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Selective and Sensitive Method for PCR Amplification of *Escherichia coli* 16S rRNA Genes in Soil

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A set of PCR primers targeting 16S rRNA gene sequences was designed, and PCR parameters were optimized to develop a robust and reliable protocol for selective amplification of *Escherichia coli* 16S rRNA genes. The method was capable of discriminating *E. coli* from other enteric bacteria, including its closest relative, *Shigella*. Selective amplification of *E. coli* occurred only when the annealing temperature in the PCR was elevated to 72°C, which is 10°C higher than the optimum for the primers. Sensitivity was retained by modifying the length of steps in the PCR, by increasing the number of cycles, and most importantly by optimizing the $MgCl_2$ concentration. The PCR protocol developed can be completed in less then 2 h and, by using Southern hybridization, has a detection limit of ca. 10 genomic equivalents per reaction. The method was demonstrated to be effective for detecting *E. coli* DNA in heterogeneous DNA samples, such as those extracted from soil.

Bacteria of the *Enterobacteriaceae* are important pathogens causing intestinal and systemic illness of humans and other animals. Recent outbreaks of gastrointestinal diseases focused public attention on one of the more widely known members, *Escherichia coli*, and the potential problems with strains of this organism as food-borne pathogens. Consumption of water polluted with fecal material is an important exposure pathway, and while monitoring of total coliforms is the standard technique, some research suggests that analysis for *E. coli* specifically may be a better indicator (3).

Traditional approaches for analysis of *E. coli* have relied on cultural techniques, and many selective-differential media have been developed. Generally, lactose fermentation is used for differentiation, sodium lauryl sulfate or bile salts are used as a selective agent, and a fluorogenic reaction is used for confirmation. β-Glucuronidase is the target enzyme for confirmation of *E. coli*, which mediates hydrolysis of 4-methylumbelliferyl-β-D-glucuronide (MUG) to a fluorescent product. However, other *Enterobacteriaceae* produce β-glucuronidase (e.g., Shigella, Salmonella, and Yersinia), not all strains of *E. coli* express the *uiaA* gene that encodes β-glucuronidase, and some *Staphylococcus* spp. hydrolyze MUG (6, 25). Biochemical analysis for an enzyme associated with a particular pathogenic trait and immunodiagnostic assays for O antigens associated with pathogenic strains have also been developed (11, 16). Again, cross-reactivity limits the utility of these techniques for identification of *E. coli*.

The emergence of DNA technology has opened new possibilities for development of methods with improved selectivity for *E. coli*. Relevant techniques include use of PCR and/or hybridization probes to detect *E. coli*-specific genes encoding invasion proteins (12, 13), toxins (17, 23, 24), catabolic enzymes (4, 8), or structural (lipo)proteins (10, 12). A drawback to these approaches has been that when cross-reactivity tests were done, target sites were often detected not only in *E. coli* but also in closely related organisms (14, 17, 18). Cross-reaction is particularly problematic with *Shigella*. This is not surprising insofar as 16S rRNA and other genome-level similarities suggest that *Escherichia* and *Shigella* are sufficiently similar for placement in a single genus (5, 19). Nevertheless, for clinical, epidemiological, and historical reasons they are regarded as different genera. Thus, for development of molecular methods, the challenge is to identify sequences conserved within *E. coli* that may be targeted to minimize false negatives yet can be distinguished from similar sequences likely to be present in *Shigella*.

The goal of this study was to design a selective and sensitive PCR method for amplification of a 16S rRNA gene region from *E. coli*. The key performance criterion for the method was to reliably amplify the targeted region from template levels equivalent to 100 or fewer *E. coli* cells without cross-reaction with similar sequences present in *Shigella*. An additional consideration was that the method be sufficiently robust for analysis of genomic DNA extracted from water or soil samples as well as that prepared from clinical isolates.

Cultures and DNA template preparation. Cultures used in this study are listed in Table 1. Genomic DNA for PCR experiments was extracted from the liquid cultures as described by Ausubel et al. (2). DNA concentration and quality were determined by UV light absorbance at 260 and 280 nm and by band intensity densitometry using NIH Image version 1.55 (National Institutes of Health, Bethesda, Md.).

Primers and probes. Primers targeting hypervariable regions of the *E. coli* 16S rRNA gene were developed by using PrimerSelect (DNAStar, Madison, Wis.). Three sets of primer pairs were designed and tested: ECP79F (forward, targeting bases 79 to 96; 5′-GAAGCTTGCTTCTTTGCT-3′)-ECR620R (reverse, targeting bases 602 to 620; 5′-GAGCCCGGGGATTTCACAT-3′); ECB75F (forward, targeting bases 75 to 97; 5′-GGAAGAAGCTTGCTTCTTTGCTG-3′-ECR620R (reverse, described above); and ECA75F (forward, targeting bases 75 to 99; 5′-GGAAGAAGCTTGCTTCTTTGCTGAC-3′)-ECR619R (reverse, targeting bases 594 to 619; 5′-AGCCCGGGGATTTCACATCTGACTTA-3′). The optimal melting temperature and expected PCR product sizes for the primer pairs were as follows: ECP79F-ECR620R, 55°C and 541 bp; ECB75F-ECR620R, 59°C and 545 bp; and ECA75F-ECR619R, 60°C and 544 bp. The probe used in Southern hybridization experiments was S-D-Bact-0338-a-A-1 (previously referred to

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TABLE 1. Cultures used in these tests[a]

Category	Isolate[a]
Enteric bacteria	
Escherichia coli	UW8002 (CDC4102-72)
	UW8009
	UW8101
	UW8204 (WSLH 10555)
	UW8410
	UW8P39
	UW8001D (ATCC 12814)
Shigella spp.	*S. dysenteriae*, serotype 4, UW8P01
	S. dysenteriae, serotype 1 (WSLH isolate)
	S. flexnerii UW8P02
	S. sonnei UW8P15
Other enteric bacteria	*Citrobacter freundii* UW8606
	Enterobacter aerogenes UW8411 (ATCC 13048)
	Enterobacter nimipressuralis UW8103 (ATCC 09912)
	Enterobacter agglomerans UW8710 (ATCC 55046)
	Klebsiella pneumoniae UW8215 (WSLH 25400)
	Proteus vulgaris UW8068 (WSLH 58224)
	Salmonella serovar Typhimurium UW8P14
	Salmonella serovar Typhimurium UW8P40
Nonenteric gamma Proteobacteria	*Pseudomonas aeruginosa* UW9020 (ATCC 10145)

[a] All cultures were obtained from either the University of Wisconsin Department of Bacteriology (UW) or the Wisconsin State Laboratory of Hygiene (WSLH). Some cultures obtained from the UW collection are also on deposit at the American Type Culture Collection (ATCC) or the Centers for Disease Control and Prevention (CDC); the culture identifiers for the latter collections are given in parentheses for cross-reference.

as EUB338 [1]). This probe targeted a 16S rRNA gene sequence conserved in the domain *Bacteria* and occurring near the center of the PCR products generated by all primer pairs. The oligonucleotide was 5′ labeled with digoxygenin by the supplier (Sigma-Genosys, The Woodlands, Tex.).

PCR and hybridization protocols. PCR protocols were developed empirically for each primer set to obtain maximum selectivity (*E. coli* versus *Shigella* and other enteric bacteria) while retaining sensitivity (desired detection level of 10 fg to 1 pg, ca. 1 to 100 cell equivalents). Optimization focused on levels of primers, DNA polymerase, and $MgCl_2$ in the reaction mixture as well as thermal cycling programs. For ECP79F-ECR620R, the reaction mixture (50 μl, total volume) contained 1:10 dilution of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15 mM $MgCl_2$, 0.01% [wt/vol] gelatin), 200 μM each deoxynucleoside triphosphate, 0.6 μM primers, and an appropriate amount of template. The thermal cycling program consisted of a hot start (5 min, 94°C) before 1.25 U of AmpliTaq DNA polymerase (PE Biosystems, Foster City, Calif.) was added per reaction. The reaction was run for 40 cycles with 45-s denaturation (94°C), 45-s annealing (50°C), 1.5-min extension (72°C), and a final extension (5 min, 72°C). For ECA75F-ECR619R and ECB75F-ECR620R, reaction mixtures (50 μl, total volume) contained 1:10 dilution of 10× PCR buffer (100 mM Tris-HCl [pH 9.0], 500 mM KCl, 0.1% Triton X-100), 200 μM each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 0.4 μM primers, bovine serum albumin (40 μg/reaction), and an appropriate amount of template. The thermal cycling program consisted of a hot start (30 s, 94°C) followed by addition of 1.5 U of native *Taq* DNA polymerase (Promega, Madison, Wis.) per reaction. The thermal cycling program was run for 40 cycles of denaturation (45 s, 94°C) and annealing-extension (45 s, 72°C) and then a final extension (10 min, 72°C).

All of the reactions were done in thin-walled 0.5-ml Eppendorf tubes (USA/Scientific Plastics, Ocala, Fla.) and were

TABLE 2. Alignment of forward primers with the 16S rRNA gene of *E. coli*, *Shigella*, and other enteric bacteria

Source	Region from bp 75–99 of 16S rRNA gene (5′-3′)[a]																								GenBank Accession no.
ECA75F-ECP79F[b]	G	G	A	A	G	A	A	G	C	T	T	G	C	T	T	C	T	T	T	G	C	T	G	A	C	
E. coli																									
rrnA operon	G	G	A	A	G	A	A	G	C	T	T	G	C	T	T	C	T	T	T	G	C	T	G	A	C	CR01508
rrnB operon	G	G	A	A	G	A	A	G	C	T	T	G	C	T	T	C	T	T	T	G	C	T	G	A	C	CR04840
rrnC operon	G	G	A	A	**A**	**C**	A	G	C	T	T	G	C	T	**C**	**T**	T	T	**C**	G	C	T	G	A	C	X80723
rrnD operon	G	G	A	A	**A**	**C**	A	G	C	T	T	G	C	T	**G**	**T**	T	T	**C**	G	C	T	G	A	C	CR05849
rrnE operon	G	G	A	A	G	A	A	G	C	T	T	G	C	T	T	C	T	T	T	G	C	T	G	A	C	CR01512
rrnG operon	G	G	A	A	G	**C**	A	G	C	T	T	G	C	T	**C**	**T**	T	**C**	**G**	**C**	**T**	**G**	**A**	**C**	C	R01932
rrnH operon	G	G	A	A	G	A	A	G	C	T	T	G	C	T	T	C	T	T	T	G	C	T	G	A	C	CR01815
Shigella boydii	G	G	A	A	G	**C**	A	G	C	T	T	G	C	T	**G**	**T**	T	T	**C**	G	C	T	G	A	C	X96965
Shigella dysenteriae	G	G	A	A	G	**C**	A	G	C	T	T	G	C	T	**G**	C	T	T	T	G	C	T	G	A	C	X80680
	G	**A**	A	A	G	**C**	A	G	C	T	T	G	C	T	**G**	**T**	T	T	**G**	**C**	**T**	**G**	**A**	**C**	**G**	X96966
	G	**A**	A	A	G	**C**	A	G	C	T	T	G	C	T	**G**	C	T	T	T	G	C	T	G	A	C	AF207826
Shigella flexnerii	G	G	A	A	G	**C**	A	G	C	T	T	G	C	T	**G**	**T**	T	T	**C**	G	C	T	G	A	C	X80679
Shigella sonnei	G	G	A	A	**A**	**C**	A	G	C	T	T	G	C	T	**G**	**T**	T	T	**C**	G	C	T	G	A	C	X80726
Salmonella LT2	G	G	A	A	G	**C**	A	G	C	T	T	G	C	T	**G**	C	T	T	T	G	C	T	G	A	C	Z49264
Citrobacter freundii	**C**	**A**	**G**	A	G	**G**	A	G	C	T	T	G	C	T	**C**	C	T	T	**G**	G	**G**	T	G	A	C	M59291
Edwardsiella tarda	G	G	A	**G**	**A**	A	A	G	C	T	T	G	C	T	T	**T**	**C**	T	**C**	**C**	**G**	**C**	**T**	**G**	**A**	AF053975
Enterobacter aerogenes	**A**	**C**	A	**G**	**A**	**G**	A	G	C	T	T	G	C	T	**C**	**T**	**C**	**G**	**G**	G	**T**	**G**	**A**	**C**	**G**	AJ001237
Enterobacter nimipressuralis	**A**	**C**	A	**G**	**A**	**G**	A	G	C	T	T	G	C	T	T	C	T	T	**C**	**A**	**G**	**G**	G	**T**	**G**	Z96077
Klebsiella pneumoniae	**A**	**C**	A	**G**	**A**	**G**	A	G	C	T	T	G	C	T	**C**	**T**	**C**	**G**	**G**	G	**T**	**G**	**A**	**C**	**G**	Y17656
Proteus vulgaris	G	G	A	**G**	**A**	A	A	G	C	T	T	G	C	T	T	**T**	**C**	T	T	G	C	T	G	A	C	J01874
Pseudomonas aeruginosa	G	**A**	A	**G**	G	**G**	A	G	C	T	T	G	C	T	**C**	C	T	**G**	**G**	**A**	**T**	**C**	**A**	**G**	C	AJ249451

[a] Boldface bases are mismatches.
[b] Primers ECA75F and ECP79F cover bases 75 to 99 and 79 to 96, respectively.

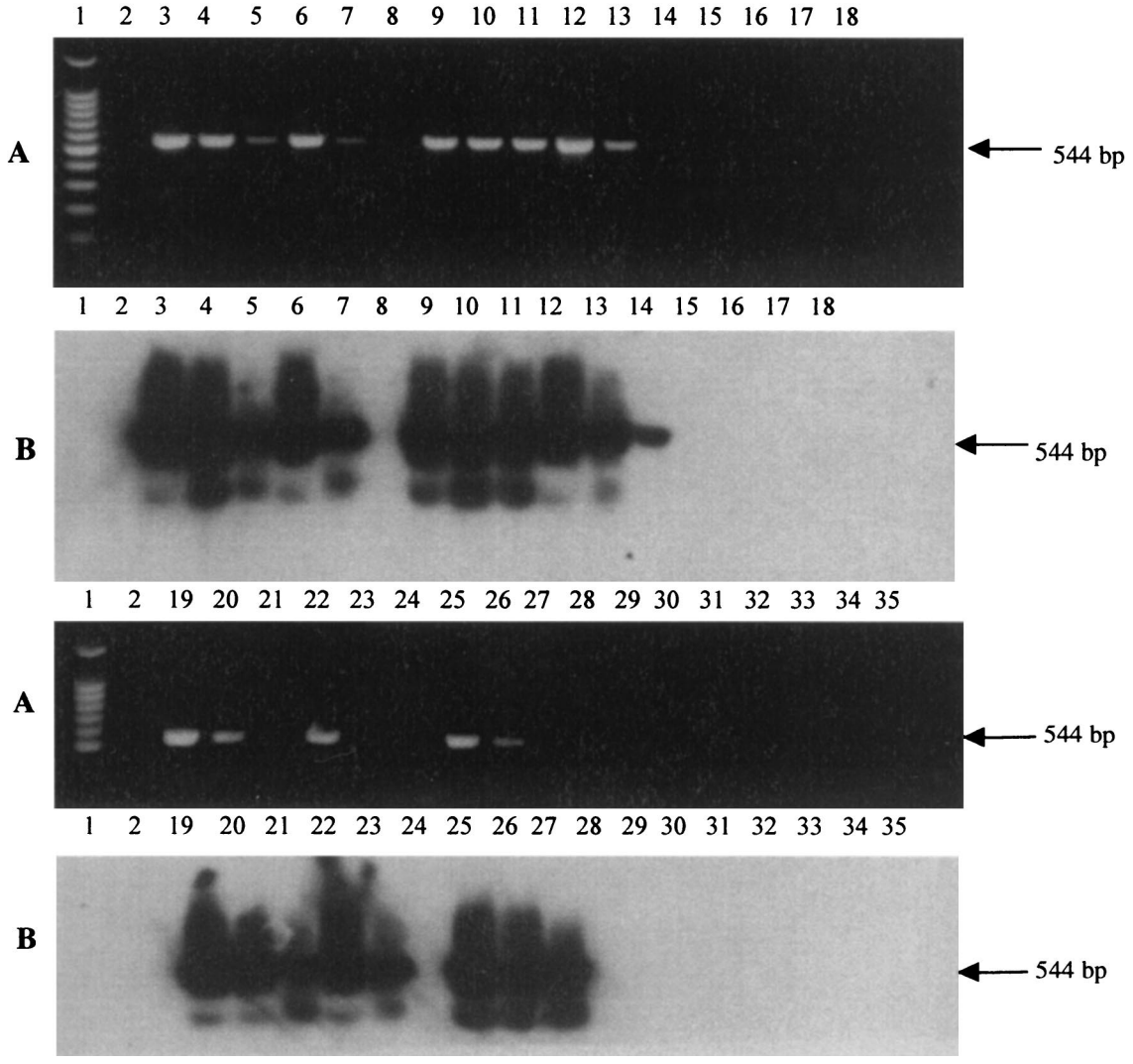

FIG. 1. Specificity of primer pair ECA75F-ECR619R in the optimized PCR for amplification of *E. coli* 16S rRNA. (A) Agarose gel separation of PCR mixtures; (B) Southern hybridization of the gel in panel A to EUB338. Lanes: 1, 100-bp DNA ladder; 2, no template; 3 to 5, 1 ng, 100 pg, and 10 pg of *E. coli* UW8101 template; 6 to 8, 1 ng, 100 pg, and 10 pg of *E. coli* UW8002 template; 9 to 11, 1 ng, 100 pg, and 10 pg of *E. coli* UW8204 template; 12 to 14, 1 ng, 100 pg, and 10 pg of *E. coli* UW8009 template; 15 and 16, 1 ng and 100 pg of *Salmonella* serovar Typhimurium UW8P14 template; 17 and 18, 1 ng and 100 pg of *Salmonella* serovar Typhimurium UW8P40 template; 19 to 21, 1 ng, 100 pg, and 10 pg of E. coli UW8410 template; 22 to 24, 1 ng, 100 pg, and 10 pg of *E. coli* UW8P39 template; 25 to 27, 1 ng, 100 pg, and 10 pg of *E. coli* UW8001D template; 28 and 29, 1 ng and 100 pg of *Shigella dysenteriae* UW8P01 template; 30 and 31, 1 ng and 100 pg of *Shigella dysenteriae* WSLH template; 32 and 33, 1 ng and 100 pg of *Shigella flexnerii* UW8P02 template; 34 and 35, 1 ng and 100 pg of *Shigella sonnei* UW8P15 template. Strain designations refer to those given in Table 1.

assembled in a CloneZone unit (USA/Scientific Plastics) to prevent airborne contamination or template carryover from previous experiments. Thermal cycling was done with a Delta-Cycler I unit (Ericomp, San Diego, Calif.). After amplification, 10 μl of each PCR mixture was analyzed by electrophoresis (8 mV/cm, 1 h) in ethidium bromide-stained agarose (2%, wt/vol) gels. A 100-bp ladder (Promega) was included for molecular weight estimation. Gels were placed on an UV transillumination unit (Fotodyne, Hartland, Wis.) for band visualization and photography. Southern hybridization to DNA immobilized on Hybond N^+ charged nylon membranes (Amersham, Arlington Heights, Il.) was done according to the manufacturer's directions. Bound probe was detected by chemiluminescence using the Genius system and CPD-Star substrate solution (Boehringer Mannheim, Indianapolis, Ind.). The membrane was then exposed to X-ray film (Eastman Kodak, Rochester, N.Y.), and the film was developed according to the manufacturer's instructions.

Detecting *E. coli* in soil by PCR. Soil was sampled from experimental plots located at the Arlington Research Station (Arlington, Wis.) and at the Lakeland Agricultural Complex (Lakeland, Wis.). The Arlington soil was a Plano silt loam (typic argiudoll; pH 6.9, 48 g of organic matter kg^{-1}), while that from Lakeland was a Griswold silt loam (aquic argiudoll; pH 7.0, 42 g of organic matter kg^{-1}). At both sites, the plots were established as continuous corn and had not been amended with animal manure for at least 12 years. The samples collected were kept on ice for transport to the laboratory and then stored frozen at −20°C. Prior to use, the soils were sieved through 2-mm mesh screen and their moisture contents were determined. DNA was extracted by a freeze-thaw method essentially as described by Tsai and Olson (22) except that

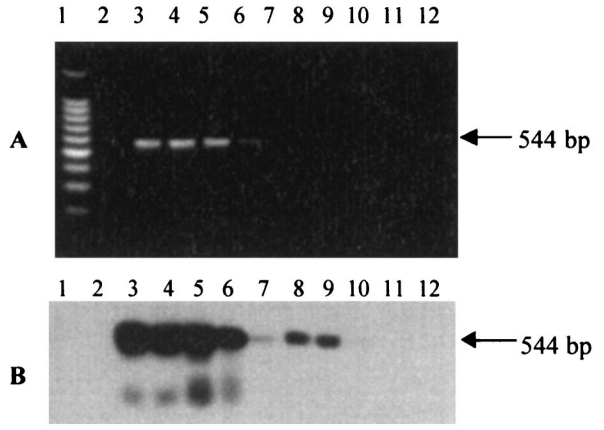

FIG. 2. Sensitivity of primer pair ECA75F-ECR619R in the optimized PCR for amplification of *E. coli* 16S rRNA DNA template. (A) Agarose gel separation of PCR mixtures; (B) Southern hybridization of the gel in panel A to EUB338. Lanes: 1, 100-bp DNA ladder; 2, no template; 3 to 12, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg, 500 fg, 100 fg, 50 fg, 10 fg, and 5 fg of *E. coli* genomic DNA.

$CaCl_2$ (final concentration of Ca^{2+}, 90 mM) was substituted for NaCl in the lysis solution. The extracts were further purified by a Wizard kit (Promega). Use of Ca^{2+} in the lysis solution precipitates humic materials and makes cleanup by the Wizard kit more effective. Concentrations of Ca^{2+} in extracts were 690 μM or less as determined by atomic absorption spectroscopy and shown not to interfere with the PCR (21). DNA purity and concentrations were determined by UV absorbance at 260 and 280 nm and by gel image analysis with NIH Image version 1.55 software (National Institutes of Health).

Two groups of experiments were done to examine the efficiency of the PCR method for amplification of *E. coli* 16S rRNA gene sequences from soil. In the first, DNA was extracted from the Arlington and Lakeland soils, and an aliquot of each purified extract (containing approximately 100 ng of DNA) was then spiked with either 1 ng or 1 pg of *E. coli* genomic DNA and used for PCR. In the second experiment, Lakeland soil (4 g, wet weight) was inoculated with *E. coli* at densities ranging from 7×10^4 to 7×10^7 cells g^{-1} (each inoculum level established in triplicate) and then incubated for 1 h. The incubation time in soil was kept relatively short to allow interaction between cells and soil particles while minimizing the extent to which inoculum densities were altered by population growth or death. Plate counts were made on Levine eosin-methylene blue (Difco Laboratories, Detroit, Mich.) agar and *Shigella-Salmonella* agar (Difco). Plate counts on the two media were not significantly different and confirmed that numbers of cells inoculated into and recovered from soil after 1 h incubation were similar. DNA was extracted from the soils as described above. The amount of DNA recovered from the noninoculated soil was approximately 7 μg g^{-1}, while that extracted from the inoculated soils ranged from ca. 7 to 10 μg g^{-1}. A total of 10 ng of DNA from each extract was used in the PCR tests.

Primer design and PCR development. The primary criterion for selection of PCR primers was the identification of 16S rRNA gene sequences that could be used to distinguish *E. coli* from its closest relative, *Shigella*. The 16S rRNA gene of *E. coli* differs from that of *Shigella flexneri*, *S. sonnei*, *S. boydii*, and *S. dysenteriae* by 4, 5, 8, and 17 bases, respectively (7, 26). When the *E. coli* and *Shigella* 16S rRNA genes are aligned, only two areas (both within hypervariable regions) have more than two mismatches over 20 contiguous nucleotides, the length of a typical PCR primer. One region spans bases 75 to 100 and was chosen as the target site for the forward primer because of its relatively high number of mismatches with *Shigella* (Table 2). The other region has substantial disagreement between reported *Shigella* sequences and was deemed unsuitable. Within the region from bp 75 to 100, a number of primers targeting various stretches of nucleotides were considered, and one (ECP79F) that presented the best balance between the desired selectivity and potential unfavorable secondary structure characteristics was chosen for further study. The reverse primer was ultimately targeted to bases 594 to 620, as mismatches in this region provide selection against other *Proteobacteria* that may cross-react with the forward primer.

The first set of tests used the primer pair ECP79F-ECR620R and the 40-cycle protocol. Amplification was very effective, and the amount of product accumulated from as little as 50 fg of *E. coli* genomic DNA (five genomic equivalents) was sufficient to allow easy visualization in ethidium bromide-stained agarose gels. However, in these tests a product of the expected size was also amplified from the water blanks. We subsequently determined that the latter originated from *E. coli* DNA present in the recombinant polymerase AmpliTaq and found that this problem could be eliminated by treatment of AmpliTaq (DNase digestion, UV light irradiation), use of purified recombinant polymerase (AmpliTaq LD), or using *Taq* DNA polymerase isolated from *Thermus aquaticus*. The latter was the most cost- and time-efficient and was used as standard practice.

Use of ECP79F-ECR609 combined with native *Taq* polymerase in the extended PCR program achieved the desired sensitivity. However, subsequent experiments demonstrated that the PCR protocol was not adequately selective for *E. coli*. In these specificity tests, there was amplification of the expected 541-bp product from other *Enterobacteriaceae* (*Citrobacter freundii*, *Proteus vulgaris*, *Enterobacter aerogenes*, and *Enterobacter nimipressuralis*) and even from *Pseudomonas aeruginosa*. Amplification could have been driven by the reverse primer alone, but this would result in linear amplification and probably not yield the amount of product observed. The annealing conditions used were optimal for the primers, so it was unlikely that amplification resulted from nonspecific annealing to and extension of regions outside the 16S rRNA gene. Selectivity was not improved by reducing template concentrations from 100 to 1 ng, although at lower levels the amount of PCR product amplified from *E. coli* template was greater than that from nontarget organisms. A variety of modifications to the PCR program's annealing temperature and the reaction mixture composition (e.g., inclusion of dimethyl sulfoxide or mod-

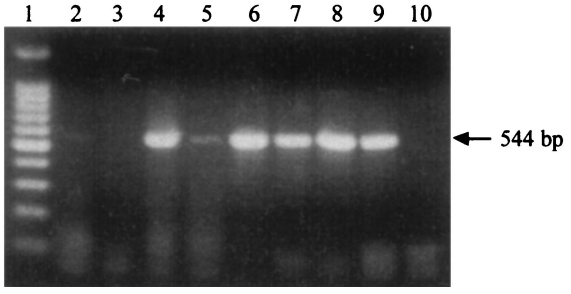

FIG. 3. Amplification of *E. coli* 16S rRNA from DNA extracted from Arlington (A) and Lakeland (L) soil and spiked with either 100 ng or 1 pg of *E. coli* genomic DNA. Lanes: 1, 100-bp DNA ladder; 2, A, nonspiked; 3, L, nonspiked; 4, A, 1-ng spike; 5, A, 1-pg spike; 6, L, 1-ng spike; 7, L, 1-pg spike; 8, 1 ng of *E. coli* genomic DNA; 9, 1 pg of *E. coli* genomic DNA; 10, no template.

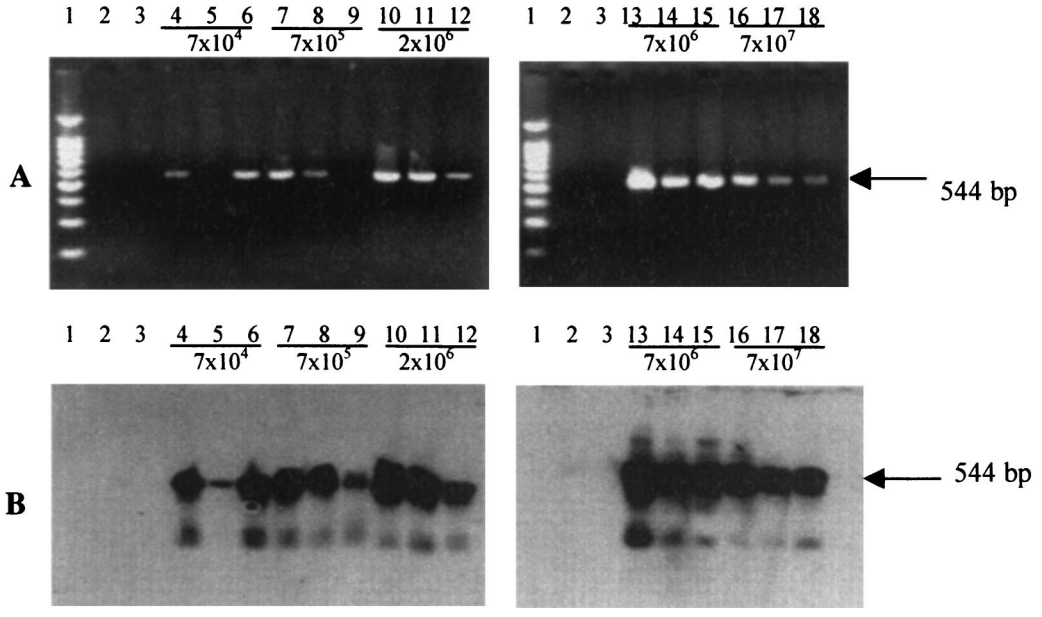

FIG. 4. Amplification of *E. coli* 16S rRNA from nonsterile soil inoculated with various densities of *E. coli*. (A) Ethidium bromide-stained agarose gel; (B) Southern hybridization of gel from panel A to EUB338. Lanes: 1, 100-bp DNA ladder; 2, no template; 3, 10 ng of DNA extract template from nonsterile, nonseeded soil sample; 4 to 18, 10 ng of DNA extract template from nonsterile soil samples seeded with stationary-phase *E. coli* at 7×10^4, 7×10^5, 2×10^6, 7×10^6, and 7×10^7 CFU g^{-1}, respectively.

ulation of $MgCl_2$ level) were also tested, but none eliminated the cross-reactivations.

The main constraint for improving PCR selectivity was restriction to a single region within the 16S rRNA gene for which *E. coli*-specific primers could be targeted. The strategy adopted was to increase stringency by increasing the annealing temperature and by designing longer forward primers (ECA75F and ECB75F) expected to have greater stability at elevated temperatures. Initial PCR experiments with ECA75F-ECR619R and ECB75F-ECR620R were run using the optimal annealing temperatures for each primer pair. Both sets showed good sensitivity, giving detectable amplification from *E. coli* DNA template levels of at least 500 fg, but cross-reaction with *Shigella* and other enteric bacteria persisted. Selective amplification of *E. coli* was attained when the annealing temperature was increased to 72°C, but only with ECA75F-ECR619R. However, while the increase in annealing temperature gave the desired selectivity, it had a serious negative impact on the sensitivity as *E. coli* template levels needed to be in the nanogram range for detection.

Subsequent tests focused on increasing sensitivity of the PCR by altering amounts of reaction mixture components. First, the $MgCl_2$ concentration was reexamined for its effects on the efficiency of amplification from low template levels (all reactions done in a total volume of 50 μl). Amplification from high amounts of template (100 ng, ca. 10^8 copies) was efficient with $MgCl_2$ levels ranging from 1.0 to 3.0 mM. However, with low amounts of template (500 fg), amplification was efficient only between 2.0 and 2.5 mM $MgCl_2$. Next, the combination of *Taq* DNA polymerase and $MgCl_2$ levels that gave the best balance in sensitivity and selectivity (*E. coli* versus *S. sonnei*) at low template levels (500 fg, ca. 500 copies) was examined and identified as 1.5 U of *Taq* and 2.0 mM $MgCl_2$. The selectivity of the optimized PCR protocol was then verified with a battery of different *E. coli*, *Shigella*, and *Salmonella* isolates (Fig. 1). The detection limit of the optimized PCR was established by Southern hybridization as 100 fg of template, which is equivalent to ca. 10 cells of *E. coli* (Fig. 2).

Specificity was also examined by using BLAST to search GenBank for sequences similar to ECA75F and ECR619R. *Vibrio gazogenes* and *Enterobacter gergoviae* have single mismatches to the 5′ end of ECA75F but several mismatches to ECR619R. There were 15 exact matches to ECA75F, 13 of these were bacteria of the *Pasteurellaceae* family (various species or subspecies of *Haemophilus*, *Mannheimia*, and *Pasteurella*), and two were unknown γ-*Proteobacteria*. All of these organisms, however, had a number of mismatches to ECR619R. The greatest similarity to our primers was with *Erwinia psidii*, *Pseudomonas flectens*, *Salmonella enterica* serovar Waycross, and *S. enterica* serovar Chingola, all of which had either a single mismatch or single point deletion with ECA75F and were exact matches to ECR619R. This last group would theoretically pose the greatest potential for cross-reaction. However, these organisms are probably not typical water or soil inhabitants (*E. psidii* and *P. flectens* are described as originating from Brazilian guava fruit and an Australian bean, respectively), nor are they common residents of the human digestive tract. Thus, based on both empirical and theoretical testing, the method is expected to be reliable for selective detection of *E. coli* in soil or water contaminated by human wastes.

We evaluated the optimized PCR protocol for detection of *E. coli* in environmental samples. Soil was selected as it is an important reservoir for *E. coli*, and its chemical complexity provides a good test of the method's robustness. Initial tests to examine matrix effects on the PCR method were done with DNA extracted from soil and spiked with *E. coli* genomic DNA. These experiments showed that method performance with a heterogeneous mixture was similar to that obtained with pure *E. coli* genomic DNA: as little as 1 pg of *E. coli* template added to the PCR mixture gave sufficient product to detect by gel electrophoresis (Fig. 3). The sensitivity was comparable to that obtained using pure *E. coli* genomic DNA (Fig. 2). In tests

to evaluate detection of *E. coli* DNA extracted from soil inoculated with various densities of *E. coli* cells, amplification was positive from all DNA extracts, and the expected product was detectable using agarose gels or Southern hybridization (Fig. 4). Furthermore, the lack of amplification from noninoculated soil, which likely had an indigenous population of enteric bacteria (e.g., *Klebsiella*), suggests that the method provided the necessary selectivity. The main difference between treatments was that with extracts prepared from the two lowest-inoculum densities, PCR products were sometimes detected only by hybridization. This probably reflects variability in *E. coli* DNA recoveries. Because many variables affect DNA extraction from bacterial cells in soil (e.g., soil type, residence time of cells in the soil, and soil biomass level), the detection limits achievable by this method will vary on a case-by-case basis.

In summary, a sensitive and robust PCR protocol was developed that discriminated *E. coli* from *Shigella* and other enteric bacteria based on selective amplification of 16S rRNA gene sequences. To the best of our knowledge, this is the first report of a PCR protocol based on amplification of a 16S rRNA that effectively distinguishes *E. coli* from these closely related bacteria. For environmental analysis, the extent to which the method's sensitivity of ca. 10 genomic equivalents can be exploited is controlled primarily by the efficiency with which DNA is extracted from the target organism, concentrated, and purified for the samples used in the PCR. For high-volume sample analysis, the protocol could be coupled with rapid methods for PCR product detection such as fluorogen labeling or enzyme-linked immunosorbent assay.

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