Simultaneous Detection and Differentiation of \textit{Escherichia coli} Populations from Environmental Freshwaters by Means of Sequence Variations in a Fragment of the \(\beta\)-d-Glucuronidase Gene

ANDREAS H. FARNLEITNER, 1* NORBERT KREUZINGER, 2 GERHARD G. KAVKA, 3 SONJA GRILLENBERGER, 4 JOHANNES RATH, 5 AND ROBERT L. MACH 1


\textit{Institute of Biochemical Technology and Microbiology 172/5, Technical University of Vienna, 1060 Vienna,} 1 \textit{Institute of Water Quality and Waste Management, Department of Chemistry and Microbiology, Technical University of Vienna, 1040 Vienna,} 2 \textit{Institute of Water Quality, Federal Agency for Water Management, 1220 Vienna,} 3 \textit{Yppenplatz 5/18, 1160 Vienna,} 4 \textit{and Institute of Zoology, University of Vienna, 1090 Vienna,} 5 \textit{Austria}

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A PCR-based denaturing-gradient gel electrophoresis (DGGE) approach was applied to a partial sequence of the \(\beta\)-d-glucuronidase gene (\textit{uidA}) for specific detection and differentiation of \textit{Escherichia coli} populations according to their \textit{uidA} sequence variations. Detection of sequence variations by PCR-DGGE and by PCR with direct sequencing correlated perfectly. Screening of 50 \textit{E. coli} freshwater isolates and reference strains revealed 11 sequence types, showing nine polymorphic sites and an average number of pairwise differences between alleles of the \textit{uidA} gene fragments (screened fragment length, 126 bp) of 2.3%. Among the analyzed strains a range of dominating to more rarely and/or uniquely observed \textit{E. coli} sequence types was revealed. PCR-DGGE applied to facally polluted river water samples simultaneously detected \textit{E. coli} and generated a fingerprint of the mixed populations by separating the polymorphic \textit{uidA} amplicons. No significant differences between non-cultivation-based and cultivation-based profiles were observed, suggesting that at least some members of all occurring sequence types could be cultivated. As \textit{E. coli} is frequently used as a fecal indicator, this work is considered an important step towards a new, practical tool for the differentiation and tracing of fecal pollution in all kinds of waters.

\textit{Escherichia coli} contamination is a widely used parameter for the examination of various kinds of waters. The detection of \textit{E. coli} in temperate freshwaters usually provides a reliable indication of fecal pollution from humans and warm-blooded animals (14, 33). Recently, efficient cultivation media for routine monitoring of \textit{E. coli} in environmental freshwaters have been developed (2, 9, 20). Those methods are generally applied in a quantitative way to estimate the actual concentration of \textit{E. coli}. No information about the qualitative composition of different strains and clones can be gained (3, 14). Differentiation is not the main purpose for most routine applications, although there is a considerable demand for techniques for the simultaneous detection and differentiation of \textit{E. coli} populations in aquatic habitats. A practical method generating a representative genetic fingerprint of population structures would enable routinely performed qualitative investigations such as the comparison of different sources of fecal emissions (e.g., effluents from different sewage treatment plants or human versus nonhuman fecal pollution).

Several methods are available for the identification, characterization, and typing of microorganisms (34). So far, serotyping, biotyping, multilocus enzyme electrophoresis (MLEE), profiling for insertion elements and plasmids, restriction fragment length polymorphism analysis, random amplified polymorphic DNA analysis, amplified restriction fragment polymorphism analysis, ribotyping, pulsed-field gel electrophoresis, DNA sequencing, and detection of a genome polymorphism marker by PCR have been used for typing of \textit{E. coli} cells, representing low- to high-range resolution techniques (8, 13, 16, 17, 19, 21, 27–29, 35, 37). Application of those methods to pathogenic and nonpathogenic \textit{E. coli} populations from humans and/or warm-blooded animals has revealed substantial information about their tremendous genetic diversity, clonality, and extent of recombination, as well as the spatial and temporal distributions of \textit{E. coli} strains in different hosts from different locations (16, 30, 39, 40). However, these techniques still require a statistically sound number of pure cultures, including laborious cultivation, isolation, and identification before typing of the respective \textit{E. coli} strains. Consequently, differentiation of strains isolated from environmental waters has been rarely undertaken, although it can be used for qualitative investigations on pollution-related issues (28, 36).

Recently, denaturing-gradient gel electrophoresis (DGGE) of PCR-amplified ribosomal or other functional gene fragments has been adapted for use in microbial ecology as a non-cultivation-based molecular approach for the analysis of microbial communities (22, 24). DGGE can detect single base substitutions in DNA by separating fragments of identical length but different sequence (25). This approach has led to new insights into the dynamics and diversity of natural microbial communities (24). These studies analyzed communities comprising microorganisms from different taxonomic units, ranging from genus specificity up to higher taxonomic ranks. In this study we applied the approach for investigations at the single-species level. PCR-DGGE technology was adapted for specific detection and profiling of \textit{E. coli} populations differing

\* Corresponding author. Mailing address: Institute of Biochemical Technology and Microbiology 172/5, Technical University of Vienna, Getreidemarkt 9, 1060 Vienna, Austria. Phone: 43 2630 30650. Fax: 43 2630 363439. E-mail: A.FARNLEITNER@aon.at.
in a fragment of the functional uidA gene. The uidA gene product is a frequently used enzymatic marker for *E. coli* identification in recently developed detection media (2, 9, 20).

The aims of this study were (i) to establish PCR-DGGE-based technology for a fragment of the *uidA* gene of *E. coli*, (ii) to screen for *uidA* gene diversity and evaluate the efficiency of the method by use of a representative set of environmental freshwater isolates, and (iii) to apply this technique to a culture-versus non-culture-based analysis of *E. coli* populations from an aquatic habitat. To our knowledge, this is the first report of a technique for simultaneous detection and differentiation of *E. coli* populations from the environment without the need for isolation and identification of pure cultures.

### MATERIALS AND METHODS

#### Sampling and sampling locations

For the recovery of *E. coli* strains from different habitats, water samples were collected aseptically in 250-cm³ glass bottles (Pyrex, Stone, United Kingdom) from a water depth of 30 cm. Samples were taken from 26 different temperate aquatic sampling sites in Austria (Table 1), immediately put in a 4°C cooling box, and processed within 6 h after collection. For comparison, four additional water samples were taken from the tropical Nakivubo channel in Kampala, Uganda, a man-made stream discharging into Lake Victoria, Uganda (10). The sampling schedule is shown in Table 1.

For simultaneous detection and differentiation of mixed *E. coli* populations obtained by culture-based versus non-culture-based approaches, sampling was carried out as described above, with the exception that 2,000-ml bottles (Pyrex) filled with 1,500 ml of water sample were used. Two samples were taken from the right bank of the River Danube at Wildungsmauer (river km 1895.0). Before the samples were processed, the bottles were shaken vigorously; aliquot volumes were then used for Sterivex filtrations and for membrane filtration techniques (see below).

### TABLE 1. Bacterial isolates and microbial pollution of sampling sites

<table>
<thead>
<tr>
<th><em>E. coli</em> strain(s)</th>
<th>Sampling site...</th>
<th>Sampling date (day/mo/yr)</th>
<th>MT¹</th>
<th>Microbial pollution of sampling site</th>
</tr>
</thead>
<tbody>
<tr>
<td>A109</td>
<td>March, Devin (R)</td>
<td>02/04/1998</td>
<td>4</td>
<td>3.0 x 10⁰</td>
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<tr>
<td>A1114</td>
<td>River Danube, km 1874</td>
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<td>A737</td>
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<td>02/04/1998</td>
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<td>7.2 x 10⁰</td>
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<tr>
<td>A552</td>
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<td>02/04/1998</td>
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<tr>
<td>A1128/1, A1128/2</td>
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<td>15/04/1998</td>
<td>4, 1</td>
<td>4.0 x 10⁰</td>
</tr>
<tr>
<td>A2005/1, A2005/2</td>
<td>River Danube, km 1994</td>
<td>15/04/1998</td>
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<tr>
<td>A1139/1, A1139/2</td>
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<td>3.2 x 10⁴</td>
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<td>A115/1, A115/2</td>
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<td>A1067/1, A1067/2</td>
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<td>A789/1, A789/2</td>
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<td>15/04/1998</td>
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<td>A1075/1, A1075/2</td>
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<td>A1024/1, A1024/2</td>
<td>River Danube, km 1939</td>
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<tr>
<td>A1124/2</td>
<td>River Danube, km 50</td>
<td>15/04/1998</td>
<td>4</td>
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<tr>
<td>A1084/1, A1084/2</td>
<td>River Danube, km 2061</td>
<td>15/04/1998</td>
<td>7, 4</td>
<td>4.0 x 10⁰</td>
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<tr>
<td>A2002</td>
<td>Salzach, Uberockern (R)</td>
<td>15/04/1998</td>
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<td>7.0 x 10⁴</td>
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<td>A1117</td>
<td>Goldbach (S)</td>
<td>20/04/1998</td>
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<td>2.1 x 10⁴</td>
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<td>A47</td>
<td>Raab (S)</td>
<td>21/04/1998</td>
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<td>9.0 x 10⁰</td>
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<td>A1149</td>
<td>Strem (S)</td>
<td>21/04/1998</td>
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<td>A905</td>
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<td>A1016</td>
<td>Pinka (S)</td>
<td>21/04/1998</td>
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<tr>
<td>A1016a</td>
<td>Lainz, Etendorf (S)</td>
<td>21/04/1998</td>
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<td>A55, A26</td>
<td>NeusiedlerSee, Apetlon (L)</td>
<td>07/05/1998</td>
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<td>2.0 x 10⁻²</td>
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<td>A10, A1, A2, A5, A6, A7, A8, A9</td>
<td>River Danube, km 1895</td>
<td>13/01/1998</td>
<td>10, 4, 4</td>
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<tr>
<td>U4, U5, U6, U7</td>
<td>Nakivubo channel</td>
<td>15/09/1998</td>
<td>4, 5, 4, 4</td>
<td>2.0 x 10⁴</td>
</tr>
</tbody>
</table>

| Strain isolation, total and fecal coliforms, and Salmonella³ |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|

*⁠, A strain isolated from Austrian waters; U, strain isolated from Ugandan waters.

¹ R, river; S, stream; L, lake.

² uidA MT of *E. coli* determined by DGGE.

³ Salmonella present (+) or absent (−) in a 1-dm³ water sample.

⁴ ND, not determined.

Reference strains. Strains were obtained from the German Culture Collection of Microorganisms and treated according to the instructions given. Strains used throughout this study were *E. coli* type strain DSM 30083, *E. coli* K-12 DSM 498, *Escherichia hermannii* DSM 4560, *Escherichia vulneris* DSM 4564, *Escherichia blattae* DSM 4481, *Shigella sonneti* DSM 5570, *Shigella flexneri* DSM 4782, *Salmonella enterica* serovar *Choleracaus* DSM 5569, and *Klebsiella oxytoca* DSM 5175.

Isolation of *E. coli* and other coliforms. Cellulose nitrate membrane filters (45-μm diameter, 0.45-μm pore size; Sartorius, Vienna, Austria) were used for membrane filtration techniques (14). Chromocoll Coloniform agar (CCA) (Merck, Vienna, Austria) and mPC agar (Biomerieux, Lyon, France) were used to detect presumptive *E. coli* and total coliforms (2, 20) and fecal coliforms (14), respectively. For the isolation and purification of single *E. coli* strains, presumptive colonies taken from the CCA were transferred first to an additional CCA plate and then to nutrient agar (Merck) incubated at 37°C for 48 h. To obtain the most dominating strains, presumptive colonies from the highest possible dilution steps were randomly chosen. Purified strains were subsequently characterized by the biochemical API 20E test system (Biomerieux) according to the manufacturer’s instructions. In addition, several *E. coli* strains, total and fecal coliforms, and other *Enterobacteriaceae* from the water sample taken at Wildungsmauer on 13 January 1998 were isolated as described above.

DNA extractions from isolated strains and MFE. Two different approaches for DNA extractions were used. (i) DNA extractions from pure cultures. Overnight cultures were grown from isolated strains and processed by a standard protocol using sodium dodecyl sulfate, protease K, hexadecyltrimethyl ammonium bromide, chloroform, phenol, isopropanol, and ethanol treatment (4). (ii) DNA extractions from membrane filter enrichments (MFE) containing all growing colonies. After membrane filtration and incubation procedures (as described for *E. coli* isolation), membrane filters were rolled and placed in sterile 5-ml vials (Nunc, Roskilde, Denmark) containing 2 ml of sterile filtered TE buffer.
buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The vials were closed immediately and incubated at room temperature for 2 h on a MultiMix roll incubator (Labin, Vienna, Austria) at 20 rpm. DNA was then extracted from an aliquot of the suspension (2 mL) according to standard protocols (4). All extracted DNAs were examined by agarose gel electrophoresis before subsequent PCR analysis.

**DNA extractions from water samples.** Recovery of microbial cells was achieved by filtering of 500-mL water samples from Wildungsmauer through Sterivex filters (Millipore, Bedford, Mass.) using a Multiplex peristaltic pump (Labin). Cell lysis and nucleic acid extraction were performed directly in the filtration unit as previously described (32), except using a 5% SET buffer (5% sucrose, 50 mM EDTA, 50 mM Tris-HCl [pH 8.0]) instead of a 20% SET buffer. The crude lysate was then purified and concentrated by standard protocols (4). In addition, the DNA extractions were further purified by anion-exchange chromatography, using the Wizard DNA Clean-Up System (Promega, Vienna, Austria).

**Generation of PCR products for DGGE.** A 0.166-kb fragment was specifically amplified from the uidA genes of *E. coli* and *Shigella* spp. (for consideration of the codetection of *Shigella*, see Discussion) by use of primers UAL-1939 and UAR-2105, previously described by Bej et al. (5). A GC clamp, previously described for DGGE analysis (31), was attached to the 5′ end of primer UAR-2105, leading to UAL-1939GCPCR. PCR touchdown and hot-start techniques were performed by the protocol of Muyzer et al. (23). Denaturation, annealing, and extension were at 94, 50, and 74°C for 60, 60, and 90 s, respectively. The reaction volume was 50 µL and included 1/4 PCR buffer, 1.5 mM MgCl₂, a 200 µM concentration of each deoxynucleoside triphosphate (Promega), a 100 µM concentration of each primer, DNA templates, and water; the reaction mixture was overlaid with a drop of mineral oil (Sigma, Vienna, Austria). One unit of *Taq* DNA polymerase (Promega) was used for PCR amplification in a Trio-Thermoblock (Biometra, Göttingen, Germany).

**Evaluation of PCR specificity for *E. coli* detection.** To evaluate the specificity of *E. coli* detection, PCR reactions using primers UAL-1939GC and UAR-2105 were performed with 47 *E. coli* freshwater isolates, previously described reference strains, and 12 non-*E. coli* environmental isolates. To determine possible interference from non-*E. coli* DNA templates with the detection of *E. coli* DNA templates, *E. coli* DNA was supplemented with non-*E. coli* DNA (containing *S. enterica* serovar Choleraesuis DSM 5569, *K. oxytoca* DSM 5175, *E. coli* strains DSM 4564, *K. blyttiae* DSM 4481, and *E. hermannii* DSM 4560 DNAs in equivalent ratios) in the ratios 1:1, 1:10, and 1:1,000. For quality control, *E. coli* type strain DSM 30083 and *K. oxytoca* DSM 5175 were used as positive and negative controls, respectively, in all performed PCRs.

**DGGE optimization.** In order to determine the optimal DGGE melting range for the amplified uidA fragment (1), the theoretical and practical melting behaviors of the sequence were analyzed. The theoretical melting graph (18) was calculated for the *E. coli* K-12 sequence by means of the software program WinMelt 2.0 (Bio-Rad, Vienna, Austria). The sequence was obtained by direct sequencing with a LI-COR (Lincoln, Nebr.) long reader 4200 L-1 using a Thermo Sequenase fluorescence-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, Buckinghamshire, United Kingdom).

**RESULTS**

**Characterization of sampling sites and identification of strains.** Forty-seven *E. coli* strains from 26 different environmental freshwater sites across Austria, including channels, streams, small and large rivers, and one lake, were isolated (Table 1). The sampled freshwater sites showed low to excessively high fecal pollution, demonstrated by concentrations of fecal coliforms and *E. coli* ranging from 2.0 × 10⁻² to 1.7 × 10⁵ CFU ml⁻¹ and from 2.0 × 10⁻² to 3.2 × 10⁴ CFU ml⁻¹, respectively. Nakivubo channel, Uganda, receiving untreated wastewater, was tremendously fecally polluted, showing average *E. coli* concentrations as high as 2.0 × 10⁴ CFU ml⁻¹ (Table 1). Consequently, due to the heterogeneity of the sampling sites in terms of aquatic habitat, geographical location, and extent of fecal pollution, the *E. coli* types expected in this study should cover a representative range of strains occurring at different freshwater sites.

In general, the biochemical identification by API 20E yielded identification probabilities of ≥0.965 for *E. coli*, except for strains A1128/1, A1084/1, A1084/2, and A719, which gave values of 0.798, 0.798, 0.798, and 0.921, respectively. Further, 12 non-*E. coli* coliform strains, including 3 *Citrobacter*, 4 *Klebsiella*, and 4 *Enterobacter* strains, were isolated from the River Danube at Wildungsmauer and were identified to the genus level by the API 20E system; the remaining strain was identified as *Salmonella* by serum agglutination.

**Detection of *E. coli* by PCR.** The primers UAR-2105 and the modified UAL-1939GC were used for PCR with 68 *E. coli* strains (including *S. sonnei* and *S. flexneri*) and non-*E. coli* strains, including environmental isolates and German Culture Collection of Microorganisms reference strains. Forty-eight of the 50 *E. coli* strains resulted in strong PCR amplification products. With the *E. coli* strain WA98-5, only a weak PCR product was obtained, irrespective of template concentrations. Only *S. flexneri* DSM 4782 consistently led to a false-negative PCR result (it should be positive because *Shigella* spp. are genetically the same as *E. coli* [see Discussion]). Supplementation of *E. coli* template DNA with non-*E. coli* template DNA in the ratios 1:1, 1:10, or 1:1,000 did not interfere with the observed reaction patterns (data not shown), and none of the 18 non-*E. coli* strains yielded a PCR product. These results showed strong evidence that the GC clamp-modified primer pair used for the PCR is a useful tool for the qualitative detection of *E. coli* DNA templates. Amplification yielded a single DNA product of approximately 200 bp, in agreement with the calculated length of 208 bp (Fig. 1). In addition, a representative set of 12 PCR products generated from reference strains, freshwater isolates, MFE, and direct DNA extractions were analyzed by PCR using DNA templates from MFE and direct DNA extractions from water samples showed an amplification pattern similar to that described above. *E. coli* concentrations of samples from Wildungsmauer taken for DNA direct extraction were in the range of 2.0 × 10⁴ CFU ml⁻¹.

**DGGE optimization.** Theoretical and empirical melting profiles were determined for the *E. coli* K-12 uidA sequence (uidA positions 1640 to 1805) including the 5′-attached GC clamp. The theoretical calculated melting graph exhibited a single flat
melting domain for the amplified sequence position within the \textit{uidA} gene (data not shown). The inferred estimated melting temperature (50\% melting probability) for the sequence region from position 1640 to 1805 is between 76 and 77°C. This finding was supported by perpendicular DGGE, which resulted in an empirically determined melting temperature of about 77°C (data not shown). A denaturing gradient difference of 20\% (1) from the top to the bottom of the gel was chosen for parallel DGGE, with the denaturant concentration ranging from 48 to 68\%.

Determination of the optimal running time for parallel DGGE was carried out by loading the gel every 1.5 h for a total period of 18 h. The best resolution of bands was obtained by running the gel for 14 to 18 h (data not shown). The fragments remained stable during the whole experiment, and dissociation was therefore considered negligible.

**Screening of \textit{E. coli} isolates by DGGE.** Forty-seven freshwater \textit{E. coli} isolates, in addition to \textit{E. coli} type strain DSM 30083, \textit{E. coli} K-12 DSM 498, and \textit{S. sonnei} DSM 5570, were screened by parallel DGGE under the conditions described above. Each analyzed PCR product led to a well-focused single band in the DGGE gel (Fig. 2). Among the 47 freshwater isolates, 10 different melting types (MTs) (i.e., bands reaching different positions in the gel) could be identified. All MTs were easily distinguished from each other, except for MT6 and MT7, which could hardly be distinguished in the 48 to 68\% denaturant gradient gel (Fig. 2). Using a 49 to 65\% DGGE gel, a clear separation of MT6 and MT7 was achieved (data not shown).

In comparisons of freshwater isolates with type strains, \textit{E. coli} K-12 DSM 498 and \textit{S. sonnei} DSM 5570 were equal MT4 and MT6, respectively. No field isolate counterpart for \textit{E. coli} type strain DSM 30083 was found, and this type was therefore designated MT11. Mixed PCR products containing different MTs could also be separated efficiently (Fig. 2, lane 6).

The abundance of MTs among the 47 environmental \textit{E. coli} isolates showed an uneven distribution (Table 1). The frequencies of observed MTs were as follows (relative frequencies are in parentheses): 27 isolates of MT4 (58\%), 5 isolates of MT7 (11\%), 4 isolates of MT10 (9\%), 3 isolates of MT2 (6\%), 2 isolates each of MT5 (4\%) and MT8 (4\%), and 1 isolate each of MT1 (2\%), MT3 (2\%), MT6 (2\%), and MT9 (2\%). Three of the four tropical \textit{E. coli} isolates from the fecally polluted Nakivubo channel belonged to the dominant MT4, and one tropical strain belonged to the rarely observed MT5.

**Sequencing and evaluation of MTs.** To evaluate the efficiency of DGGE in screening and separating \textit{uidA} fragments with different sequences, a representative set of 32 selected \textit{E. coli} isolates and reference strains (\textit{E. coli} type strain DSM 30083, \textit{E. coli} K-12 DSM 498, and \textit{S. sonnei} DSM 5570) were sequenced. This included 10 strains of MT4, 5 strains of MT7, and all strains of the remaining and more rarely observed MTs. Sequence information derived from amplicons of the \textit{uidA} fragments matched perfectly with their migration patterns in DGGE. Different identified MTs were due to different sequence types (STs), and all identical STs belonged to the same MT. The results are based on two independently performed PCR amplifications, to eliminate artifacts derived from PCR copy errors.

The 11 different STs derived from the \textit{E. coli} strains differ in single base substitutions (GenBank accession no. AF173317 to 173327). In general, the 32 analyzed sequences of 126 bp \textit{uidA} gene positions 1660 to 1785) showed nine polymorphic sites and an average of 2.3\% pairwise differences between STs. The variable sites of the 126-bp fragment are located at relative base pair positions 9, 30, 42, 45, 54, 57, 75, 105, and 125.

**Simultaneous detection and differentiation of \textit{E. coli} isolates.** The described PCR-DGGE detection system was applied to a culture- versus a non-culture-based analysis of \textit{E. coli} populations from a particular aquatic habitat. Therefore,
DNA was extracted from (i) several single *E. coli* strains isolated from MFE, (ii) the whole MFE by using two different media, and (iii) direct water filtrates, using for all fractions the same water sample from the fecally polluted sampling site of River Danube km 1895 taken on 13 January 1998.

(i) DGGE analysis of eight isolated *E. coli* strains (A10, A1, A2, A5, A6, A7, A8, and A9) showed the following distribution: five strains were of MT4, one strain was of MT5, and two strains were of MT10 (Table 1; Fig. 3, lanes 1 to 3).

(ii) DGGE analysis of PCR products from MFE yielded five and four distinct bands for mFC agar and CCA, respectively (Fig. 3, lanes 4 to 6 and 7 to 9). All analyzed replicate PCR products at 10-fold template dilutions gave consistent results. The MTs of the eight isolated single strains corresponded well with the band patterns of the MFE analysis, except for two bands. This clearly can be demonstrated by the MFE-DGGE analysis of the mFC agar, which included all MTs from the isolated strains (Fig. 3, bands 2, 4, and 5 in lanes 5 and 6). The band patterns of the MFE of the CCA also matched with the MTs of the isolated strains, except for MT5 (Fig. 3, lanes 7 to 9); however, this *E. coli* MT was isolated only on mFC agar.

The DGGE analysis of both MFE resulted in two additional bands (Fig. 3, bands 1 and 3 in lanes 4 to 9), most likely from additional culturable *E. coli* clones differing in their *uidA* fragments.

(iii) DGGE analysis of PCR products from direct DNA extractions of the water sample gave results consistent with the MFE CCA analysis (Fig. 3, lanes 11 and 12). In contrast to the PCR-DGGE band patterns from MFE mFC extracts (Fig. 3, lanes 4 to 6), the band corresponding to MT5 could not be detected in direct extracts. This was probably due to lower DNA template concentrations and/or lower PCR amplification efficiencies for direct extracts compared to MFE extracts. PCR-DGGE analysis of direct DNA extracts from the second sample taken at the same sampling location at River Danube km 1895 gave the same band patterns as described above (data not shown).

**DISCUSSION**

For specific detection of *E. coli* DNA templates, the primers UAR-2105 and UAL-1939 were used (5). We clearly demonstrated that modification of the primer UAL-1939 to make it UAL-1939GC for DGGE neither affected the reported specificity for PCR-based *E. coli* detection (5) nor caused multiple bands on DGGE due to the attachment of a GC clamp leading to incomplete strand synthesis (26). Furthermore, *E. coli* could also be detected directly in contaminated river water, as previously reported (15). It should be noted that primers UAR-2105 and UAL-1939GC also detected *Shigella* spp. (5). This is reasonable, because there is no real difference between *Shigella* and *E. coli* in terms of biochemical, serological, genetic, and even pathogenic traits (6). Therefore, *Shigella* should be treated as a distinct clone of *E. coli* (40). However, for historical reasons, *Shigella* continues to be reported as a distinct genus in clinical laboratories (38). DGGE efficiently separated the 208-bp *uidA* fragments, differing by one to seven single base substitutions, from each other. These results are consistent with previous reports, where DGGE detected nearly all single base substitutions (25, 31).

The 50 screened *E. coli* strains (including reference strains) from different aquatic habitats with different levels of fecal pollution (Table 1) revealed 11 STs within the analyzed 126-bp *uidA* fragments (i.e., the effectively analyzed fragment size, excluding primer sequences and the GC clamp). The STs showed nine polymorphic sites and an average number of pairwise differences between alleles (ANPDAs) (40) of 2.3%. Comparison of these results with currently available data from allelic variations in nucleotide sequences of 15 other protein-encoding chromosomal genes or gene fragments among wild strains of *E. coli* (40) indicates that the *uidA* fragment represents medium sequence diversity. Except for the highly conserved glyceraldehyde-3-phosphate dehydrogenase gene, showing a value of 0.24%, literature values for ANPDAs range from 1.11% for malate dehydrogenase gene up to 7% for the highly variable 6-phosphogluconate dehydrogenase gene, according to a recent review (40). In comparison, phenotypic analysis of the genetic diversity *h* (*h* = 1 − Σxᵢ², where *x*ᵢ is the frequency of the *i*th allele at the locus) of 35 *E. coli* enzymes from various strains by MLEE, including β-D-glucuronidase, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, and 6-phosphogluconate dehydrogenase, yielded values of about 0.17, 0.02, 0.28, and 0.71, respectively (30). Extreme *h* values of almost 0 and 0.82 were calculated for nucleoside phosphorylase and β-D-galactosidase, respectively. Taking into account the methodological differences between MLEE and DNA sequence analyses (40), as well as the fact that sequence analyses were not always performed on the entire gene, the results of both approaches are surprisingly congruent. However, in relation to the other mentioned enzymes, the value for the genetic diversity (*h*) of the β-D-glucuronidase was somewhat lower than the ANPDA value for the *uidA* fragment.

The uneven frequency of the observed STs from different strains and habitats was in accordance with data from MLEE analysis of *E. coli* populations derived from within and between host family investigations of different areas (12) and from a study of the temporal variations in the *E. coli* population of a single human host (11). These studies showed diverse *E. coli* populations (i.e., a multiclonal distribution of strains), ranging from dominant to more rarely observed types in the human feces, undergoing strong dynamic fluctuations. Furthermore, some MLEE types were observed in all investigated families, and some strains were more restricted to individual hosts (12). However, a previous study suggested that transitions of cells from the primary habitat (colonies of warm-blooded animals and humans) to the secondary habitat (the environment) might involve dramatic changes in the genetic compositions of *E. coli* populations (39). STs of the four tropical *E. coli* isolates from...
a highly anthropogenically polluted channel in Uganda matched the sequences of the STs of Austrian freshwater isolates, supporting the hypothesis that some clones may have a worldwide distribution (30). Interestingly, three of the four tropical isolates from the highly polluted environment belonged to the most dominant ST4, suggesting that ST4 is of ubiquitous occurrence.

Application of PCR-DGGE to fecally polluted river water samples containing mixed *E. coli* populations (i) specifically detected the presence of *E. coli* cells in the water samples and (ii) generated a fingerprint of the population by separating the uidA amplicons according to their sequence variations. The method could be applied to a culture-based as well as a nonculture-based DNA extraction method and enable the comparison of both techniques. The results were obtained without the need for isolation, purification, and identification of pure cultures of *E. coli*. However, data were evaluated by comparison with *E. coli* isolates retrieved from the same sample, and all of them correlated well with the simultaneous analysis of mixed populations, hence indicating that possible PCR-related artifacts were negligible (41). No significant differences between the fingerprints of the cultivation-based and the non-cultivation-based profiles could be observed. This shows that at least some members of all detected *E. coli* STs could be cultivated, which is in line with the hypothesis that enteric bacteria do not enter the viable but nonculturable cell state (7). In comparisons of direct DNA extracts from water samples to DNA extracts from MFE, the detection sensitivity of PCR-DGGE analysis was increased for extracts from MFE. This finding has a particular practical implication, as membrane filtration techniques are routinely used for the determination of *E. coli* and fecal coliform concentrations (3, 14). Representative filters can be frozen for long-term storage and analyzed without the need for any further laborious and expensive sampling and filtration procedures. Compared to environmental samples (41), DNA extracted by standard methods from MFE contains only negligible concentrations of PCR inhibitors and can therefore be subjected to PCR directly, avoiding any further purification procedures. However, in some particular circumstances it may be desirable to use direct DNA extracts, avoiding a cultivation-dependent step.

The use of PCR-DGGE in this study was focused on the development of a practical method for the simultaneous detection and differentiation of *E. coli* populations from environmental freshwater samples without the need for pure and identified cultures. It could be clearly demonstrated that PCR-DGGE technology can be used to generate a species-specific community fingerprint of *E. coli*. The developed method is currently being further tested for its potential for discrimination of *uidA* profiles of mixed *E. coli* populations from different sources. The development of a differential diagnostic marker need not necessarily be based on the *uidA* fragment described, but a broad range of *E. coli* loci with low to high sequence variation can be employed. We consider the present study an important step towards a new practical tool for qualitative comparisons and for the tracing of fecal pollution in all kinds of waters.

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