Development of Enterobacterium-Specific Oligonucleotide Probes Based on the Surface-Exposed Regions of Outer Membrane Proteins

GONNIE SPIERINGS,1,2* HARM HOFSTRA,1 JOS HUIS IN‘T VELD,3 WIEL HOEKSTRA,2 and JAN TOMMASSEN1,2

Institute of Molecular Biology and Medical Biotechnology1 and Department of Molecular Cell Biology,2 State University of Utrecht, Padualaan 8, 3584 CH Utrecht, and Department of Microbiology, Division for Nutrition and Food Research TNO, P.O. Box 360, 3700 AJ Zeist,3 The Netherlands

Received 8 August 1989/Accepted 23 September 1989

Outer membrane proteins of members of the family Enterobacteriaceae consist of conserved membrane-spanning segments and hypervariable, surface-exposed regions. We demonstrate that the hypervariable DNA segments corresponding to the surface-exposed regions of these proteins can be used to develop specific DNA probes for the identification of members of the family Enterobacteriaceae.

The identification of microorganisms is essential in a variety of types of studies, including fundamental research and applied research in the medical, environmental, and agricultural sectors. Existing identification procedures are generally slow and laborious. DNA hybridization techniques could potentially increase the rapidity and decrease the labor intensiveness of microbial identification. In addition, it could increase precision, because it is independent of the phenotypical expression of identification markers.

The first step in developing specific DNA probes is to find a piece of DNA unique to the organism to be identified. An ideal DNA probe for any particular organism has to recognize all strains and serotypes of the organism, but it must not cross-react with other bacteria. In this study we investigated whether the genes encoding outer membrane proteins of members of the family Enterobacteriaceae can be used to develop specific probes.

Under standard growth conditions, Escherichia coli K-12 synthesizes two pore-forming outer membrane proteins, OmpC and OmpF. The synthesis of another porin, PhoE, is induced by growth under phosphate starvation (9). The genes encoding these three porins have been sequenced, and comparison of the deduced amino acid sequences revealed an overall homology of approximately 60% (7). A model for the topology of PhoE protein in the outer membrane has been proposed (12, 16). According to this model, the polypeptide traverses the outer membrane 16 times in an antiparallel \( \beta \)-sheet structure. Eight areas are exposed at the cell surface. Comparison of the primary structures of OmpC, OmpF, and PhoE showed that the membrane-spanning segments are conserved, whereas the surface-exposed regions are hypervariable. The \( \text{phoE} \) genes of Klebsiella pneumoniae and Enterobacter cloacae have also been sequenced (15). Comparison of the primary structures of the different PhoE proteins revealed a high degree of homology (81%). Four hypervariable regions were discerned, all of which corresponded to regions predicted to be cell surface exposed (15).

OmpA protein is an outer membrane protein which is not related to the porins, and a model for the folding of this protein has been proposed (8). Of OmpA proteins of several members of the family Enterobacteriaceae showed that the cell-surface-exposed regions are hypervariable (1).

Emanating from the idea that probes based on DNA sequences encoding surface-exposed regions of outer membrane proteins could be specific, we designed a 23-mer oligodeoxynucleotide (Fig. 1A) based on the fifth cell surface-exposed part of the PhoE protein of \( E. \) coli K-12 and tested its specificity in slot-blot hybridization experiments. The oligodeoxynucleotide was synthesized on a Biosearch 8600 DNA synthesizer, purified by high-pressure liquid chromatography, and labeled by the enzymatically catalyzed transfer of \([\gamma-32P]ATP\) (3,000 Ci/mmol; Amersham International) with \( T4 \) polynucleotide kinase according to the procedure described by Maniatis et al. (6).

The sensitivity and specificity of the probe was tested in slot-blot hybridization assays (Fig. 2). Strains were grown overnight at 37°C in L broth (14). Approximately 10⁸ cells were filtered onto nitrocellulose filters (BA85; Schleicher & Schuell, Inc.) in a slot-blot apparatus (Minifold II; Schleicher & Schuell). The blots were prepared as described by Carter et al. (3). The DNA was fixed onto the filters by 4 min of UV irradiation (\( \lambda = 320 \) nm). The blots were prehybridized at 60°C for 45 min in 0.25% Protifar (Nutricia N.V., Zoetermeer, Holland), 6x SSC (900 mM sodium chloride, 90 mM sodium citrate, pH 7.0). After 20 pmol of radioactively labeled oligodeoxynucleotides was added, the filters were hybridized for 1.5 h at 60°C. The blots were washed twice for 15 min in 6x SSC at 60°C and autoradiographed.

The sensitivity assay, which tests the capacity of the

<table>
<thead>
<tr>
<th>A</th>
<th>K. pneumoniae</th>
<th>ACCGCGGCACGCGTCCCACACCTTT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( E. ) coli</td>
<td>TTTCGGCCACGCTGCTCTGCCA probe</td>
</tr>
<tr>
<td></td>
<td>( E. ) cloacae</td>
<td>ACCGCCGACCGCTCCCCTGTCCTTT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>K. pneumoniae</th>
<th>GAACCTATGATTTGCTGCTCTGTAG probe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( E. ) coli</td>
<td>AACCTAAATATATACCTACATAA</td>
</tr>
<tr>
<td></td>
<td>( E. ) cloacae</td>
<td>GAACCGATGCTGCTGCTACATAA</td>
</tr>
</tbody>
</table>

FIG. 1. Comparison of the DNA sequences of (A) the \( E. \) coli probe and (B) the \( K. \) pneumoniae and \( E. \) cloacae probes with the corresponding sequences of the other PhoE genes.
probe to recognize all different strains within a single species, was performed with *E. coli* strains with a variety of O and K serotypes (the serotypes are described in reference 10). Since *E. coli* and *Shigella* species belong to the same species according to Bergey's *Manual of Systematic Bacteriology* (2), different serovars of *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, and *Shigella sonnei* were also tested (Fig. 2). The probe recognized all *E. coli* K-12 CE1194, which carries a deletion of the *phoE* gene (13).

A successful DNA probe should not only show a high degree of sensitivity but it should also be very specific, i.e., it should not cross-react with other bacteria. Figure 2B shows that the probe has a high degree of specificity within the family *Enterobacteriaceae*, since it did not react with strains other than *E. coli* or *Shigella* strains. Therefore, we did not expect to find cross-reactions with nonmembers of the family *Enterobacteriaceae*. This was tested for a few species, i.e., *Aeromonas hydrophila*, *Bacillus cereus*, *Bacteroides anthratum*, *Pseudomonas aeruginosa*, *Sarcina flava*, and *Staphylococcus aureus*. Indeed, no reactions were observed (data not shown). The results of these experiments show that the designed probe is specific for the species *E. coli*-Shigella.

To test the general applicability of the idea that specific DNA probes can be developed on the basis of cell surface-exposed regions of outer membrane proteins, two other probes were tested. These probes were based on the DNA encoding the eight cell surface-exposed regions of the PhoE proteins of *K. pneumoniae* and *E. cloacae* (Fig. 1B). Figure 3 shows the specificities of these probes. The tests were performed under the same conditions as those for Fig. 2 except that the hybridizations and the wash steps were performed at 63°C. The *Klebsiella* probe reacted exclusively with the *K. pneumoniae* strain. The *Enterobacter* probe only reacted with *E. cloacae* and not with other members of the family *Enterobacteriaceae*, including *Enterobacter aerogenes*.

In conclusion, it appears that DNA segments corresponding to cell surface-exposed regions of outer membrane proteins can be used to develop genus- and species-specific probes for the identification of members of the family *Enterobacteriaceae* and possibly also for other gram-negative bacteria. These probes can be used for taxonomic research and for monitoring specific (genetically engineered) microorganisms in the environment. In addition, these probes can be used for the detection and identification of microorganisms in clinical material, food, and feed.

---

**FIG. 2.** Autoradiograms of slot-blot hybridizations using the *E. coli* probe. *E. coli* K-12 CE1194 carries a *phoE* deletion and CE1195 is its *phoE*’ derivative (13). *E. coli* B and *E. coli* C were from our laboratory stocks. F, S, and U denote *E. coli* strains isolated from feces of healthy volunteers, from blood cultures of patients with bacteremia, and from urine of patients with urinary tract infections, respectively (10). Strain EIEC, an enteroinvasive *E. coli*, and the *Shigella* (Sh) strains were obtained from the Institute for Public Health, Bilthoven, The Netherlands. *Salmonella typhimurium* SJ2353 (11) and the other enterobacterial strains (4) have been described previously.

**FIG. 3.** Autoradiograms of slot-blot hybridizations using the *Enterobacter* and *Klebsiella* probes.
We thank D. van Brenk, J. Dankert, and T. Sato for providing different strains.
This project was supported by the Netherlands Technology Foundation (S.T.W.).

LITERATURE CITED