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Detection with Synthetic Oligonucleotide Probes of Nucleotide Sequence Variations in the Genes Encoding Enterotoxins of *Escherichia coli*

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We examined variations in the genes encoding heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) in 88 strains of *Escherichia coli* isolated from individuals with traveler's diarrhea to find suitable sequences for use as oligonucleotide probes. Four oligonucleotide probes of the gene encoding ST of human origin (STIb or STh), one oligonucleotide probe of the gene encoding ST of porcine origin (STIa or STp), and three oligonucleotide probes of the gene encoding LT of human origin (LTIh) were used in DNA colony hybridization tests. In 15 of 22 strains possessing the STh gene and 28 of 42 strains producing LT, the sequences of all regions tested were identical to the published sequences. One region in the STh gene examined with a 18-mer probe was relatively well conserved and was shown to be closely associated with the enterotoxicity of the *E. coli* strains in suckling mice. This oligonucleotide, however, hybridized with strains of *Vibrio cholerae* O1, *V. parahaemolyticus*, and *Yersinia enterocolitica* that gave negative results in the suckling mouse assay.

Enterotoxigenic *Escherichia coli* strains induce diarrhea in humans by producing heat-stable enterotoxins (ST) and/or heat-labile enterotoxins (LT). ST can be classified into two types, methanol-soluble STI (STa) and methanol-insoluble STII (STb), but the latter may not be associated with human diarrhea (3). STI can be further subdivided into two forms, STIa (STp) and STIb (STh), originally found in a porcine strain and a human strain, respectively. There have been many attempts to determine the capacities of *E. coli* isolates to produce enterotoxins.

One line of research has been focused on the development of methods to detect genetic potentials for producing the enterotoxins. Cloned gene probes and synthetic oligonucleotide probes have been used to examine *E. coli* isolates from patients with diarrhea, and, in general, results have been consistent with those obtained by methods for detecting the enterotoxins produced by the isolates (1–6, 10, 12, 14, 15, 18–20, 22, 23). However, discrepant results have been obtained with a few isolates (4, 5, 15, 18, 20). The following possibilities may explain these discrepant results. The sensitivities of the methods may be different. Unlike genes stably maintained in the chromosome, the genes encoding ST and LT (ST genes and LT genes, respectively) are plasmid borne and could be lost by segregation during bacterial growth. Growth conditions that might influence gene expression as well as plasmid segregation during assays could affect the sensitivity of the assays. Random mutations in the genes encoding the enterotoxins may influence the phenotypes. This last point in particular must be considered when specific oligonucleotide probes are used. For development and utilization of oligonucleotide probes, it would be helpful to know the extent of variation of the genes. However, so far, there has been no report on variations in the ST genes and LT genes in enterotoxigenic *E. coli* strains. In this study, we used oligonucleotide probes representing various regions of the genes for this purpose and compared the results with enterotoxin production by the test strains.

Unlike LT, ST are small polypeptides, their extracellular forms consisting of 18 or 19 amino acid residues (27). Thus, detection of ST is very difficult, and until recently the suckling mouse assay has been the main method used for identifying ST-producing *E. coli* strains of human origin. This method is effective for the direct assay of enterotoxic activity of *E. coli* isolates, but it is cumbersome. Hybridization tests with oligonucleotide probes would be a better method, provided that the results were closely correlated with those obtained by the suckling mouse assay. Oligonucleotide probes have many advantages over cloned gene probes; e.g., they can be synthesized automatically and in large amounts, and their hybridization reactions are fast and highly specific. In addition, nonisotopically labeled oligonucleotide probes for the ST genes have been developed, and simple hybridization methods for these probes have been devised (9, 11, 12, 15). Furthermore, oligonucleotide probes can represent relatively large portions of the nucleotide sequences coding for ST, because extracellular ST are encoded on polynucleotide sequences of less than 60 base pairs (13, 21). Therefore, there is a good chance that an oligonucleotide probe may represent a region that is closely associated with the toxic activity of ST. In this study, we evaluated the specificities of the oligonucleotide probes used for identifying suckling-mouse-positive *E. coli* strains on the basis of our results.

MATERIALS AND METHODS

Bacterial strains. *E. coli* strains isolated from patients with traveler's diarrhea (19) and maintained on Dorset egg medium were used in this study. The *E. coli* strains were examined for ST production by the suckling mouse assay

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TABLE 1. Oligonucleotide probes used in this study

Designation	Nucleotide base sequence (5′ to 3′)	Location in the gene[a]	Calculated T_d (°C)[b]
STH1	CTTCAAAAGAAAAAATCACAC	27–34	54
STH2	CACACTAGAATCAAAAAAATG	32–39	52
STH3	CTGCTGTGAATTGTGTTG	58–64	52
STH4	CCTGCTTGTACCGGGTGC	66–71	60
STP	CTGCTGTGAACTTTGTTG	58–64	52
LT1	GAGCACAATATATCTCAGG	224–230	54
LT2	GAACTATGTTCGGAATATCG	28–34	56
LT3	GACCGAGACCAAAATTGATAA	98–105	58

[a] Locations correspond to the positions of the amino acid residues from the N termini of the polypeptides (including signal peptides) reported in the literature (13, 29). The LT1 pobe is for the A subunit of LT, and the LT2 and LT3 probes are for the B subunit.

[b] T_d values, at which 50% of the probe is dissociated, were calculated by the method of Suggs et al. (24).

and for LT production by the Biken test as described previously (7). The suckling mouse assay was performed simultaneously with DNA colony blot preparation. The presence of genes encoding STh and STp (the STh gene and STp gene, respectively) was examined by the DNA colony hybridization test with cloned gene probes as reported previously (15). Reference strains other than *E. coli* have been stored in our laboratory.

Oligonucleotide probes. Oligonucleotide probes representing four regions of the STh gene (STH1, STH2, STH3, and STH4), one region of the STp gene (STP), and three regions of the LT gene of human origin (LT1, LT2, and LT3) were used (Table 1). Their nucleotide sequences were taken from published data (13, 21, 29) and selected to have minimum homology with other known bacterial gene sequences by computer search (European Molecular Biology Laboratory Nucleotide Sequence Data Library). The oligonucleotides were synthesized by the phosphotriester method in a Shimazu automated DNA synthesizer NS-1 (Shimazu, Kyoto, Japan) and purified in a high-performance liquid chromatography apparatus (LC-6A; Shimadzu) equipped with a reversed-phase column (Cosmosil C18). The synthetic oligonucleotides were labeled at the 5′ end by transfer of ^{32}P from [γ-^{32}P]ATP with T4 polynucleotide kinase and purified by chromatography on SEP-PAK C_{18} Cartridges (Waters Associates, Inc., Milford, Mass.). Their specific activities were about 10^8 cpm/μg of probe.

DNA colony hybridization with oligonucleotide probes. Test organisms were spot inoculated onto nitrocellulose filters placed on LB agar, and DNA colony blots were prepared as described previously (16). The hybridization technique allows the detection of even a 1-base-pair mismatch as a reduced hybridization signal after the hybridized blot was washed at a stringent temperature (28). Our previous DNA colony hybridization study (16) indicated that stringent washing temperatures were close to T_d minus 5°C, where T_d is the temperature at which 50% of the probe is dissociated and can be calculated from the G+C content (24). The calculated T_d values of the probes listed in Table 1 ranged from 52 to 60°C. Therefore, a washing temperature of 50°C was used in the initial hybridization test with all the test organisms. When positive hybridization signals were obtained, the test was repeated with a washing temperature of 55°C. The results obtained under the two sets of conditions were the same.

TABLE 2. Detection of ST gene sequences by hybridization with cloned gene probes and oligonucleotide probes in *E. coli* isolates from individuals with traveler's diarrhea

No. of strains	Result in suckling mouse assay[a]	Presence[b] of:		Hybridization with following oligonucleotide probe[c]:				
		STh gene	STp gene	STH1	STH2	STH3	STH4	STP
15	+	+	−	+	+	+	+	−
1	+	+	−	+W	+	+	+	−
1	+	+	−	+	+W	+	+	−
1	+	+	−	+	+W	+W	+	−
1	+	+	−	+	+	+W	+	−
2	+	+	−	−	−	+	+	−
1	+	+	−	+W	+W	+W	+W	−
1	+	−	−	+W	+	+W	+	−
1	+	−	−	+W	+W	+W	+W	−
5	+	−	+	−	−	−	−	+
1	+	−	−	−	−	−	−	+
1	+	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	+
55	−	−	−	−	−	−	−	−

[a] Performed as described previously (19). Symbols: +, positive; −, negative.

[b] Detected with the cloned gene probes by the DNA colony hybridization test as described previously (15). Symbols: +, positive; −, negative.

[c] Nucleotide sequences of the probes are listed in Table 1. Symbols: +, positive; +W, weakly positive; −, negative.

RESULTS AND DISCUSSION

Of 88 *E. coli* strains examined, 31 produced ST detectable by the suckling mouse assay. Of these suckling-mouse-positive strains, 22 possessed the STh gene, only 5 had the STp gene, and 4 had neither gene (Table 2). Hybridization tests with oligonucleotide probes revealed nucleotide sequence diversity in the STh gene. Of 22 STh gene-positive strains, 15 gave positive hybridization signals with all four oligonucleotide probes for STh, whereas the other 7 had nucleotide sequences that varied from the published sequences, as evidenced by weakly positive or negative hybridization signals, in one or more regions examined. Interestingly, two suckling-mouse-positive strains did not react with either of the cloned gene probes but exhibited either positive or weakly positive signals with four oligonucleotide probes for the STh gene. Of the four regions within the STh gene, the one corresponding to the STH4 oligonucleotide probe was the most highly conserved. One oligonucleotide probe (STP) for the STp gene was also used to demonstrate the specificity of the oligonucleotide probes for the STh gene. The STH3 and STP oligonucleotide probes detect analogous regions within the respective ST genes. They differ by two bases (Table 1) but encode identical amino acid sequences (13). The STP oligonucleotide probe did not hybridize with any of the strains that reacted with the cloned gene probe or oligonucleotide probes for the STh gene. STp gene-bearing strains, which constituted only a minority of the suckling-mouse-positive strains, were all identified with the STP oligonucleotide probe. In addition, the STP oligonucleotide probe hybridized to three strains that did not react with any of the cloned gene probes or oligonucleotide probes for the STh gene. Only one of these three strains, however, gave a positive reaction in the suckling mouse assay, indicating that the STP oligonucleotide probe is not specifically associated with positive results in the suckling mouse assay. The STh and STp genes both encoded polypeptides composed of 72 amino acid residues, and their

TABLE 3. Detection of LT gene sequences by hybridization tests with oligonucleotide probes in *E. coli* isolates from individuals with traveler's diarrhea

No. of strains	Result of Biken test for LT[a]	Hybridization with following oligonucleotide probe[b]:		
		LT1	LT2	LT3
28	+	+	+	+
1	+	+	+	−
1	+	+	+W	+
1	+	+W	+	+W
2	+	−	+	+
1	+	+W	+	+W
2	+	−	−	+
1	+	+W	+W	+W
1	+	−	+W	−
1	+	−	−	+W
3	+	−	−	−
46	−	−	−	−

[a] Performed as previously described (7). Symbols: +, positive; −, negative.
[b] Nucleotide sequences of the probes are listed in Table 1. Symbols: +, positive; +W, weakly positive; −, negative.

nucleotide sequences showed high homology (13). Extracellular forms of STh and STp are shorter polypeptides (18 or 19 amino acid residues). Within the amino acid sequences of STh produced extracellularly, a stretch of 13 amino acid residues (located between residues 59 and 71 in the published amino acid sequence [13]) was shown to be essential for the full enterotoxic activity of STh (30). A particular sequence, Asn-Pro-Ala, within the stretch (located between residues 65 and 67 in the published sequence [13]) may be associated with the enterotoxic activity of STh (S. Yamasaki, H. Ito, T. Hirayama, Y. Takeda, and Y. Shimonishi, Adv. Res. Cholera Relat. Diarrheas, in press). The STH3 and STH4 oligonucleotide probes detect regions of the STh gene (residues 58 to 64 and 66 to 71, respectively, in the published sequence [13]) that cover most of the stretch of 13 amino acid residues. In particular, the STH4 oligonucleotide probe detects a region corresponding to Pro-Ala (residues 66 and 67 in the published sequence) of the Asn-Pro-Ala sequence. This may explain why the nucleotide sequence of this region was most highly conserved and closely associated with enterotoxicity in suckling mice. Therefore, this region appears suitable for identifying strains capable of producing biologically active STh. The same region in the STp gene could probably be used to identify strains producing biologically active STp.

One suckling-mouse-positive strain did not hybridize with any of the probes used in this study. This strain may produce a factor(s) that induces fluid accumulation in suckling mice, but that is different from ST, as indicated in a previous study (15). Of 55 strains that gave negative results in the suckling mouse assay, 9 were originally recorded as suckling mouse positive, but they did not give a positive hybridization reaction with cloned gene probes in this study. These strains did not hybridize with any of the oligonucleotide probes for the STh gene or STp gene. They probably lost the ST gene-bearing plasmids by segregation during storage.

Next, we examined nucleotide sequence variation in the LT gene in the *E. coli* strains (Table 3). Oligonucleotide probes representing three regions of the genes encoding LT of human origin (LTIh), the LT1 oligonucleotide probe from the coding region of the A subunit and the LT2 and LT3 oligonucleotide probes from the coding region of the B subunit, were used in hybridization tests. Of the 88 strains, 42 produced LT detectable by the Biken test. Of these 42 Biken test-positive strains, 28 had identical sequences to that reported for the human LT gene. Sequence variations were found in one or more regions of the other 14 strains. The three Biken test-positive strains that did not hybridize with any of the three oligonucleotide probes may have lost the LT gene-bearing plasmid while being grown for the hybridization assay, but this possibility was not investigated. The frequencies of variation in the three test regions were not significantly different.

Our results indicate the existence of considerable nucleotide sequence variation in the ST and LT genes in *E. coli* isolates from patients with traveler's diarrhea. The results seem to substantiate the idea of Medon et al. (12) of using mixtures of oligonucleotide probes covering various regions to detect enterotoxin genes in *E. coli* isolates. This method should detect genes even if they have a silent mutation(s)

TABLE 4. Results of the hybridization test with oligonucleotide probes on enteric pathogens other than *E. coli*

Strain	Hybridization with following oligonucleotide probe[a]:							
	STH1	STH2	STH3	STH4	STP	LT1	LT2	LT3
Salmonella enteritidis 1933001	−	−	−	−	−	−	−	−
Salmonella typhi 1984003	−	−	−	−	−	−	−	−
Salmonella typhi 1984026	−	−	−	−	−	−	−	−
Salmonella typhimurium 1985002	−	−	−	−	−	−	−	−
Shigella dysenteriae 3101003	−	−	−	−	−	−	−	−
Shigella dysenteriae 3101009	−	−	+W	−	−	−	−	−
Shigella flexneri 3102011	−	−	−	−	−	−	−	−
Shigella sonnei 3104011	−	−	−	−	−	−	−	−
Shigella sonnei 3104012	−	−	−	−	−	−	−	−
V. cholerae O1 2203239	−	−	−	+W	−	−	−	−
V. cholerae O1 2243148	+W	−	+W	+	−	−	−	+W
V. cholerae O1 2203120	−	−	+W	+W	+W	−	−	+W
V. cholerae O1 2203240	−	−	+W	+W	−	−	−	+W
V. parahaemolyticus WP1	+	−	+	+W	−	−	−	+W
V. parahaemolyticus WP28	−	−	+	+	−	−	−	+W
Yersinia enterocolitica 2501003	−	−	+W	+	−	−	−	+
Staphylococcus aureus 3109003	−	−	−	−	−	−	−	−
Staphylococcus aureus 3109007	−	−	−	−	−	−	−	−

[a] Nucleotide sequences of the probes are listed in Table 1. Symbols: +, positive; +W, weakly positive; −, negative.

and should be more sensitive than one in which a single oligonucleotide probe is used. However, oligonucleotide probes are short and are more likely to show nonspecific hybridization reactions than longer probes are. Thus, the use of a mixture of oligonucleotide probes increases the chance of obtaining false-positive reactions. In fact, the oligonucleotide probes used in this study were found to hybridize to some enteric pathogens other than *E. coli* (Table 4). Some strains of *Vibrio cholerae* non-O1 and *Yersinia enterocolitica* have been shown to produce a ST similar to that of *E. coli* (25, 26). It is tempting to speculate that the positive reactions seen with vibrios and *Y. enterocolitica* may be due to the presence of ST genes in these organisms. However, the ST produced by *V. cholerae* non-O1 or *Y. enterocolitica* can be detected by the suckling mouse assay (8, 17), whereas none of the test strains listed in Table 4 gave a positive result in this assay. The hybridization reactions seen in Table 4 are probably false-positive reactions, although no definite conclusion is possible because the nucleotide sequences of the ST genes present in non-*E. coli* organisms have not been determined. Even if some of the probe-positive strains have ST genes, the probes may have detected mutated genes. Therefore, we believe that it is better to use a limited number of oligonucleotide probes with high specificity in testing for enterotoxin activity under stringent conditions. The region in the ST genes corresponding to the STH4 oligonucleotide probe was shown to be closely correlated with enterotoxic activity. However, the STH4 oligonucleotide probe also hybridized to the non-*E. coli* enteric pathogens mentioned above. Sommerfelt et al. used 33-mer oligonucleotide probes for the STh and STp genes, which represented regions in these genes (22, 23). The sequence in the STh gene, for example, included a considerable portion of the STH3 oligonucleotide probe sequence and all the STH4 oligonucleotide probe sequence. However, the 33-mer probes did not hybridize to *V. cholerae* and *Y. enterocolitica* (22, 23). By using longer probes, reactions with other organisms may be avoided, but correlation with enterotoxic activity may become weaker. Sommerfelt et al. detected one STp gene-positive but suckling-mouse-negative enterotoxigenic *E. coli* strain with their 33-mer STp oligonucleotide probe (22). This observation is comparable to our finding that the STP oligonucleotide probe hybridized to two suckling-mouse-negative strains of *E. coli*. We are currently investigating whether the use of oligonucleotide probes that are a few bases longer than the 18-mer STH4 probe can avoid hybridization to non-*E. coli* pathogens but still give reactions that are highly correlated with suckling mouse activity.

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