Molecular Identification of Bacteria by Fluorescence-Based PCR–Single-Strand Conformation Polymorphism Analysis of the 16S rRNA Gene

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PCR–single-strand conformation polymorphism (PCR-SSCP) analysis is a rapid and convenient technique for the detection of mutations and allelic variants. We have adapted this technique for the identification of bacteria by PCR with fluorescein-labeled primers chosen from the conserved regions of the 16S rRNA gene flanking a variable region. The PCR product was denatured, separated on a nondenaturing gel, and detected by an automated DNA sequencer. The mobility of the single-stranded DNA is sequence dependent and allows the identification of a broad panel of bacteria. A single nucleotide difference in the amplified region was sufficient to obtain different PCR-SSCP patterns. The simultaneous amplification of multiple polymorphic regions by multiplex PCR with subsequent multiplex SSCP increased the discriminatory power of PCR-SSCP. A broad range of gram-negative and gram-positive bacteria were tested by PCR-SSCP, including, e.g., *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Haemophilus* spp., *Neisseria* spp., *Staphylococcus* spp, *Streptococcus* spp., *Enterococcus* spp., and *Bacillus* spp. In total, a panel of 178 strains of bacteria representing 51 species in 21 genera was examined. Although a limited number of strains from each species were tested, the strains tested gave species-specific patterns, with only one exception: *Shigella* species were indistinguishable from *E. coli*. PCR is a sensitive technique; as few as 10 CFU of *E. coli* was sufficient to produce PCR-SSCP patterns suitable for identification. The whole fluorescence PCR-SSCP procedure takes approximately 8 h for the detection and identification of low numbers of bacteria. Fluorescence PCR-SSCP seems to be a promising method for the differentiation of a broad range of pathogens found in usually sterile clinical sites, such as blood and cerebrospinal fluid.

The use of PCR as a diagnostic tool for the detection of pathogens has been expanding during the past few years. Most PCR detection systems described are based on the use of PCR with genus- or species-specific primers (20). These specific detection systems are not suitable for the detection of pathogens in usually sterile clinical sites, such as blood and cerebrospinal fluid, since a broad spectrum of pathogens can be expected. A more universal method for the detection of microorganisms is based on the use of broad-range conserved primers for PCR (7, 23). Although this format is able to detect a pathogen in a usually sterile site, the identity of the microorganism remains unknown. Probe hybridization requires an extensive panel of specific probes (12), and sequencing (3, 5) is not a suitable tool for routine diagnostics. Amplification of the 16S/23S rRNA gene spacer region by using conserved primers generated multiple PCR products with size distribution profiles that might be used for the differentiation of bacterial species (16).

Recently, we have presented a new, general approach for directly determining the identities of bacteria that is based on the principle of single-strand conformation polymorphism (SSCP) electrophoresis (19) of PCR-amplified 16S rRNA gene fragments (22). PCR of bacterial cell lysates was performed with conserved primers flanking a variable region. The amplified PCR product was denatured to two single-stranded DNAs (ssDNAs) and subjected to nondenaturing polyacrylamide gel electrophoresis. SSCP patterns were detected by silver staining the nucleic acids (1). The mobility of the ssDNA is sequence dependent and could be used to identify the unknown bacteria. The feasibility of the technique was demonstrated for a broad panel of gram-negative and gram-positive bacteria. We tested over 100 strains of bacteria representing 15 genera and 40 species and obtained species- or genus-specific patterns.

However, in conventional SSCP, the examination of patterns is visual and correction for lane-to-lane and gel-to-gel variation is not possible. Also, silver staining of nucleic acids has a poor reproducibility. PCR-SSCP with fluorescence-labeled primers and analysis on an automated DNA sequencer could overcome these disadvantages (11, 17). The separated strands are detected during electrophoresis as laser-excited fluorescence at the bottom of the gel, and differences in the mobilities of the ssDNAs are detected as differences in retention times. The use of an automated sequencer permits strict control of the gel temperature, which is an important variable when PCR-SSCP is performed (13). Furthermore, the sequencer is coupled to a computer, which enables direct data management by computer.

In this report, we describe the use of fluorescence PCR-SSCP, using conserved 16S rRNA gene primers, for the detection and identification of bacteria. We focused in this study on the identification of a broad range of bacterial pathogens, including bacterial species causing bacteremia. The general features of fluorescence PCR-SSCP are described, and the application of (multiplex) fluorescence PCR-SSCP for the discrimination of 178 bacterial strains is shown.

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TABLE 1. Primers located in the 16S rRNA gene
used for PCR-SSCP

Oligonucleotide (sequence)	Position[a]	Fragment size (bp)
P11P (5′-GAG GAA GGT GGG GAT GAC GT)	1173–1192	217
P13P (5′-AGG CCC GGG AAC GTA TTC AC)	1370–1389	
ER10 (5′-GGC GGA CGG GTG AGT AA)	103–119	255
ER11 (5′-GAC TGC TGC CTC CCG TAG) or	341–357	
ER11G (5′-G GAC TGC TGC CTC CCG TAG)		
ER14 (5′-GCT AAC TCC GTG CCA GCA)	506–523	305
ER15 (5′-GCG TGG ACT ACC AGG GTA TC)	791–810	

[a] Based on the *E. coli* sequence as a reference (GenBank accession no.
JO1695).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used were either clinical isolates collected from the University Hospital Utrecht (Utrecht, The Netherlands), clinical isolates from the National Institute for Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands), or isolates obtained from the American Type Culture Collection. After observation of colony morphology and Gram staining, the clinical isolates were identified either by the automated Vitek system (bioMérieux Vitek Inc., Hazelwood, Mo.) or by the Enterotube II system (Roche Diagnostic Systems, Nutley, N.J.) in combination with the API Staph or API 20S system (bioMérieux Vitek Inc.). For both systems, supplementary well-established biochemical tests were performed (14).

Bacterial strains were cultured overnight at 37°C on blood agar plates, scraped from the plates, and lysed in 10% Chelex 100 (Bio-Rad, Richmond, Calif.)–0.03% sodium dodecyl sulfate–1% Tween 20–1% Nonidet P-40 for 5 min at 95°C. After centrifugation for 10 s, 5 μl of the supernatant was directly used for PCR amplification.

The number of CFU was determined by plating on blood agar and culturing for 16 h at 37°C.

DNA amplification. The (multiplex) PCR mixture (50 μl) contained 50 mM Tris-HCl (pH 9.0), 50 mM KCl, 7 mM MgCl$_2$, 2 mg of bovine serum albumin per ml, 16 mM (NH$_4$)$_2$SO$_4$, 100 μM each deoxynucleoside triphosphate (dNTP), 0.4 μM each primer, and 0.1 U of Super-*Taq* polymerase (HT Biotechnology, Cambridge, England). The target DNA sequence was the 16S rRNA gene, and conserved sequences were selected for oligonucleotide primers (Table 1). The primers were either obtained commercially (Pharmacia, Roosendaal, The Netherlands) or synthesized on a Pharmacia Gene Assembler Plus (Pharmacia LKB, Upsala, Sweden) for primers labeled with a 5′-fluorescein phosphoramidite, which was characterized by an extended spacer arm (Fluorescein-ON; Clonetech, Palo Alto, Calif.).

The PCR was performed for 25 cycles of 1 min at 94°C, 10 s at 72°C, and 1 min at 55°C on a DNA thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). After amplification, 5 μl of the amplified product was run on a 1.5% agarose gel in 0.5× TBE. DNA bands were detected by ethidium bromide staining and visualized by UV light photography. Other thermostable DNA polymerases used were *Tth* (Boehringer, Mannheim, Germany) Ampli-*Taq* (Perkin-Elmer Cetus), Ampli-*Taq* Stoffel fragment (Perkin-Elmer Cetus), and *Pfu* (Stratagene, La Jolla, Calif.) with buffers as supplied by the manufacturers.

For determining the sensitivity of PCR-SSCP, Ampli-*Taq* Stoffel fragment was used in the buffer supplied by the manufacturer with the addition of 10 mM MgCl$_2$. The whole PCR mixture, without the target DNA, was incubated with DNase I (Boehringer) for 15 min at room temperature and then subjected to DNase I inactivation by an incubation of 10 min at 95°C. Five microliters of cell lysate was then added to the PCR mixture, and PCR was subsequently performed for 35 cycles.

PCR product polishing. After PCR amplification, the mixture was precipitated with ethanol, resuspended in distilled water, and incubated for 1 h in the appropriate DNA polymerase buffer with 100 μM dNTPs and 5 U of DNA polymerase. Both the *Pfu* DNA polymerase (Stratagene) and T4 DNA polymerase (Boehringer) enzymes possess 3′-to-5′ proofreading exonuclease activity, according to the instructions of the manufacturers. Incubation was at 72°C for *Pfu* DNA polymerase or at 37°C for T4 DNA polymerase. The reaction was stopped by the addition of sequencing sample buffer (5 mM EDTA and 0.05% blue dextran in formamide), and the products were subsequently analyzed on an SSCP gel.

SSCP electrophoresis on an automated sequencer. SSCP electrophoresis was performed on a Pharmacia A.L.F. DNA sequencer. After thermal cycling, 0.5 μl of the PCR mixture was added to 10 μl of sequencing sample buffer and heated for 3 min at 95°C. The denatured DNA was then placed directly on ice for 5 min before being loaded onto the gel. The SSCP gel composition was 0.5× MDE gel (J. T. Baker, Phillipsburg, N.J.), 10% glycerol, and 0.6× Tris-borate-EDTA. Electrophoresis was done at 30°C with 30 W for 360 min for a gel of 20 by 34 by

0.05 cm. Electrophoresis was evaluated with the Fragment Manager Software (Pharmacia LKB).

Manual SSCP electrophoresis. SSCP electrophoresis was performed at room temperature with an overnight run on a Bio-Rad Protean II electrophoresis apparatus on a gel of 16 by 20 cm with 0.75-mm spacers at 5 W. The gel matrix and running buffer were the same as those for the automated sequencer. Electrophoresis and detection of the nucleic acids by silver staining were as previously described (22).

Internal control for automated SSCP electrophoresis. Two internal controls were added to each SSCP sample lane, with the toxin B gene of *Clostridium difficile* as the target sequence. PCR was performed with a toxigenic *C. difficile* strain (ATCC 9689) with PCR conditions as described above. A multiplex PCR was performed with one fluorescein-labeled primer (5′-GTC AGA GAA TAC TGT AGT CG, positions 508 to 527) (2) and two unlabeled primers, generating either a 150-bp fragment (fragment I) (primer 5′-CTT TAG CTC TAA TAC TTC TG, positions 638 to 657) or a 257-bp fragment (fragment II) (primer 5′-CTA TTT ACA TCT TTC CAT TG, positions 755 to 764). For each lane, 0.5 μl of the PCR product was added to the sequencing sample buffer as an internal control. The peak positions of the curves from the shorter ssDNA fragment I and larger fragment II were set arbitrarily at 100 and 200, respectively, in the Fragment Manager Software. All retention times were correlated to those for the internal standards.

RESULTS

Fluorescence PCR-SSCP. PCR amplification with universal bacterial primers is hampered by low-level DNA contaminations in the PCR reagent mixture, especially in *Taq* DNA polymerase (21). Even the low-DNA *Taq* polymerase preparation still contains low levels of DNA contaminants (18). A PCR of 25 cycles did not show contaminating DNA in the controls without added DNA by agarose gel electrophoresis or SSCP analysis. When a greater number of cycles was used, PCR components had to be treated to reduce the amounts of contaminating DNA. Therefore, we performed a short DNase I treatment for the whole PCR mixture, excluding the target DNA, followed by heat inactivation of the DNase (22). This protocol required a high magnesium concentration (7 to 10 mM) (4) and was feasible only in combination with Super-*Taq* and Ampli-*Taq* Stoffel fragment DNA polymerases. With this pre-PCR sterilization protocol included, the sensitivity of the fluorescein PCR-SSCP was investigated. Serial dilutions of *Escherichia coli* cells were made and subjected to a 35-cycle PCR with 5′-fluorescein-labeled primers (P11P and P13P), and 5 μl of PCR product was applied to an SSCP gel on an automated sequencer. The resulting electropherogram (Fig. 1) is normalized by using the internal-standard fragments to correct for the lane-dependent mobility. The mobility profile resulted in two major bands representing the two ssDNAs amplified by PCR. As few as 10 CFU of *E. coli* resulted in SSCP patterns suitable for identification.

The mobilities of the ssDNAs on a manual SSCP electrophoresis gel were altered when 5′-fluorescein-labeled primers instead of nonlabeled primers, which were used in a previous study (22), were used for PCR amplification (data not shown). In addition, we studied the effect of the spacer length between the primer and the fluorescein label. FLU1 labeled primers carry the normal spacer arm, whereas FLU2 labeled primers carry an extended spacer arm. A small difference in retention times was observed for the two fluorescein-labeled primers P11P and P13P; e.g., the *E. coli* relative retention times for FLU1 primers were 135.1 and 122.0, while FLU2 primers showed retention times of 135.6 and 122.5 (results are the means of three independent experiments). Comparison of both types of fluorescein-labeled primers in PCR-SSCP on an automated sequencer for a larger panel of bacteria showed similar resolutions for both types of primers. Further research was performed with the commercially available fluorescein primers with a normal spacer arm (FLU1).

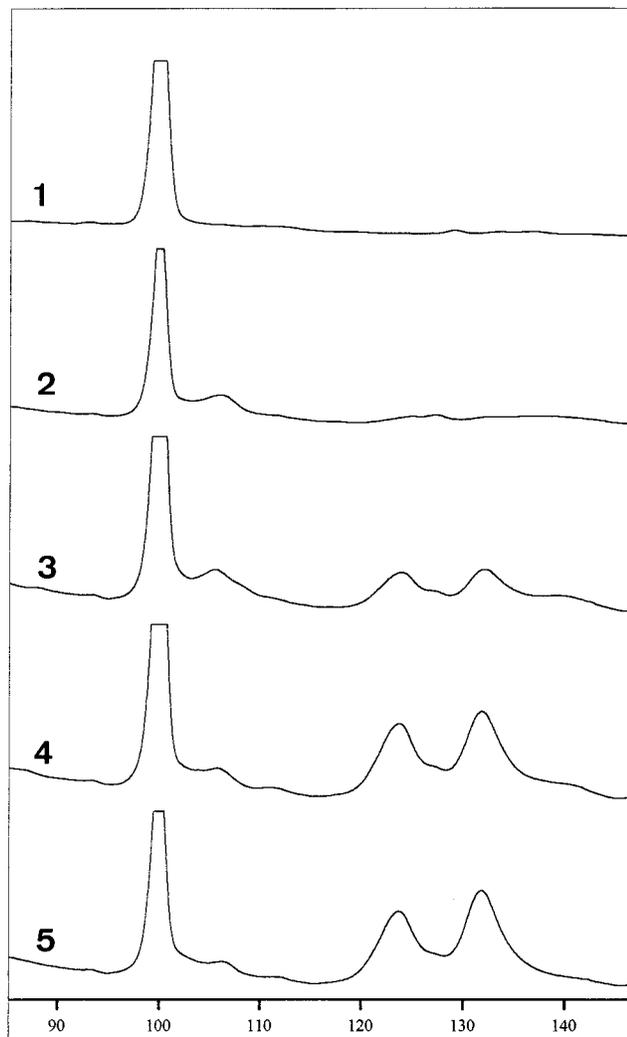Automated PCR-SSCP enables strict temperature control of

FIG. 1. Sensitivity of fluorescence PCR-SSCP with P11P-P13P. A serial dilution of *E. coli* cells ranging from 0 to 100 CFU was tested with a 35-cycle PCR. Curves 1 to 5, 0, 3, 10, 30, and 100 CFU of *E. coli* cells, respectively. Retention times are correlated to those for the internal controls, which were assigned values of 100 and 200.

electrophoresis, an important variable in PCR-SSCP, and in each lane internal controls can be added to increase reproducibility. To investigate the reproducibility of the automated SSCP, the day-to-day variation and lane-to-lane variation for *E. coli* SSCP patterns were investigated. When 20 different *E. coli* strains were tested on the same gel, automated PCR-SSCP proved to be quite accurate, with a lane-to-lane variation of less than 0.15%. The day-to-day variation for 5 consecutive days was less than 0.5%. The same numbers of bands were reproducibly observed. In some cases multiple SSCP bands from one ssDNA were consistently observed, reflecting the fact that an ssDNA possesses multiple conformations during migration on a nondenaturing gel.

Effect of different thermostable DNA polymerases on SSCP patterns. PCR amplification by different thermostable DNA polymerases might influence SSCP patterns because of different enzymatic characteristics of the polymerases. Therefore, the PCR-SSCP patterns obtained for the *Taq*, *Tth*, and *Pfu* DNA polymerases with primer set P11P-P13P were investigated. Strikingly, a minor difference between *Taq* and *Pfu*

DNA polymerases was observed for both *E. coli* and *Staphylococcus epidermidis* cell lysates. One explanation might be the terminal deoxynucleotide transferase activity of *Taq* DNA polymerase, which is mostly limited to the extension of a single nucleotide, predominantly a single A nucleotide, whereas *Pfu* DNA polymerase possesses 3′-to-5′ exonuclease proofreading activity. This hypothesis was tested by polishing the PCR fragments with *Pfu* and T4 DNA polymerase; both enzymes possess 3′-to-5′ exonuclease proofreading activity in the presence of dNTPs (10). Treatment of *Taq* DNA polymerase-amplified PCR products with either T4 DNA polymerase or *Pfu* DNA polymerase resulted in SSCP patterns similar to those obtained with *Pfu* DNA polymerase (Table 2). Thus, a single overhanging 3′ nucleotide has a significant effect on the retention times of the ssDNAs.

PCR-SSCP for bacterial identification. PCR products obtained from 119 bacterial strains belonging to 27 species in 9 genera were tested with the primer set P11P-P13P. Twenty different patterns were observed; in most cases these were species-specific patterns, and in some cases they were genus-specific patterns. For example, species-specific patterns were observed for *Enterobacter* and *Klebsiella* spp., and genus-specific patterns were observed for *Proteus* spp. The closely related *E. coli*, *Shigella*, and *Salmonella* species all gave identical PCR-SSCP patterns. PCR-SSCP with primer set P11P-P13P gave marginal differences in retention times for some species, which hampered the identification of species.

To increase the resolution of PCR-SSCP, an additional conserved primer set, ER10-ER11, was developed for use in a multiplex fluorescence PCR-SSCP. For example, the P11P-P13P amplification products with *Citrobacter* species showed a minor difference between the patterns of *C. freundii* and the conserved patterns of *C. diversus* and *C. amalonaticus*, whereas the second primer set, ER10-ER11, resulted in three distinct species-specific patterns (Fig. 2). Also, PCR-SSCP with these two primer sets enabled the distinct discrimination of all *Listeria* species (species-specific patterns for *L. innocua*, *L. monocytogenes*, *L. ivanovii*, *L. murray*, *L. welshimeri*, and *L. seeligeri* were observed), whereas the use of either primer set alone did not result in species-specific patterns. Fluorescence-based PCR-SSCP was capable of reproducibly detecting a 2-nucleotide difference between *L. innocua* and *L. ivanovii* in the amplified region of P11P-P13P (9). For some species the fluorescence signal of the ssDNA extended from the fluorescein-labeled ER11 primer was very weak. This reduction of signal was overcome by the insertion of an extra G base at the 5′ end of the primer, as described by Makino et al. (17). Also, this single addition of a base altered the retention times slightly because of the presence of an extra base.

When we first tested the applicability of primer set ER10-

TABLE 2. Effect of post-PCR modification on SSCP patterns of *E. coli* with primer set P11P-P13P

DNA polymerase used in PCR	DNA polymerase used for post-PCR treatment	Relative retention times[a]
Taq	None	121.98, 135.66
	Pfu	120.94, 133.12
	T4	120.95, 133.52
Pfu	None	120.63, 133.53
	Pfu	120.80, 133.28
	T4	120.82, 133.43

[a] Retention times are correlated to those for the two internal controls, which were assigned values of 100 and 200. Results are means of two independent assays.
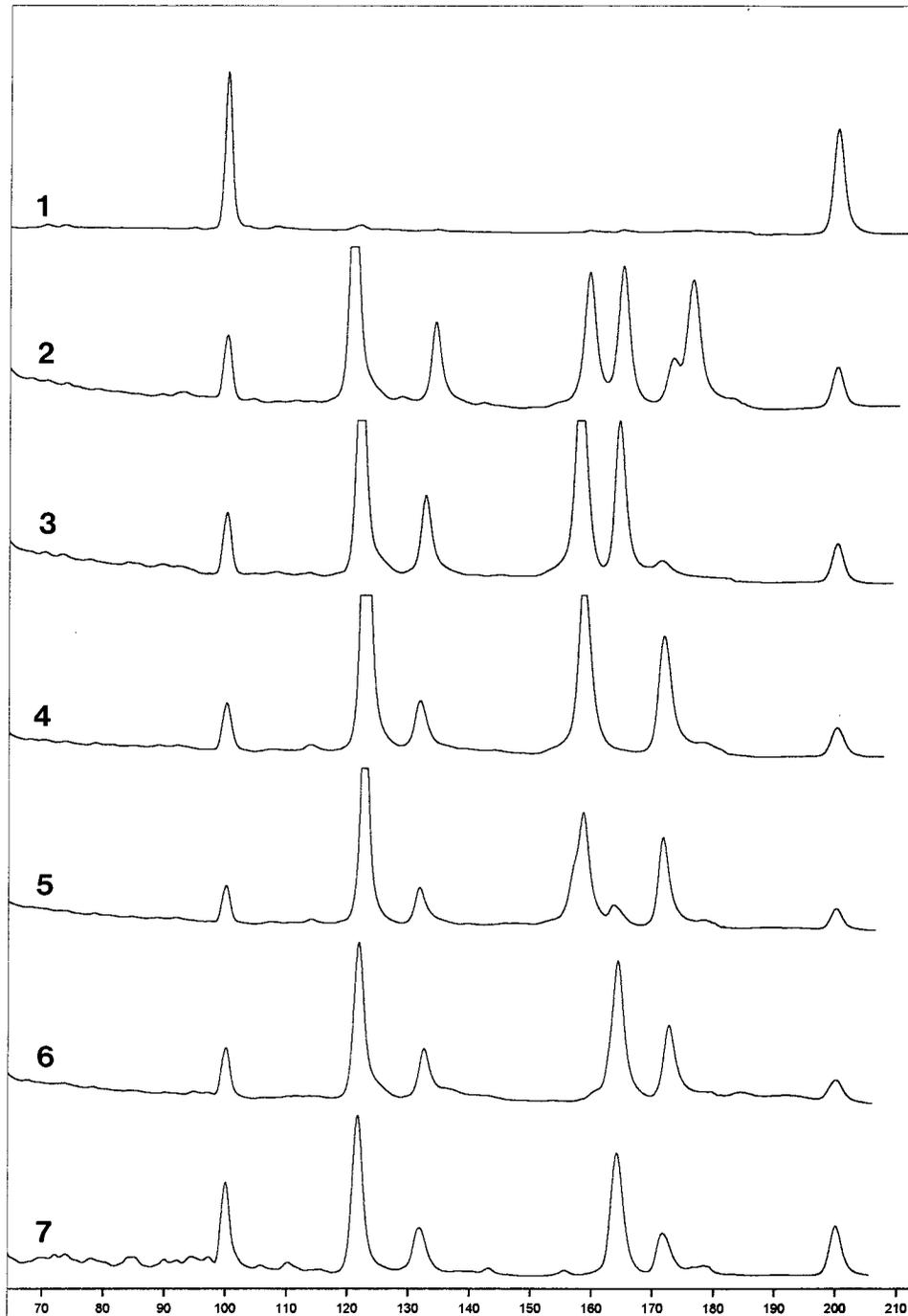
FIG. 2. Multiplex fluorescence PCR-SSCP for *Citrobacter* species with P11P-P13P and ER10-ER11. Curves: 1, internal control; 2, *E. coli* reference; 3, *C. amalonaticus*; 4 and 5, *C. freundii*; 6 and 7, *C. diversus*.

ER11 with a broad panel of microorganisms, we also tested three strains of *E. coli*, four species of *Shigella*, and four strains of *Salmonella*. For these strains we obtained distinct genus-specific patterns. However, when we tested 30 *E. coli* strains, although there was a conserved pattern for the P11P-P13P region, polymorphism was observed within the ER10-ER11 region, yielding five different patterns. Remarkably, one of these patterns was similar to the genus-conserved pattern of *Shigella* spp. Polymorphism in the ER10-ER11 region was also

observed for *Proteus vulgaris*. Thus, this region is less conserved for some species than the P11P-P13P region. This polymorphism makes identification based on SSCP patterns more difficult; therefore, we developed another primer set located in the 16S rRNA sequence, ER14-ER15, which proved to be more conserved than ER10-ER11. ER14-ER15 was capable of discriminating *E. coli* and *Shigella* species from *Salmonella* species and of distinguishing *P. vulgaris* from *Proteus mirabilis* while showing conserved patterns.

TABLE 3. Multiplex fluorescence PCR-SCCP with primer sets
P11P-P13P, ER10-ER11G, and ER14-ER15

Group and pattern no.	Species	No. of strains
Gram-negative bacteria		
1	*Acinetobacter anitratus*	3
2	*Acinetobacter calcoaceticus*	4
3	*Acinetobacter lwoffi*	3
4	*Arcanobacterium haemolyticus*	2
5	*Bacteroides fragilis*	2
6	*Citrobacter amalonaticus*	1
7	*Citrobacter diversus*	2
8	*Citrobacter freundii*	3
9	*Enterobacter aerogenes*	2
10	*Enterobacter agglomerans*	3
11	*Enterobacter cloacae*	3
12	*Enterobacter gergoviae*	1
13	*Enterobacter sakazakii*	1
14	*Escherichia coli*[a]	12
	Shigella boydii	2
	Shigella sonnei	2
	Shigella flexneri	2
	Shigella dysenteriae	2
15	*Haemophilus influenzae*	4
16	*Haemophilus parainfluenzae*	2
17	*Klebsiella oxytoca*	3
18	*Klebsiella pneumoniae*	2
19	*Neisseria gonorrhoeae*	3
20	*Neisseria meningitidis*	3
21	*Pseudomonas aeruginosa*	4
22	*Proteus mirabilis*	4
23	*Proteus vulgaris*[a]	7
24	*Salmonella enterica*	4
25	*Yersinia enterocolitica*	3
26	*Xanthomonas malthophilia*	3
Gram-positive bacteria		
27	*Bacillus cereus*	3
28	*Bacillus subtilis*	2
29	*Corynebacterium diphtheriae*	4
30	*Corynebacterium jeikeium*	4
31	*Clostridium difficile*	4
32	*Enterococcus faecalis*	4
33	*Enterococcus faecium*	5
34	*Listeria innocua*	4
35	*Listeria ivanovii*	3
36	*Listeria monocytogenes*	5
37	*Listeria murray*	6
38	*Listeria seeligeri*	5
39	*Listeria welshimeri*	2
40	*Staphylococcus aureus*	9
41	*Staphylococcus epidermidis*	7
42	*Staphylococcus saprophyticus*	3
43	*Staphylococcus warneri*	2
44	*Streptococcus agalactiae*	3
45	*Streptococcus pyogenes*	3
46	*Streptococcus pneumoniae*	4
47	*Streptococcus suis*	4

[a] Shows polymorphic patterns for ER10-ER11G.

PCR-SSCP with a multiplex PCR based on three regions of
the 16S rRNA was tested on 178 bacterial isolates (Table 3).
This panel included a large number of gram-negative and
gram-positive bacteria. The universal primers used for PCR
were highly conserved, and amplification was observed for all
species tested. Besides the species mentioned above, species-
specific SSCP patterns were observed for the following gram-
negative genera: *Acinetobacter* (*A. anitratus*, *A. calcoaceticus*,
and *A. lwoffi*), *Enterobacter* (*E. aerogenes*, *E. agglomerans*, *E.*

cloacae, *E. gergoviae*, and *E. sakazakii*), *Haemophilus* (*H. influ-
enzae* and *H. parainfluenzae*), *Klebsiella* (*K. oxytoca* and *K.
pneumoniae*), and *Neisseria* (*N. gonorrhoeae* and *N. meningiti-
dis*). Species-specific PCR-SSCP patterns were observed for
the gram-positive genera *Bacillus* (*B. cereus* and *B. subtilis*),
Corynebacterium (*C. diphtheriae* and *C. jeikeium*), *Enterococcus*
(*E. faecalis* and *E. faecium*), *Staphylococcus* (*S. aureus*, *S. epi-
dermidis*, *S. saprophyticus*, and *S. welshimeri*), and *Streptococcus*
(*S. agalactiae*, *S. pyogenes*, *S. pneumoniae*, and *S. suis*). *Ar-
canobacterium haemolyticus*, *Bacteroides fragilis*, *Salmonella
enterica*, *Pseudomonas aeruginosa*, *Xanthobacter malthophilia*,
Yersinia enterocolitica, and *C. difficile* each gave conserved pat-
terns different from those observed for the other species. The
178 tested strains belonging to 51 species resulted in 47 differ-
ent PCR-SSCP patterns, although a limited number of strains
per species were tested.

DISCUSSION

The currently used molecular biological approach for the
detection and identification of a broad spectrum of microor-
ganisms involves the use of PCR with conserved 16S rRNA
primers followed by sequencing or hybridization with a large
panel of probes (5, 12). Recently, we have developed a method
based on PCR-SSCP analysis for directly determining the iden-
tity of a bacterium (19, 22). PCR was performed with con-
served primers located in the 16S rRNA gene sequence flank-
ing a variable region. The amplified products contained
specific sequences which were utilized for identification by
SSCP. The initial PCR-SSCP protocol (22) has been modified
by labeling the PCR product with fluorescein-labeled primers
instead of silver staining the nucleic acids in the SSCP gel. A
major advantage of fluorescein PCR-SSCP over silver staining
is that the system is coupled to a computer, which enables data
management by computer. The fluorescein label changed the
mobility of the ssDNA slightly, but the observed patterns of
fluorescence PCR-SSCP are in accordance with the results
obtained by manual PCR-SSCP with silver staining. The addi-
tion of internal standards to each SSCP lane made the proce-
dure highly reproducible, with an interassay variation of less
than 0.15% and an intra-assay variation of less than 0.5%. This
reproducibility is most likely sufficient for the discrimination of
different species.

The conserved primers chosen for PCR amplification were
highly conserved as shown by the amplification of DNAs from
the panel of 178 bacterial strains from phylogenetically diver-
gent bacteria. However, the use of these universal primers is
hampered by low-level contamination of PCR reagents with
DNA. For the detection of low numbers of pathogens, a pre-
treatment of these reagents is necessary. We successfully per-
formed a DNase treatment of the whole PCR mixture (omit-
ting the target DNA) followed by heat inactivation of this
enzyme. The PCR-SSCP sensitivity obtained with this pretreat-
ment was the detection of as few as 10 CFU of *E. coli*.

Manually performed SSCP detects ssDNA bands after elec-
trophoresis, which makes the running time crucial for the dis-
crimination of closely related species, such as *L. innocua* and
L. ivanovii, which differ by only 2 nucleotides in the noncon-
served region of P11P-P13P (9). The observed differences were
marginal and could not always be visualized. Automated fluo-
rescence SSCP, which detects fluorescence at the bottom of the
gel during electrophoresis, was able to detect these small dif-
ferences reproducibly. Also, fluorescence PCR-SSCP with the
same primer set could discriminate *C. freundii* from *C. diversus*
and *C. amalonaticus*, whereas silver-stained PCR-SSCP prod-
ucts showed a genus-conserved pattern (22) Automated SSCP

could detect a single nucleotide difference. The SSCP patterns of PCR fragments amplified by *Taq* DNA polymerase, which extends the amplified sequence by a single A nucleotide (8), could easily be distinguished from those of fragments amplified by *Pfu* DNA polymerase, which possesses 3′-to-5′ proofreading. One of the fluorescence-labeled primers yielded a low fluorescence signal with some species. The efficiency of the fluorescence emission might be diminished by the secondary structure of the ssDNA. The addition of a single G nucleotide at the 5′ end, as suggested by Makino et al. (17), increased the fluorescence signal. Also, the addition of this single nucleotide altered the retention times slightly.

Automated SSCP detects the ssDNA during electrophoresis and makes optimal use of the separation capacity of the SSCP gel, and it is therefore suitable for multiplex PCR-SSCP. Multiplex PCR-SSCP (15) makes a further differentiation of species which show conserved SSCP patterns with a PCR with a single primer set. For example, the genus *Proteus* is conserved for P11P-P13P but showed species-specific patterns for ER14-ER15. Also, PCR-SSCP with P11P-P13P gave proximate retention times for some species, whereas retention times with ER10-ER11 and ER14-ER15 were more distant. We were able to discriminate the 178 bacterial strains tested to the species level with a multiplex PCR-SSCP, with only one exception: the genus *Shigella*, which is conserved and showed patterns similar to those of *E. coli*. These species are closely related and might even be considered to belong to the same genotype, as suggested by some authors (6). A broad range of bacterial pathogens was tested by PCR-SSCP, including those frequently encountered in bloodstream infections, such as *E. coli*, *Enterobacter* spp., *Klebsiella* spp., *Haemophilus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., and *Bacillus* spp.

The choice of conserved primers is crucial. First, they must be conserved for a broad range of microorganisms. Second, the conserved primers have to include a 100- to 400-bp region to obtain good PCR-SSCP resolution (13). Third, the flanking region has to contain a species- or genus-conserved region. Too much polymorphism within a certain species in the amplified region will lead to different SSCP patterns for one species, which makes interpretation more difficult. In the future, direct comparisons of the patterns obtained with those present in a computer data bank, and thereby direct identification of the bacterium, will be possible.

The combined use of PCR and SSCP on an automated sequencer made the total time for PCR-SSCP only 8 h (2 h for PCR plus 6 h for SSCP), compared with 20 h for the manual PCR-SSCP with an overnight SSCP run and an additional silver staining of nucleic acids. Automated SSCP offered a substantial time gain, better sample reproducibility, and higher resolution than manual SSCP. Multiplex PCR and subsequent multiplex SSCP enabled the discrimination of species on the basis of multiple polymorphic sites, thereby increasing the discriminating power. Fluorescence PCR-SSCP is capable of identifying as few as 10 CFU of *E. coli* within 8 h and seems to be a promising method for the identification of pathogens in usually sterile clinical sites, such as blood and cerebrospinal fluid.

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