-HP), Paris, France

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An anonymous 14.9-kb *rrn*-containing HindIII fragment is strongly linked to *Escherichia coli* strains causing neonatal meningitis. We show in this report that this fragment does not encode new virulence factors but lacks *arpA*, a gene common in avirulent *E. coli* strains, and we developed a PCR test to detect this fragment.

After group B streptococci, *Escherichia coli* is the second-leading cause of neonatal bacterial meningitis in industrialized countries. It may soon become the leading cause, with worldwide implementation of per partum antibacterial prophylaxis against group B streptococci (14). Better molecular characterization of *E. coli* neonatal meningitis (ECNM) strains is needed to develop new preventive strategies.

Known virulence factors alone cannot explain the pathogenicity of *E. coli* strains causing neonatal meningitis (3, 6, 9). Ribotype analysis of 67 ECNM isolates with the restriction enzyme HindIII identified a 14.9-kb *rrn*-containing fragment in 73% of cases, compared to only 32% of blood culture isolates and 19% of commensal strains (2). A recent study of 134 neonatal meningitis strains confirmed the high prevalence of this fragment (73%) in French strains (6). This anonymous 14.9-kb fragment was harbored by most ECNM strains belonging to the highly virulent phylogenetic groups D (62%) and B2 (82%) but was absent from ECNM strains belonging to the less pathogenic groups A and B1 (6). In contrast, the fragment was found in only 20% of group B2 and 42% of group D strains in the *E. coli* reference (ECOR) collection (8). The significant difference ($P < 0.001$) in the prevalence of the 14.9-kb fragment between ECNM and ECOR group B2 strains made this fragment an anonymous marker strongly linked to ECNM strains, and its presence in the most virulent ECNM strains suggested that it might contain virulence genes.

We examined this fragment for new potential virulence determinants and developed a PCR method for rapid identification of mothers and neonates harboring ECNM strains.

The 14.9-kb fragment contains the *rrnE* operon. To identify putative virulence determinants, we first had to determine which of the seven *rrn* operons was contained in the 14.9-kb fragment. To target candidate *rrn* operons, we therefore used the published genomic sequence of *E. coli* strain K-12 (4) and the results of previous work on the construction of a subtractive library (5). The *rrnE* operon emerged as the best candidate, being located in an ECNM-specific chromosome region known to bear, among other genes, *ibeA*, which is involved in blood-brain barrier penetration (5). We therefore chose two genes, *purH* and *yjaA* (Fig. 1B), located upstream and downstream of the *rrnE* operon, respectively, and used them to prepare probes with which to hybridize the ribotyping membranes. A ribosomal 16-23S RNA probe was labeled by random oligonucleotide priming, and the *yjaA* and *purH* probes were produced by PCR (see primers listed in Table 1) according to the instructions provided with the PCR digoxigenin probe synthesis kit (Roche, Meylan, France) and as previously described (1). Membrane hybridization with HindIII-restricted DNA from a panel of strains showed that the *yjaA* probe hybridized (i) to the anonymous 14.9-kb fragment in strains bearing this fragment (as shown by ribotyping), (ii) to a fragment of a different size in 14.9-kb fragment-negative strains, and (iii) to a fragment of 11.5 kb in the *E. coli* K-12 reference strain MG1655; the size of the latter fragment was that expected from the published sequence (4), thus validating the use of our probe (Fig. 1A). These results showed that the anonymous 14.9-kb fragment contained the region downstream of the *rrnE* operon.

The 14.9-kb fragment is characterized by a single gene deletion. To explain the length difference between the 11.5-kb *rrnE*-containing fragment of *E. coli* K-12 and the 14.9-kb fragment, we performed primer walking along the chromosome of the *E. coli* K-12 MG1655 chromosome and the reference meningitis strain C5 (which harbors the 14.9-kb fragment) (8). Intergenic PCR with the sequence and annotation of the K-12 chromosome (available at http://genolist.pasteur.fr/colibri [data and primers not shown]) was used to compare the sizes of the amplification products. No difference between the two strains was observed up to the *aceK* gene. In contrast, amplification failed between *aceK* and *arpA* in strain C5, the latter gene bearing the next three HindIII sites in *E. coli* K-12 (Fig. 1B). We then showed that the failure of amplification between these two genes was due to total deletion of the *arpA* gene. This was confirmed by PCR between the *aceK* and *iclR* genes, which frame *arpA* in *E. coli* K-12 (data not shown). No other differences were noted between the two strains up to the *metH* gene, which bears the next HindIII site (Fig. 1B).

Development of PCR-based test for the 14.9-kb fragment. In strain C5, the 14.9-kb fragment—the length of which was approximated from the molecular marker used in our ribotyping method (2)—was in fact 15.8 kb. This latter length was de-

* Corresponding author. Mailing address: Service de Microbiologie, Hôpital Robert Debré, 48 Blvd. Sérurier, 75395 Paris cedex 19, France. Phone: 33-1-40-03-23-40. Fax: 33-1-40-03-24-50. E-mail: edouard.bingen@rdb.ap-hop-paris.fr.

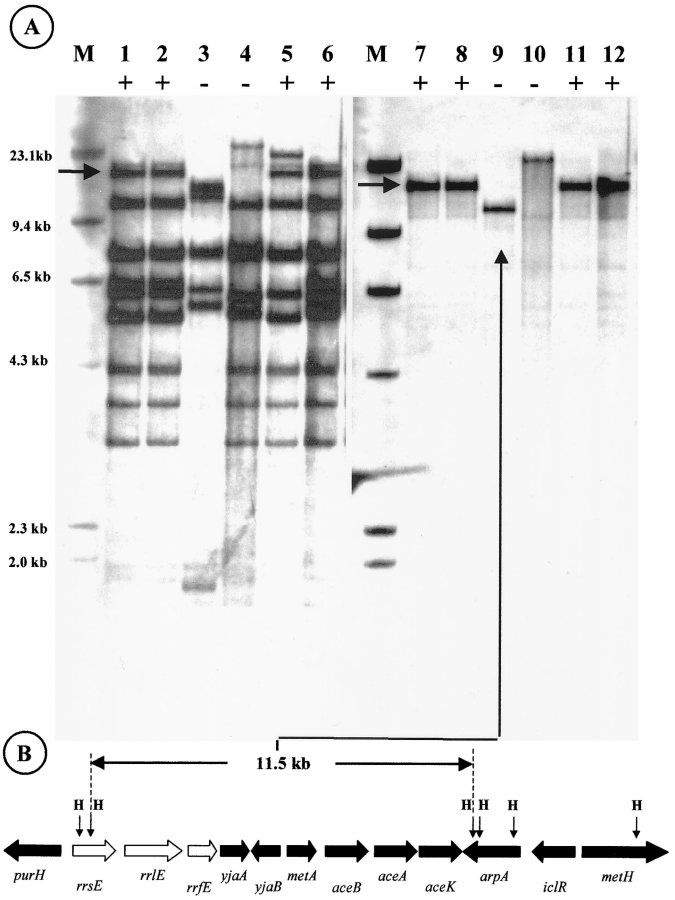

FIG. 1. Determination of the *rrn* operon containing the 14.9-kb fragment. (A) Hybridization with an *rrn* probe (lanes 1 to 6) and a *yjaA* probe (lanes 7 to 12) of the same Southern blot-analyzed membrane. Lanes 1 and 7, C5 (14.9-kb fragment positive, group B2); lanes 2 and 8, S88 (14.9-kb fragment positive, group B2); lanes 3 and 9, K-12 (14.9-kb fragment negative, group A); lanes 4 and 10, J96 (14.9-kb fragment negative, group B2); lanes 5 and 11, S5 (14.9-kb fragment positive, group B2); lanes 6 and 12, S15 (14.9-kb fragment positive, group B2); lane M, size marker. Horizontal arrows indicate the 14.9-kb fragment. (B) A genomic map of the region framing the *rrnE* operon in *E. coli* strain K-12. The *rrnE* operon contains the genes *rrsE*, *rrlE*, *and rrfE* (white horizontal arrows). Vertical arrows indicate HindIII restriction sites.

duced from the length of the *E. coli* strain K-12 DNA region between *rrsE* and *metH* (20 kb) and lacking *arpA* (4.2 kb). The past designation "14.9 kb fragment" is still used for more clarity and in keeping with previous reports (2, 6). We then examined the distribution of the *arpA* gene deletion in 133 neonatal meningitis strains (6) and the 72 ECOR strains (13). Surprisingly, *arpA* was absent from all group B2 strains and also from group D strains of serotype K1, whatever their origin and whether or not they harbored the 14.9-kb fragment, whereas the fragment was present in all group A and B1 strains. Group B2 strains lacking the 14.9-kb fragment possess a larger *rrn*-containing fragment, and we postulated that this length difference was due to abolition of the HindIII restriction site in the *metH* gene by a point mutation (Fig. 1B). We thus sequenced the *metH* gene in eight strains belonging to groups B2 and D, four harboring and four lacking the 14.9-kb fragment (Eurogentec, Seraing, Belgium). Several differences were found, including a point mutation in the HindIII site of the B2

TABLE 1. Primers used in this study

Designation	Sequence	Target	PCR product size (bp)	Source or reference
yjaA.1	5′-TGAAGTGTCAGGAGACGCTG-3′	*yjaA*	211	7
yjaA.2	5′-ATGGAGAATGCGTTCCTCAAC-3′			7
purH.1	5′-TCTCAACCGCATCTTCCAGC-3′	*purH*	190	This study
purH.2	5′-GAGATGATGGATGGACGCGT-3′			This study
arpA.1	5′-TAGCGTACGGTATGGGGAGA-3′	*arpA*	320	This study
arpA.2	5′-TCCCGGTTTGTTTTCAGCGATA-3′			This study
metH.1	5′-AACGCTGAATCCGCGTGGCG-3′	*metH*	195	This study
metH.HindIII	5′-GTAATCTGCTTTACCAGAAAG-3′			This study

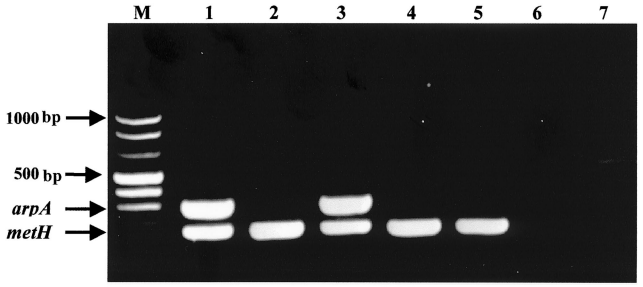

FIG. 2. Multiplex *arpA*/*metH* PCR detecting the 14.9-kb fragment. Pattern 1 is obtained with group A and B1 strains (*arpA* positive, HindIII site in the *metH* gene). Pattern 2 is obtained with group D strains possessing the 14.9-kb fragment (*arpA* negative, HindIII site in the *metH* gene). Pattern 3 is obtained with group D strains not harboring the 14.9-kb fragment (*arpA* positive, HindIII site in the *metH* gene). Patterns 4 and 5 correspond to group B2 strains possessing the 14.9-kb fragment (*arpA* negative, HindIII site in the *metH* gene). Patterns 6 and 7 correspond to group B2 strains not harboring the 14.9-kb fragment (*arpA* negative, no HindIII site in the *metH* gene). Lane M, size marker.

strains, accounting for the absence of the 14.9-kb fragment. We therefore designed an oligonucleotide (metH.HindIII) specific for the nonmutated sequence and then optimized a multiplex PCR assay in order to identify strains harboring the 14.9-kb fragment without the need for ribotyping (Fig. 2). This two-step PCR assay involves simultaneous amplification of the *arpA* gene and the *metH* gene containing the wild-type HindIII restriction site (primers listed in Table 1). Therefore, strains harboring the 14.9-kb fragment would be associated with both negative amplification of the *arpA* gene and positive amplification of the wild-type HindIII restriction site.

PCR was carried out in a 20-μl reaction volume containing 2 μl of 10× buffer (supplied with *Taq* polymerase), 20 pmol of each primer, a 2 μM concentration of each deoxynucleoside triophosphate, 2.5 U of *Taq* polymerase (Sigma-Aldrich, Saint-Quentin Fallavier, France), and 3 μl of bacterial lysate. The cycling conditions were as follows: denaturation for 4 min at 94°C, 30 cycles of 5 s at 94°C and 10 s at 59°C, and a final extension step of 5 min at 72°C. When applied to the 72 strains of the ECOR collection and the 134 meningitis strains, the results of this PCR assay showed perfect agreement with the ribotyping data.

In conclusion, we show that the 14.9-kb fragment does not harbor new specific virulence determinants and differs from the corresponding nonpathogenic *E. coli* strain K-12 DNA region by the lack of *arpA*, a gene of unknown function. We found that *arpA* was absent from all group B2 and most group D meningitis strains, while it was present in all group A and B1 strains. This finding is in accordance with work by Nelson et al. on the *acek-iclR* intergenic region (12). One attractive explanation for this selective distribution of *arpA* is that the lack of this gene in extraintestinal virulent *E. coli* strains may correspond to a "black hole" as described in the theory of Maurelli et al. (11). Nevertheless, the *arpA* G+C content is only 37.7%, compared to 50.8% in *E. coli* K-12, and the most plausible hypothesis is that this gene was acquired by nonpathogenic strains after the separation of groups A and B1 from groups B2 and D (10). At all events, *arpA* may be incompatible with full expression of virulence of extraintestinal pathogenic *E. coli*. This hypothesis is currently being tested in our laboratory. We also developed a PCR-based screening test for the 14.9-kb fragment that obviates the need for ribotyping. This assay could serve to identify asymptomatic neonates, colonized by highly virulent isolates of *E. coli*, who qualify for early treatment.

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