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Development of Primers to O-Antigen Biosynthesis Genes for Specific Detection of *Escherichia coli* O157 by PCR

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The chemical composition of each O-antigen subunit in gram-negative bacteria is a reflection of the unique DNA sequences within each *rfb* operon. By characterizing DNA sequences contained with each *rfb* operon, a diagnostic serotype-specific probe to *Escherichia coli* O serotypes that are commonly associated with bacterial infections can be generated. Recently, from an *E. coli* O157:H7 cosmid library, O-antigen-positive cosmids were identified with O157-specific antisera. By using the cosmid DNAs as probes, several DNA fragments which were unique to *E. coli* O157 serotypes were identified by Southern analysis. Several of these DNA fragments were subcloned from O157-antigen-positive cosmids and served as DNA probes in Southern analysis. One DNA fragment within plasmid pDS306 which was specific for *E. coli* O157 serotypes was identified by Southern analysis. The DNA sequence for this plasmid revealed homology to two *rfb* genes, the first of which encodes a GDP-mannose dehydratase. These *rfb* genes were similar to O-antigen biosynthesis genes in *Vibrio cholerae* and *Yersinia enterocolitica* serotype O:8. An oligonucleotide primer pair was designed to amplify a 420-bp DNA fragment from *E. coli* O157 serotypes. The PCR test was specific for *E. coli* O157 serotypes. PCR detected as few as 10 cells with the O157-specific *rfb* oligonucleotide primers. Coupled with current enrichment protocols, O157 serotyping by PCR will provide a rapid, specific, and sensitive method for identifying *E. coli* O157.

Enterohemorrhagic *Escherichia coli* (EHEC) has become the leading cause of hemorrhagic colitis and hemolytic-uremic syndrome in the United States and Canada. The challenge to clinical and food microbiologists is to differentiate *E. coli* O157:H7 from other commensal *E. coli* isolates present within the gastrointestinal tract. The transmission of EHEC occurs by the consumption of improperly cooked beef products, person-to-person contact, or drinking of unpasteurized milk or water (14, 15, 28). Dairy and beef cattle have been identified as one reservoir for *E. coli* O157:H7 (31, 50), although a survey of meat products, including pork, poultry, and lamb, suggests that other animals may also harbor *E. coli* O157:H7 (18, 39).

Identification of *E. coli* O157:H7 relies on a combination of biochemical and serological tests. *E. coli* O157:H7 can be biochemically distinguished from commensal *E. coli* isolates based on the absence of sorbitol fermentation and β-glucuronidase activity (17). Colony immunoblots or slide agglutination tests with O157 antiserum are subsequently used for confirmation of *E. coli* isolates as the O157 serotype (17). Current identification schemes, therefore, involve an enrichment step, a screen for sorbitol fermentation, and a final serological confirmation of *E. coli* O157:H7 (35). The current EHEC isolation and enrichment scheme occasionally results in the report of false positives. Cross-reactivity to O157 polyclonal antisera has been reported for *Citrobacter freundii*, *Yersinia enterocolitica*, *Pseudomonas maltophilia*, *Brucella abortus*, *Brucella melitensis*, *Escherichia hermanii*, *Hafnia alvei*, *Morganella morganii*, and *Salmonella* group N (5, 9, 11). The serological and biochemical basis for the cross-reactivity is the presence of 4-amino-4,6-dideoxy-D-mannopyranosyl as a constituent sugar of the lipo-polysaccharide (LPS) (36). Cross-reactivity of O157 antisera to *E. hermanii* is a particular problem, because the biochemical profile of *E. hermanii* is similar to that of *E. coli* O157:H7. Other EHEC O157 strains that are sorbitol negative and H negative have also been identified (24). Although several enteric bacteria exhibit cross-reactivity to O157-typing sera, a thorough analysis of genes necessary for O-antigen synthesis may identify gene sequences unique to *E. coli* O157:H7.

The chemical composition of each O antigen is a reflection of the unique DNA sequences that are collectively responsible for the synthesis of this polysaccharide. The enzymes involved in the synthesis, polymerization, and attachment of the O-antigen subunits to the core LPS structure are specified by the *rfb* operon. The number of genes present within this operon varies depending on the sugar compositions and complexities of the O antigens found in the LPSs of *Salmonella* serovars and *E. coli* O serotypes (41). The *rfb* gene cluster has been cloned from a number of gram-negative bacteria, including *Salmonella* spp. (10, 23, 53), various *E. coli* O serotypes (25, 42, 44, 46), *Shigella* spp. (26, 32, 43, 48), *Klebsiella pneumoniae* (12), *Y. enterocolitica* (1, 22, 55), *Vibrio cholerae* (6, 33), and *Pseudomonas aeruginosa* (19).

Comparisons of DNA sequences within *Salmonella* rfb operons have identified regions that are conserved and regions that are unique to a given *Salmonella* serovar (10, 29, 47, 53, 54). Conservation of *rfb* gene sequences reflects the presence of common sugars present in O antigens in some members of the *Enterobacteriaceae* family. For example, *S. typhimurium*, *S. paratyphi*, and *S. typhi* produce a common core O-antigen subunit consisting of rhamnose, galactose, and mannose. It is therefore not surprising that at the genetic level the *rfb* operon is similar in *S. typhimurium*, *S. paratyphi*, and *S. typhi*. In fact, the genes *rfbBCAD*, which are responsible for the synthesis of TDP-rhamnose, not only are found in *Salmonella* serovars Typhimurium, Paratyphi, and Typhi but also have been iden-

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tified in other *rfb* operons encoding proteins in which rhamnose is a constituent sugar of the O antigen (38). Serological differences observed among *S. typhimurium*, *S. paratyphi*, and *S. typhi*, however, reflect the presence of the unique dideoxy sugars abequose, paratose, and tyvelose, respectively (41). The *rfb* genes, which specify the synthesis of these unique sugars, have proven to be useful as DNA-based markers for identification and differentiation of *Salmonella* serogroups A, B, C2, and D (30). The O157 *rfb* operon also contains gene sequences that specify enzymes that are either unique to *E. coli* O157 serotypes (7) or evolutionarily divergent enough to serve as molecular markers for *E. coli* O157 (this study). In this study, a region within the *E. coli* O157:H7 *rfb* operon which was specific to *E. coli* O157 was identified from Southern analysis. DNA sequence analysis revealed similarity to two previously identified *rfb* genes from *Y. enterocolitica* and *V. cholerae*. Sufficient difference in the nucleotide sequence was present to design oligonucleotides specific to *E. coli* O157 serotypes. *E. coli* O157 serotypes were identified by PCR with these primers. PCR was able to detect as few as 10 *E. coli* O157 cells.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* O157 serotypes examined in this study were isolated from several sources, including humans and animals. The *E. coli* O157 serotypes in this study included H7 and other H serotypes. The additional *E. coli* O serotypes O11, O26, O55, and O111, as well as *Salmonella enteriditis* and *S. typhimurium*, were used to test the specificity of O157 gene probes. Also included in this test were *M. morganii*, *H. alvei*, and *C. freundii* (5), bacteria that exhibit cross-reactivity with polyclonal O157 antisera.

Creation of an *E. coli* O157:H7 genomic library and screening of the library for O-antigen-positive clones. Genomic DNA from *E. coli* O157:H7 ATCC 31350 was isolated by cetyltrimethylammonium bromide-NaCl-proteinase K extraction (3) and further purified by CsCl-ethidium bromide gradient ultracentrifugation (40). Chromosomal DNA from *E. coli* O157:H7 was partially digested with the restriction enzyme *Sau*3AI under conditions that yielded large fragments of DNA (30 to 40 kb) (37). The DNA fragments were ligated into the *Bam*HI site of pcos2EMBL (37). The ligated DNA was packaged into λ phages with an in vitro λ phage extract (Gigapack II XL; Stratagene, La Jolla, Calif.) and introduced into competent *E. coli* K-12 by λ-mediated transduction.

The library was screened with polyclonal O157-specific antiserum (Difco, Detroit, Mich.) by the procedure of Morona et al. (33). Nitrocellulose filters were incubated overnight at room temperature with the primary antibody diluted 1/2,000. An anti-rabbit immunoglobulin M–alkaline phosphatase conjugate (Sigma, St. Louis, Mo.), diluted 1/1,000, was subsequently applied to the filters and incubated at room temperature for 30 min. The development of the blots was done with Fast Red TR/Napthol AS-MX (Sigma).

O157-positive *E. coli* K-12 cosmid clones were checked for antigen expression prior to every small- or large-scale extraction procedure for plasmid DNA or LPS by slide test agglutination with polyclonal O157 antiserum. Cosmid DNA was isolated from *E. coli* K-12 by sodium doedecyl sulfate lysis (40) and purified by CsCl-ethidium bromide gradient ultracentrifugation.

E. coli O157-antigen-specific sera included rabbit polyclonal O157-specific antiserum (Difco Laboratories), which was used in the initial screen of the *E. coli* O157:H7 genomic library, and a monoclonal O157-specific antibody, 8-9H (Per-Immune Inc., Rockville, Md.). The monoclonal antibody specifically recognizes the O-antigen epitope unique to the O157 serotype in *E. coli*. This antibody was used to confirm O-antigen-positive cosmid clones identified from the initial screen with polyclonal O157 antiserum.

Characterization of the LPS from *E. coli* O157-positive cosmid clones. LPS was extracted from 1.5-ml overnight cultures by proteinase K treatment (21). The LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide resolving gel, 4% separating gel) at 75 V. Polysaccharide bands were visualized on the gels by using the periodate-silver staining method (45). For Western analysis, gels were transferred onto nitrocellulose membranes by electroblotting for 1 h at 100 V according to the manufacturer's specifications for the MiniTrans Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, Calif.). The membranes were prepared for Western analysis by the same procedure described for colony blotting.

Southern analysis. Chromosomal DNA (1 μg) was digested with 3 U of selected restriction enzymes at 37°C for 2 h. The DNA was separated on a 0.5% agarose gel (1× Tris-borate-EDTA buffer [40] and ethidium bromide [5 μg/ml]) and transferred to a nylon membrane (40). Single-stranded DNA was cross-linked to membranes by using UV light (Fisher Biotech UV Crosslinker, optimal cross-linking setting). Probes were labeled with digoxigenin (DIG)-labeled deoxynucleoside triphosphates by random primer extension (Genius 3; Boehringer Mannheim, Indianapolis, Ind.). DNA probes were prepared from pDS300 sub-

clones as follows. Plasmid DNA was cut with the appropriate restriction enzyme(s), which excised the insert from the plasmid. The DNA was separated on a 0.5% agarose gel (1× Tris-acetate-EDTA buffer [TAE] (40), and the DNA insert was extracted from the gel and purified by using a GenElute Agarose Spin Column (Supelco, Bellefonte, Pa.). The DNA was labeled with DIG-tagged dioxynucleoside triphosphates by using the Genius 3 system. Procedures for DNA-DNA hybridizations and detection were performed as specified in the protocol for the Genius 3 kit. The annealing temperature for hybridizations and washes was 68°C (40).

Strategy for subcloning and identification of *E. coli* O157-specific DNA probes. Cosmid pDS300 was digested with a variety of restriction enzymes, including *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, and *Pvu*II. DNA was separated on a 0.5% agarose gel (1× TAE) (40), and DNA bands that corresponded in size to *E. coli* O157-specific DNA fragments that were identified from Southern blots of *E. coli* and *Salmonella* genomic DNAs were extracted from the agarose gel. DNA was extracted from agarose gel slices by using the Supelco GenElute Agarose Spin Column. Extracted DNA was ligated to plasmid DNA pZERO (Invitrogen, San Diego, Calif.) by using T4 DNA ligase (Promega, Madison, Wis.). Competent *E. coli* TOP10F′ (OneShot TOP10F′; Invitrogen) was transformed with ligated DNA (20), plated on L agar containing Zeocin (200 μg/ml), and incubated overnight at 37°C. The plasmid pZERO is a cloning vector which selects for recombinants via the disruption of *ccdB*, a lethal gene in *E. coli* TOP10F′ cells (4). Plasmid DNA was isolated from transformants by alkaline lysis (8). Each DNA insert was subsequently tested for *E. coli* O157 specificity by Southern analysis.

DNA sequencing. A plasmid, pDS306, was identified from Southern analysis as being *E. coli* O157 specific. Approximately 5 μg of plasmid DNA was submitted to the Molecular Genetics Instrumentation Facility at the University of Georgia for sequencing. An ABI automated sequencer for double-stranded DNA sequencing was used to sequence the plasmid insert. Primer walking was used to generate the complete DNA sequence. DNA sequences were entered into the National Center for Biotechnology Information (34) Blast search program, which was queried for any homology between the query nucleotide sequence and reported gene sequences (2).

PCR. One hundred nanograms of *E. coli* chromosomal DNA served as the template in 10 μl of PCR mix. This reaction mix consisted of 0.2 mM deoxynucleotides, 2.0 mM MgCl₂, 1× PCR buffer (50 mM Tris, pH 7.4), bovine serum albumin (0.25 mg/ml), 50 pmol of forward (O157PF8 [CGTGATGATGTTGAGTTG]) and reverse (O157PR8 [AGATTGGTTGGCATTACTG]) PCR primers, and 0.5 U of *Taq* DNA polymerase (Boehringer Mannheim). The PCR reaction mix was drawn up into 10-μl-capacity thin-walled capillary tubes, and the ends were heat sealed. The capillary tubes were placed in a Rapidcycler (Idaho Technology, Idaho Falls, Idaho) (52). The program parameters for the hot-air thermocycler were (i) 94°C for 0 s, (ii) 55°C for 0 s, and (iii) 72°C for 15 s for 30 cycles (the thermocycler quickly heats the capillary tubes to 94° and 55°C and holds the reaction temperature at 72°C before repeating the cycle). Thirty cycles of PCR were completed in 10 min. DNA products from PCR were analyzed by gel electrophoresis. DNA was separated on 1.5% agarose–1× TAE gels at 70 V. A 100-bp ladder (Promega) served as a molecular size standard for determining molecular sizes of PCR products. PCR products were purified by using a WIZARD Magic PCR column (Promega). DNA was sent to the Molecular Genetics Instrumentation Facility for double-stranded DNA sequencing. Oligonucleotides serving as forward and reverse PCR primers were also used as primers for sequencing double-stranded DNA.

To test the sensitivity of PCR, *E. coli* O157:H7 ATCC 31350 cells were serially diluted in 1 ml of saline, starting with 10⁹ CFU/ml. A 10-μl aliquot from each 10-fold dilution was spotted, in triplicate, onto 1% sorbitol–MacConkey agar and incubated at 37°C to enumerate the cells. Each dilution was boiled for 10 min, and the samples were centrifuged at 12,000 rpm for 30 min. A 100-μl aliquot was taken from each sample, diluted 1/10 in saline, and stored at −20°C. One microliter of boiled cells from each dilution served as the template in PCRs with DIG-labeled nucleotides (Boehringer Mannheim) included in the PCR mixtures. A control lacking template was included with each reaction as a check for PCR contamination. The DNAs were separated on 1.5% agarose gels (1× TAE plus 5 μl of ethidium bromide per 100 ml) and transferred to nylon membranes (40). Membranes were blocked with casein, probed with anti-DIG antibody–alkaline phosphatase conjugate, and developed by using 4-nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indoyl phosphate solution as outlined in the Genius 3 kit (Boehringer Mannheim).

PCR was applied to the detection of *E. coli* O157 in material where the organism may be found. We chose milk and bovine feces for this purpose. An overnight culture of *E. coli* O157 (1.4 × 10⁹ CFU/ml) was diluted 10-fold in whole milk. Samples were spun at 10,000 × *g* at room temperature for 15 min. The top fat layer and underlying water were removed, and the pellet was resuspended in distilled water (dH₂O). The DNA template was then prepared by boiling the suspension for 10 min and separating the supernatant from milk proteins and cell debris by a brief centrifugation. One microliter of this supernatant served as the template in the PCR. Bovine feces were also inoculated with *E. coli* O157:H7 and used to determine the ability of the PCR to detect *E. coli* O157 present in these samples. Two hundred milligrams of bovine feces was added to a 10⁹-CFU/ml culture of *E. coli* O157:H7. The sample was vigorously vortexed for 5 min. The large insoluble particles present in the sample were
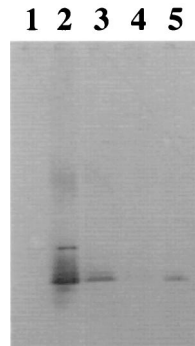
FIG. 1. Immunological analysis of LPS produced by O157-antigen-positive clones by Western blotting. Lane 1, *S. typhimurium* (wild type); lane 2, *E. coli* O157:H7 ATCC 35150; lane 3, *E. coli* LE392 with pDS23; lane 4, *S. typhimurium* *rfc*; lane 5, *E. coli* LE392 with pDS300. LPS was separated on 18% acrylamide resolving gels (75 V/gel), electrophoretically transferred to nitrocellulose membranes, and probed with O157-specific monoclonal antibody 8-9H. The primary antibody was detected by using rabbit anti-mouse immunoglobulin G antibody–alkaline phosphatase conjugate and FAST RED alkaline phosphatase substrate.

removed by a low-speed centrifugation at 3,000 × *g* for 10 min. The supernatant was boiled for 10 min. The sample was centrifuged again to pellet cell debris. The supernatant was diluted 10-fold in dH₂O and served as a template for PCR.

Nucleotide sequence accession number. Nucleotide and amino acid sequence data have been deposited in the NIH/GenBank database under accession no. AF049343.

RESULTS

Identification of unique gene sequences within a cosmid specifying O157 antigen in *E. coli* K-12. The *rfb* operon from *E. coli* O157:H7 was successfully cloned into *E. coli* K-12 host strain LE392. The cosmid library was screened with polyclonal O157-specific antiserum, resulting in the identification of 5 O-antigen-positive clones among the 5,692 clones screened. The O157-specific monoclonal antibody 8-9H reacted with low- and high-molecular-weight LPS from *E. coli* O157-antigen-positive clones (Fig. 1).

The cosmid DNAs from these O157-positive clones contained DNA inserts of between 30 and 40 kb in size. These DNA inserts probably contain not only the *rfb* genes necessary for O-antigen synthesis but genes flanking this operon as well. The genetic map of the *rfb* locus (*his-rol-gnd-rfb-cps*) is conserved among all *E. coli* O serotypes and is similar in its genetic organization to the *S. typhimurium rfb* locus (41). There is also conservation in *rfb* gene sequences for O serotypes that specify sugars commonly found in different O serotypes (41). Among these conserved gene sequences, we also anticipated finding O-serotype-specific genes that encode glycosyltranserase, reductases, epimerases, isomerases, and O-antigen polymerase, which are collectively responsible for the unique carbohydrate chemistry present in *E. coli* O157:H7. In order to generate O-serotype-specific DNA probes, we therefore first needed to distinguish the unique O157 DNA fragments from the conserved gene sequences present within the O157-positive cosmid (Fig. 2).

Experiments were therefore initiated to identify the regions within the DNA inserts that are unique to *E. coli* O157 serotypes. Southern analysis of genomic DNAs from a number of enteric bacteria was performed with the O-antigen-positive cosmids as DNA probes (Fig. 3). With selected restriction enzymes, *E. coli* O157-specific DNA bands were detected by Southern blotting. *E. coli* O157-specific bands that ranged in size from 7.5 to 2.3 kb were identified by DNA probe pDS300 with *Hin*dIII restriction enzyme digests of *E. coli* and *Salmo-*
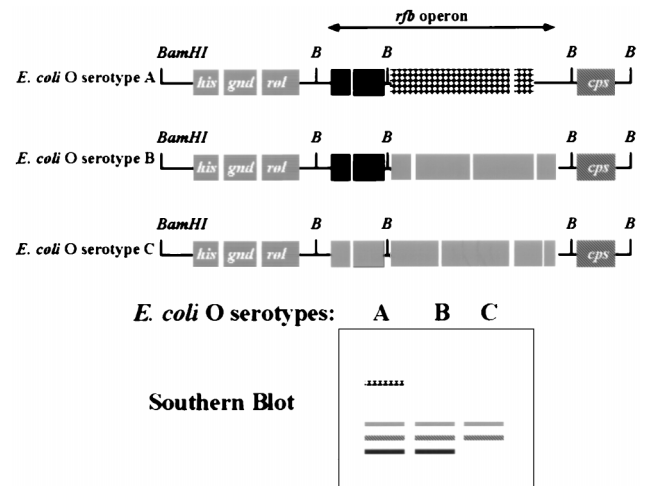

FIG. 2. Strategy for identifying gene sequences unique to the *E. coli* O157 *rfb* operon. Cosmid DNAs from O-antigen-positive clones were used as DNA probes in Southern analysis of the *Enterobacteriaciae* genome. The genes flanking the *rfb* operon are highly conserved between *E. coli* and *S. typhimurium*. If the DNA inserts contain the *rfb* operon and genes upstream or downstream of this operon, then Southern analysis will identify similar-size DNA fragments (gray bands with horizontal or diagonal stripes) in all *E. coli* O serotypes and other enteric bacteria included in the study. The *rfb* operons will also contain genes whose products synthesize a sugar common to many O serotypes. The cosmid DNA probes are expected to recognize similar-size DNA fragments (black) present in only those *E. coli* O serotypes that synthesize a sugar(s) common to both. Finally, DNA sequences within the DNA insert that are unique to a specific *E. coli* O serotype will recognize only DNA fragments in that O serotype (diamond pattern) when used as a DNA probe in Southern blotting.

nella genomic DNAs (Fig. 3). No bands were observed for genomic DNAs of *E. hermanii* and *M. morganii*, organisms that cross-react with the polyclonal O157 antiserum (data not shown). Cosmid pDS300 was digested with a number of different restriction enzymes, DNA fragments were separated by gel electrophoresis, and selected DNA bands were extracted
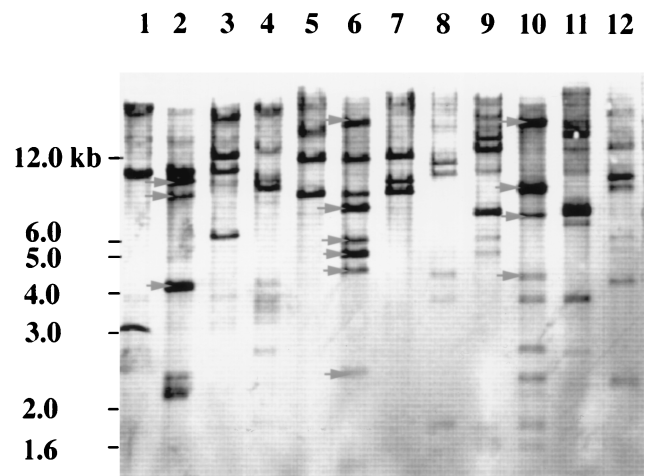

FIG. 3. Distribution of *E. coli* O157 *rfb* gene sequences present on O-antigen cosmid pDS300 among *E. coli* O serotypes and *S. typhimurium*. Lanes 1, 5, and 9, *E. coli* O26 ATCC 11840; lanes 2, 6, and 10, *E. coli* O157:H7 ATCC 35150; lanes 3, 7, and 11, *E. coli* K-12; lanes 4, 8, and 12, *S. typhimurium*. Chromosomal DNA was digested with restriction enzyme *Eco*RI (lanes 1 to 4), *Hin*dIII (lanes 5 to 8), or *Pst*I (lanes 9 to 12). DNA fragments were separated on 0.5% agarose gels, transferred to nylon membranes, and hybridized with DIG-labeled DNA probe pDS300.
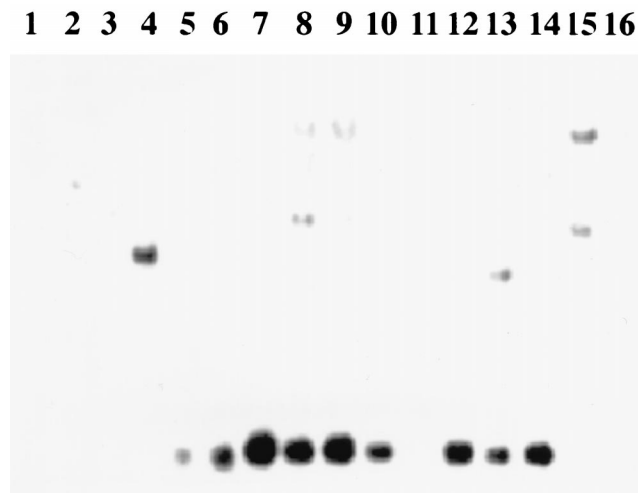
FIG. 4. Assessing pDS306 as an *E. coli* O157 serotype-specific DNA probe by Southern blotting. Lane 1, *S. typhimurium*; lane 2, *S. enteriditis*; lane 3, *E. coli* K-12; lane 4, *E. coli* O26 (ATCC 11840); lane 5, *E. coli* O157:H7 (ATCC 35150); lane 6, *E. coli* O157:H7; lane 7, *E. coli* O157:H7; lane 8, *E. coli* O157:H11; lane 9, *E. coli* O157:H16; lane 10, *E. coli* O157:H42; lane 11, *E. coli* O157:H7; lane 12, *E. coli* O157:H7; lane 13, *E. coli* O157:H7; lane 14, *E. coli* O157:H7; lane 15, *E. coli* O55:H7; lane 16, *C. freundii*. Chromosomal DNA was digested with the restriction enzyme *Eco*RI. DNA fragments were separated on 0.5% agarose gels, transferred to nylon membranes, and hybridized with DIG-labeled DNA probe pDS306.
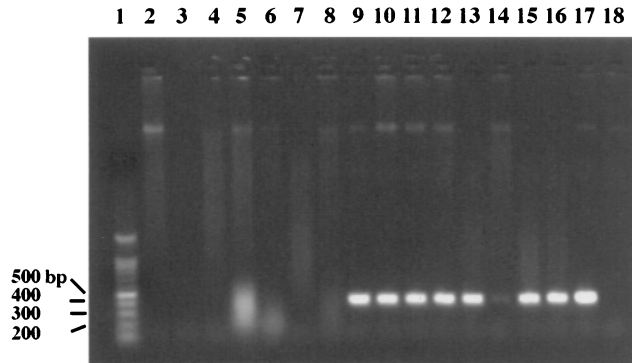

FIG. 5. Identification of *E. coli* O157 serotypes by PCR with *E. coli* O157 *rfb*B-specific primers. Lane 1, 100-bp ladder (Promega); lane 2, *S. enteriditis*; lane 3, *S. typhimurium* UK1; lane 4, *E. coli* K-12 LE392 (O16); lane 5, *E. coli* O11; lane 6, *E. coli* O18; lane 7, *E. coli* O26 ATCC 11840; lane 8, *E. coli* O111; lane 9, *E. coli* O157:H7; lane 10, *E. coli* O157:H7; lane 11, *E. coli* O157:H11; lane 12, *E. coli* O157:H16; lane 13, *E. coli* O157:H42; lane 14, *E. coli* O157:H7; lane 15, *E. coli* O157:H7; lane 16, *E. coli* O157:H7; lane 17, *E. coli* O157:H7; lane 18, *C. freundii* (O157$^+$). DNA fragments were separated on 1.5% agarose–1× TAE gels at 100 V.

from the agarose gel and cloned into DNA sequencing vector pZERO. The DNA fragments that corresponded in size to *E. coli* O157-specific DNA fragments identified from the first Southern blot were selected. The specificities of the resulting subclones were later assessed by Southern analysis. We identified a number of plasmids with inserts which were specific for *E. coli*, *E. coli* O26/O157, and *E. coli* O157 as DNA probes in Southern analysis. Plasmid pDS306 appeared to be specific for *E. coli* O157. In Southern analysis, pDS306 identified a 1.9-kb *Eco*RI DNA fragment in 9 of 10 *E. coli* O157 serotypes. Although one *E. coli* O157 isolate did not produce as strong a signal as the other O157 serotypes by Southern blotting, this isolate was weakly positive by PCR (see below). No 1.9-kb *Eco*RI DNA fragment was observed for other *E. coli* O serotypes, *S. typhimurium*, *S. enteriditis*, and *C. freundii* under stringent hybridization and wash conditions (Fig. 4).

The DNA sequence of the insertion in the plasmid pDS306 was determined and exhibited significant homology with reported gene sequences (Table 1). pDS306 has 75 and 69% identity with *rfb* genes which encode a GDP-mannose dehydratase (Gmd) and WcaG, respectively. The GC content of the *E. coli* O157 *rfb* genes was 39%, similar to low GC content present in *Salmonella* and *E. coli* *rfa* and *rfb* genes (41). The 1.4-kb *Eco*RI DNA insert present in pDS306 contains two

open reading frames (*rfb*A and *rfb*B) which correspond in amino acid sequence to a GDP-mannose dehydratase, Gmd (nucleotide positions 1 to 600), and a capsule biosynthesis gene product, WcaG (nucleotide positions 839 to end), respectively.

Specificity and sensitivity of rfb_{O157}-designed primers for detecting *E. coli* O157 serotypes by PCR. The nucleotide sequence present in the pDS306 DNA insert was searched for potential primer sequences. With primer pair O157PF8 and O157PR8, a 420-bp DNA fragment was observed for all *E. coli* O157 serotypes examined, while no product was observed for other *E. coli* O serotypes and *C. freundii*, an O157 antiserum-positive isolate (Fig. 5). The nucleotide sequences of eight *E. coli* O157 DNA fragments amplified by PCR with primers O157PF8 and O157PR8 were determined and were found to be similar to the original *rfb* gene sequence obtained for pDS306. There were a total of only four base pair changes in the nucleotide sequence among the eight *E. coli* O157 420-bp PCR products (data not shown). We were able to detect as few as 10 *E. coli* O157:H7 cells by PCR with these primers (Fig. 6).

Application of a PCR test with rfb_{O157}-specific primers to the detection of *E. coli* O157 in milk and cattle feces. PCR with this O157-specific *rfb* primer pair was assessed as to its ability to detect *E. coli* O157 in samples contaminated with the organism. Whole milk was seeded with *E. coli* O157 and serially diluted 10-fold. The PCR was able to detect as few as 10^7 cells/ml in the milk. Similarly, the PCR was also applied to the detection of *E. coli* O157:H7 in cow feces. A fecal specimen was seeded with 10^9 CFU/ml. The feces were plated onto MacConkey agar and contained both lactose-positive and -negative colonies. Product from sample diluted 1:10 and 1:100 was

TABLE 1. DNA sequence identity between *E. coli* O157 *rfb* gene sequences (plasmid pDS306) and reported bacterial gene sequences

| *E. coli* O157 *rfb* gene	Gene identified from BLAST search	Function	Identity (%)
ORF1 (*rfb*A; positions 1 to 601)	*Y. enterocolitica* O:8 *rfb* (ORF 13.7)	GDP-mannose dehydratase	73
	V. cholerae *rfb*D	GDP-mannose dehydratase	74
	E. coli K-12 *cps* operon (ORF 0.0)	GDP-mannose dehydratase	75
ORF2 (*rfb*B; positions 837 to 1465)	*E. coli* K-12 *cps* operon (ORF 0.9)	Unknown	69
	Y. enterocolitica O:8 *rfb* (ORF 14.9)	Unknown	65

A

B
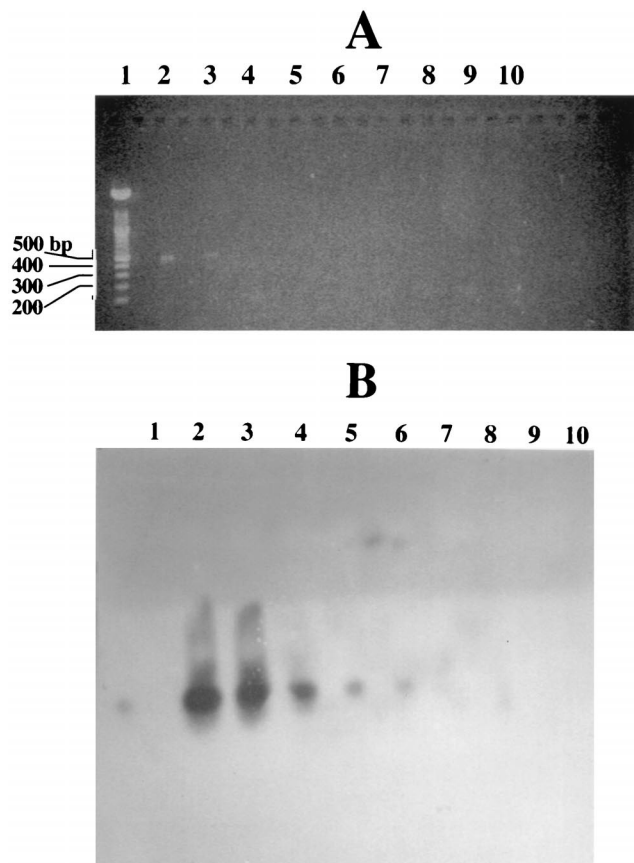
FIG. 6. Sensitivity of *E. coli* O157 *rfbB* primers in detecting *E. coli* O157:H7 by PCR. Lanes 1, 100-bp ladder; lanes 2, 10^{-1} dilution (10^5 cells); lanes 3, 10^{-2} (10^4 cells); lanes 4, 10^{-3} (10^3 cells); lanes 5, 10^{-4} (10^2 cells); lanes 6, 10^{-5} (≤ 10 cells); lanes 7, 10^{-6} (<10 cells); lanes 8, 10^{-7} (<10 cells); lanes 9, no DNA; and lane 10, *E. coli* K-12 LE392. PCR was performed with serial 10-fold dilutions of *E. coli* O157:H7 ATCC 31350 (10^9 CFU/ml) and *E. coli* O157 *rfbB*-specific primers. DNA fragments were separated on 1.5% agarose–1× TAE gels at 100 V. One microliter of the boiled sample served as the DNA template in the 10-μl PCR mixture. DIG-labeled nucleotides were incorporated to heighten the sensitivity of the PCR. (A) Agarose gel. (B) Nylon membranes probed with anti-DIG antibody.

observed, while no PCR amplicon was observed in the undiluted fecal sample that was spiked with *E. coli* O157:H7. The 1:100 dilution represented 10^7 CFU of *E. coli* O157:H7 per ml in the sample (Fig. 7).
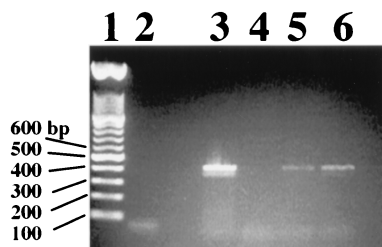

FIG. 7. Detection of *E. coli* O157:H7 in cow feces by PCR. Cow feces was inoculated with 10^9 CFU of *E. coli* O157:H7 per ml. Debris was removed by a low-speed centrifugation, and template was prepared for PCR by boiling the supernatant for 10 min. Undiluted samples and samples diluted 10-fold in dH₂O served as DNA templates for PCR. Lane 1, 100-bp ladder; lane 2, no-DNA control; lane 3, *E. coli* O157:H7 positive control; lanes 4 to 6, undiluted cow feces (lane 4) and cow feces diluted 10-fold (lane 5) and 100-fold (lane 6) that were inoculated with 10^9 CFU of *E. coli* O157:H7 per ml.

DISCUSSION

In this study, we created an *E. coli* O157:H7 cosmid library, identified O157-positive cosmid clones, and eventually identified serotype-specific DNA sequences. From the O157 cosmid library, five O157 positive cosmid clones were identified. When an O157-antigen-positive cosmid was used as a DNA probe, the probe failed to recognize the DNA sequences present within the genomes of *H. alvei*, *M. morganii*, and *C. freundii*. These bacteria exhibit cross-reactivity with polyclonal O157 antiserum and a monoclonal antibody that specifically recognizes O157 antigen among the many distinct *E. coli* O serotypes. An O157-serotype-specific DNA probe was identified. The DNA insert for pDS306 has homology to known *rfb* and capsule genes present in *E. coli* K-12 (42), *Y. enterocolitica* O:8 (55), and *V. cholerae* (6). The nucleotide sequences of *E. coli* O157 *rfb* genes were sufficiently different from those of *E. coli* K-12 to distinguish *E. coli* O157 serotypes from other *E. coli* serotypes and enteric bacteria like *Salmonella* and *Citrobacter* by Southern blotting and PCR. The *rfb* sequence chosen for design of PCR appears to be ideal, since there was no significant divergence in the nucleotide sequences among the *E. coli* O157 serotypes. This is an ideal target for PCR, since the LPS enzymes responsible for O-antigen synthesis are not subjected to the same selection pressure as surface proteins like H antigen.

By PCR, *E. coli* O157:H7 was detected in milk and cow feces spiked with the organism. We were able to detect *E. coli* O157:H7 in milk and feces at 10^7 cells/ml in samples laced with this organism. This was especially remarkable for the fecal sample, since no additional steps for concentrating the template were included in this procedure. The sensitivity of the PCR in general was increased 1,000-fold by including DIG-labeled nucleotides in the PCR. A PCR–enzyme-linked immunosorbent assay will increase the sensitivity of the test as well as make processing of large samples more tenable (27), and we are currently developing a PCR–enzyme-linked immunosorbent assay just for this purpose. There were PCR inhibitors present in fecal specimens. Feces are notorious for the presence of PCR inhibitors (51). Further DNA purification will eliminate any additional interference with the PCR.

Probe-based methods have revolutionized detection of salmonellae and other important enteric pathogens in clinical and food specimens. The basis for the molecular diagnosis of infectious diseases is the use of selective PCR amplification in the identification of the variable genetic regions unique for a given serotype or pathogenic group like EHEC. A PCR-based detection system has been established for the specific detection of *Salmonella* serogroups A, B, C2, and D (30). The advantage of this PCR test over current tests focusing on *E. coli* O157 virulence genes is because of the genetic instability and sporadic distribution observed for these markers among *E. coli* isolates that are pathogenic or nonpathogenic for humans (13, 16). The strategy outlined in this study will prove beneficial in the generation of new molecular probes for identifying specific pathogenic *E. coli* O serotypes. Long PCR has already proven to be effective in the cloning and sequencing of the complete *rfb* operon of *E. coli*, including *E. coli* O157 (49). However, by combining long-PCR products of the *rfb* operon as DNA probes in Southern analysis of various *E. coli* O serotypes, unique DNA fragments can be quickly identified for sequencing. Instead of bidirectional sequencing of PCR amplicons of 18 kb or greater, we can focus our attention on smaller DNA fragments of 1 to 2 kb, as demonstrated in this study, which will save time and expense in development of new molecular probes. Our work further supports the hypothesis that unique sequences within a pathogenic *E. coli* O serotype, such as those

located within the *rfb* gene cluster, can be used as specific DNA probes regardless of the biochemical and serological nature of other microorganisms present within the gastrointestinal tract. Even with an initial enrichment, this O157-specific, PCR-based test will detect *E. coli* O157 serotypes in less time than the current microbiological methods, while eliminating false positives.

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