Nucleotide Sequence Analysis of Enteropathogenic Escherichia coli (EPEC) Adherence Factor Probe and Development of PCR for Rapid Detection of EPEC Harboring Virulence Plasmids

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The 1-kb BamHI-SalI fragment from plasmid pMAR2 termed the enteropathogenic Escherichia coli (EPEC) adherence factor (EAF) probe was cloned in pUC19 and pK18. The nucleotide sequence of this fragment was determined, and a set of primers was designed to amplify a 397-bp region associated with pMAR2 by PCR. An analysis of the whole EAF sequence with database libraries indicated no significant homology to any known genes. However, between bases 701 and 787 of the fragment, an 82.8% homology between the EAF and the insertion sequence IS630 of Shigella sonnei exists. The results of PCR with primers of the EAF sequence demonstrated that all of the 151 EAF probe-positive EPEC strains with localized adherence to HEp-2 cells yielded positive EAF PCR results. In contrast, none of the 277 EAF probe-negative strains reacted to the EAF PCR. In addition, the PCR assay was successfully used to generate vector-free digoxigenin-labeled EAF fragments that gave valid results in colony blot hybridization assays. The EAF PCR appears to be a specific and efficient method for the detection of EPEC strains carrying the EAF plasmids.

Certain serotypes of enteropathogenic Escherichia coli (EPEC) have the ability to form adherent microcolonies in a localized pattern on the surfaces of tissue culture cells (2). EPEC strains with the capability to form this localized adherence pattern have been shown to be an important cause of infantile diarrhea in developing countries (5, 11). Since there are generally higher hygienic standards in industrialized countries, outbreaks of EPEC have been rare but these strains continue to cause sporadic cases of diarrhea (10). Molecular analysis of EPEC pathogenesis has progressed and enabled several loci potentially involved in virulence to be identified (recently reviewed by Donnenberg and Kaper [4]). From these analyses, it has been shown that both chromosomal and plasmid-encoded virulence determinants are involved in EPEC pathogenesis (4, 7). The eae gene cluster necessary for attaching and effacing is located on the chromosome (9). The bfpA gene, located on a large plasmid, encodes the bundle-forming pilis (3). From studies with human individuals fed either E. coli O127:H6 wild-type strain E2348/69 or its plasmid-cured derivative, Levine et al. demonstrated that the 90-kb plasmid termed pMAR2 or EPEC adherence factor (EAF) plasmid was necessary for this EPEC strain to have full virulence (12). The results from studies on the pathogenesis of EPEC infection also assisted in the diagnosis of EPEC infection. Nataro et al. (14) were the first to construct a DNA probe consisting of a 1-kb BamHI-SalI fragment of pMAR2 to detect EPEC strains with localized adherence. This probe, termed EAF, has shown its usefulness in several studies worldwide, demonstrating 96 to 100% correlation between probe reactivity and the capability of a strain to cause localized adherence (5, 12). Despite this high correlation, the function of the EAF sequence is still unknown.

Three considerations motivated us to sequence the EAF probe and to subsequently develop a PCR assay for the detection of EAF-harboring E. coli. First, preparing the EAF probe from cloned restriction fragment probes requires vector purification and has a potential for vector contamination. A PCR assay would be a quicker method to label sequences of EAF with digoxigenin. In addition, the demonstration of EAF sequences in EPEC strains by PCR could rapidly identify strains harboring the large virulence plasmid. Finally, since little information on the sequences of the virulence plasmids of EPEC is available, knowledge of the EAF sequence could benefit our understanding of this plasmid's functions.

MATERIALS AND METHODS

Bacterial strains. We analyzed the 608 E. coli strains summarized in Table 1. Strains were kindly provided by M. M. Levine, Center of Vaccine Development, Baltimore, Md., and J. Bockemühl, Hygienisches Institut, Hamburg, Germany, or were from the strain collections of the Nationales Referenzzentrum für Escherichia coli, Berlin, Germany, and the University Hospital, Würzburg, Germany. In addition, 17 strains belonging to the family Enterobacteriaceae (including Yersinia enterocolitica, Yersinia pseudotuberculosis, Citrobacter freundii, Citrobacter diversus, Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter aerogenes, Enterobacter cloacae, Serratia marcescens, Proteus vulgaris, Proteus mirabilis, Morganella morganii,
TABLE 1. Rates of EAF PCR positivity for various categories of E. coli

<table>
<thead>
<tr>
<th>Class of E. coli</th>
<th>No. of strains tested</th>
<th>No. of strains reactive with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EAF probe</td>
</tr>
<tr>
<td>Class I EPEC with localized adherence</td>
<td>151</td>
<td>151</td>
</tr>
<tr>
<td>EPEC with diffuse or no adherence</td>
<td>72</td>
<td>2</td>
</tr>
<tr>
<td>Enterohemorrhagic E. coli</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td>Enteroinvasive E. coli</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Enterotoxigenic E. coli</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>E. coli from urinary tract infections</td>
<td>125</td>
<td>2</td>
</tr>
<tr>
<td>Normal intestinal flora E. coli</td>
<td>125</td>
<td>0</td>
</tr>
</tbody>
</table>

*The same strains were also positive with the EAF probe.

Providencia rettgeri, Salmonella enteritidis, Salmonella typhimurium, Shigella flexneri, and Shigella sonnei isolates) were investigated. All strains mentioned above were analyzed in three independent laboratories by the methodology described below.

Cloning and sequencing of the EAF probe. The 1-kb BamHI-SalI fragment originating from plasmid pMAR2 (15) was cloned into vectors pK18 and pUC19. Sequence analysis was performed with the M13 pUC sequencing and reverse sequencing oligonucleotides (Boehringer GmbH, Mannheim, Germany) and with internal primers for the middle part of the DNA fragment. A total of 2 μg of double-stranded plasmid DNA was subjected to Taq cycle sequencing reactions by using the Prism Ready Reaction Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s instructions. The separation of sequencing products was performed on 7% denaturing polyacrylamide gels with an automatic sequencer (model 373 A; Applied Biosystems). Nucleotide sequencing was carried out in triplicate and analyzed by the DNASIS program, version 2.0, from Hitachi Software (San Bruno, Calif.).

PCR and labeling of the EAF fragment. PCR was performed with primers EAF1 (5'-CAG GGT AAA AGA AAG ATG ATA A-3') and EAF25 (5'-TAT GGG GAC CAT GTA TTA TCA-3') (8) for 30 cycles. Samples were incubated at 96°C for 40 s to denature the DNA, at 60°C for 1 min to anneal the primers, and then at 70°C for 45 s to extend the annealed primers. Labeling of the PCR-generated EAF fragment was performed with a digoxigenin labeling and detection kit (Boehringer GmbH) according to the manufacturer’s instructions.

Colony hybridization with the EAF probe and the PCR-generated 397-bp fragment. The 1-kb BamHI-SalI EAF probe (14) which was random labeled with digoxigenin and the PCR-derived EAF fragment were used for colony hybridization. Filters were prepared and hybridized as previously described (15).

Restriction enzyme analysis. Following PCR with primers EAF1 and EAF25, restriction analysis of the PCR amplification products was carried out with Alul and NcoI. On the basis of the EAF sequence (see Fig. 1), Alul should yield two fragments of 308 and 89 bp, whereas the restriction of PCR products by NcoI should yield fragments of 191 and 206 bp.

Sequence analysis of the EAF PCR fragments. To further confirm the identities of the 397-bp fragments obtained by EAF PCR, these fragments were purified with the Prep-A-Gene purification kit, as described by the manufacturer (Bio-Rad, Munich, Germany), and then subjected to sequence analysis by the Taq cycle sequencing described above.

Adhesion assays. The adhesion capabilities of strains belonging to the various O groups were assayed as previously described (10).

Nucleotide sequence accession number. The complete nucleotide sequence of the EAF probe was submitted to the EMBL database library and assigned the accession number X76137.

RESULTS

Nucleotide sequence analysis of the EAF probe. The BamHI-SalI fragment of pMAR2 was cloned into pUC19 and pK18, and the complete nucleotide sequence of the EAF probe (1,056 bp) was determined (Fig. 1). Comparisons of the whole EAF sequence with sequences in the EMBL database library revealed no striking similarities to published genes (i.e., less than 50%). In a smaller region, between bases 701 and 787 of EAF, an 82.8% homology with the insertion sequence IS630 of Shigella sonnei was found.

Selection of oligonucleotide primers for amplification of EAF fragments. The precise locations of oligonucleotides EAF1 and EAF25 within the EAF sequence are depicted in Fig. 2. This primer pair produced a single 397-bp fragment when analyzed on agarose gels (Fig. 3, lanes 1 to 3); these PCR products resulted from the amplification of strains belonging to three different serotypes, O114:H2, O111:H,-, and O128: H, respectively. The identities of these fragments were shown by endonuclease digestion with Alul, yielding the two expected fragments of 308 and 89 bp (Fig. 3, lanes 4 to 6), and with NciI, generating fragments of 206 and 191 bp (Fig. 3, lanes 7 to 9).

Sequence analysis of the fragments generated by EAF PCR. In addition to restriction enzyme analysis, nucleotide sequence analysis of a 308-bp stretch of each PCR fragment was performed, confirming that these fragments were indeed derived from the EAF sequence. The PCR product of strain E2348/69 revealed 100% homology with the sequence in Fig. 1. With the EAF PCR fragment from E. coli O111 strain 11-1, a 99% sequence homology was found.

Sensitivity of EAF PCR. To determine the sensitivity of PCR, serially diluted broth cultures of E. coli E2348/69 were subjected to amplification. A visible band was seen with even 10 bacteria. This result was also obtained when these bacteria were mixed with 109 organisms of EAF PCR-negative strains.

Evaluation of EAF PCR with different categories of E. coli. When they were analyzed, all of the EAF probe-positive class I EPEC strains gave positive EAF PCR results (Table 1). Unexpectedly, two E. coli strains of serogroup O6 from patients with urinary tract infections gave positive EAF PCR results and also hybridized with the EAF probe. Both strains showed localized adherence. All enterohemorrhagic, enteroinvasive, and enterotoxigenic E. coli strains revealed negative EAF PCR results (Table 1). Moreover, all of the EAF probe-negative strains belonging to the different species of the family Enterobacteriaceae listed above were also demonstrated to be PCR negative. These results were confirmed by all three independent laboratories.

Colony blot hybridization with the digoxigenin-labeled 397-bp EAF fragment generated by PCR. Because PCR-derived digoxigenin-labeled 397-bp fragments of the EAF probe could represent an alternative to the conventional EAF probe, both probes were compared in colony blot hybridization assays. Therefore, single colonies of all the strains listed in Table 1 and of all the different strains of Enterobacteriaceae species listed above were tested in parallel with the two probes.
by colony hybridization. Identical results with respect to EAF probe-positive and -negative strains were found. In general, the PCR-labeled 397-bp fragment produced clearer results than the 1-kb EAF probe in that the hybridization signals were stronger.

**DISCUSSION**

Several large-scale epidemiological studies have shown that local adherent *E. coli* strains are significantly associated with infantile diarrhea (11). For the detection of these pathogens, the EAF probe has successfully been used. However, the function of the EAF sequence remains unknown and could not be clarified in this study. Despite complete nucleotide sequence analysis of the probe, there was no homology to any known gene sequences. Only a small region of the EAF probe showed 82.8% homology to an insertion sequence of *Shigella sonnei* (13). Determining the nucleotide sequences of the regions flanking the EAF probe would clarify whether the EAF sequence is a component of other genes. It could then be clarified whether these genes are involved directly in adhesion or have a regulatory role. From the EAF sequence, we were able to construct various oligonucleotides which were subsequently evaluated for PCR use. The most specific primer pair used to detect EAF plasmid-harboring *E. coli* consisted of oligonucleotide EAF1, as constructed here, and EAF25, an oligonucleotide designed by Jerse et al. (8). These primers were successfully used in a highly sensitive and specific PCR assay which can be recommended for the rapid diagnosis of EPEC infections. As a result of our analysis with the EAF probe, we observed that all EAF probe-positive EPEC strains demonstrated positive PCR results. However, the correlation was not as strong when the results from adherence assays were compared with the PCR results. Two strains which were EAF PCR positive were found to be nonadherent. These strains may be mutated in other genes necessary for localized adhesion.

The pioneer work of Baldini et al. (1) and Nataro et al. (14) revealed that large plasmids present in EPEC strains encode localized adherence to HEp-2 cells. Moreover, Levine et al. (12) demonstrated that these plasmids are a necessary component for EPEC to be virulent. In a three-stage model for pathogenesis recently proposed by Donnenberg and Kaper (4), it was suggested that bundle-forming pilus play an important role in the initial stage of infection. The structural gene *bfpA* has been cloned, and a 0.85-kb fragment of this gene has been used for colony hybridization. Girón et al. (6) recently compared the results of this *bfpA* probe with those of the EAF probe. The results obtained with the two probes and from adherence assays were found to be highly correlated but not identical. Among the 1,801 *E. coli* strains studied, 137 strains hybridized with both probes and 1 strain hybridized with the EAF probe but not with the *bfpA* probe. However, 10 strains hybridized with the *bfpA* probe but not with the EAF probe. Of these 10 *bfpA* probe-positive and EAF probe-negative *E. coli* strains, only 4 showed localized adherence on HEp-2 cells. Because we have chosen a region of the EAF probe which is

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**FIG. 1.** Nucleotide sequence of the 1,056-bp EAF probe derived from pMAR2 of *E. coli* O127:H6 strain E2348/69.

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**FIG. 2.** Scheme of the EAF probe and localization of the primers EAF1 and EAF25 used to amplify the 397-bp fragment by PCR. The restriction sites of the endonucleases *Alu I* and *Nco I* within the PCR product are indicated.
highly conserved among EAF plasmids, it would be interesting to investigate the bfpA probe-positive and EAF probe-negative strains with the EAF PCR method described here. DNA hybridization with the EAF probe has been demonstrated to be an attractive alternative to adherence tests (8, 14). Unfortunately, colony hybridization methods prove to be cumbersome and personnel intensive. If samples are screened by PCR, only positive samples have to be analyzed by colony hybridization to identify infectious strains. This strategy may well prove to be of more practical use in the clinical laboratory. PCR screening may be performed either directly on stool samples or with colonies grown on MacConkey agar plates. One argument for performing PCR with colonies grown on MacConkey agar plates is that laborious DNA purification procedures are not essential and that only viable bacteria are detected.

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REFERENCES