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Separation of transfer RNA and 5S ribosomal RNA using capillary electrophoresis

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Abstract

Capillary gel electrophoresis and capillary electrophoresis using entangled polymer solutions was investigated for their applicability for the separation of low-molecular-mass RNAs (transfer RNA and 5S ribosomal RNA), with a size range of 70–135 nucleotides, from bacteria. Cross-linked polyacrylamide gel-filled capillaries (3 and 5%) were used for capillary gel electrophoresis. Good resolution was obtained using gel-filled capillaries only for small tRNAs with lengths to 79 nucleotides, larger tRNAs and 5S rRNA could not be resolved using this method. Buffers containing sieving additives were employed to improve separations of RNA by capillary electrophoresis using entangled polymer solutions. The use of linear sieving polymers in buffers resolved 5S rRNA and tRNAs, even when they possessed only different secondary structure or small differences in length (1–5 nucleotides).

1. Introduction

The rapid detection, identification and classification of bacteria is a continuing, world-wide scientific goal, particularly for medical and ecological purposes. The classical methods of bacterial identification are based primarily on phenotypic and physiological characteristics (e.g., growth on different media) and do not examine directly the genotypic characteristics of bacteria. Recently, a variety of molecular and genetic techniques have been introduced for the identification and classification of bacteria. These methods include DNA/DNA hybridisation [1], 5S, 16S and 23S rRNA and rRNA gene sequencing [2,3], as well as fingerprinting of proteins and DNA [4,5]. The only direct, genotypic fingerprinting technique using RNA is the low-molecular-mass RNA (low-M_r RNA) profiling method [7]. This new RNA fingerprinting method generates one-dimensional band patterns for three groups of molecules of taxonomic significance: 5S rRNA, large tRNAs and small tRNAs (size range 70 to 135 nucleotides). These band patterns allow an identification of bacterial strains due to species- and genus-specific tRNA bands and group-specific 5S rRNA bands and can also be applied directly to analyses of environmental samples [8].

The standard technique for low-M_r RNA profiling employs slab polyacrylamide gel electrophoresis (PAGE), followed by silver stain detection [7]. While this has been accepted as a standard methodology, several drawbacks exist. Slab gel electrophoresis is time-consuming, labour intensive and difficult to automate. Capil-

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lary electrophoresis (CE) provides an attractive alternative to the conventional method, because of shortened separation times, on-line detection, automated sample injection and reduced amounts of sample needed.

Separation of DNA fragments has been demonstrated using different CE techniques [9–16]. In most cases, gel-filled capillaries with different concentrations of cross-linked polyacrylamide have been used. Such separations of DNA poly- and oligonucleotides, as well as of polymerase chain reaction (PCR)-amplified DNA fragments, are well described and investigated [9–11]. Besides cross-linked polyacrylamide matrices, separation media containing agarose [12] and non-cross-linked linear polyacrylamide have been used [13]. In all cases, problems exist for the preparation of capillaries filled with such separation media (e.g., polyacrylamide, agarose), as well as in their handling. Additionally, the capillaries have a finite shelf-life as well as a low reproducibility. For example, bubbles may form within the capillaries by sudden current or temperature fluctuations, due to either a point of high resistance within the column, such as a build-up of high-molecular-mass sample residue or non-uniform temperature control, and finally gel degradation occurs. The capillaries then become useless and must be discarded.

As an alternative to gels, solutions of entangled polymers can be used as macromolecular sieving media [14–16]. These include water soluble, viscous, linear polymers such as hydroxypropylmethylcellulose (HPMC) or hydroxyethylcellulose (HEC). These polymers produce a sieving effect, can be replenished after each run, and are easy to handle. The capillaries used are coated, open tubular, and possess a long lifetime. Generally, these non-cross-linked polymer sieving systems possess separation efficiencies nearly equivalent to those obtained with gel-filled capillaries for the separation of DNA fragments [14–16].

High reproducibility, as well as potential for automation, were required for a routine application of the separation of low-M_r RNA by CE. Although, an easy transfer of low-M_r RNA profiling from conventional gel electrophoresis to capillary gel electrophoresis (CGE) was expected. This has proven to be difficult. In this paper we present the development and optimisation of a separation methodology for different, pure low-M_r RNA standards and the determination of whole low-M_r RNA profiles using polymer solutions, as well as gel-filled capillaries. Finally, we compare the resolutions of the low-M_r RNA fingerprints, as determined by conventional slab gel electrophoresis and by CE.

2. Experimental

2.1. Materials

Tris(hydroxymethyl)aminomethane (Tris), ammonium peroxodisulphate, N,N,N′,N′-tetramethylethylenediamine (TEMED) were obtained from Sigma (St. Louis, MO, USA). Acrylamide was purchased from Amresco (Solon, OH, USA) and 3-(trimethyoxysilyl)propylmethacrylate (Bind Silane) from LKB (Bromma, Sweden). Ethylenediaminetetraacetic acid (EDTA) and boric acid were obtained from Riedel–de Haen (Deelze, Germany). The ready available PCR product analysis buffer with 7 *M* urea was purchased from Bio-Rad (Richmond, CA, USA). The polymer HPMC, with a viscosity rating of 4000 cP for a 2% solution at 25°C, was obtained from Sigma.

$tRNA_{Phe}$ and $tRNA_{Tyr}$ from *Escherichia coli* MRE 600 were supplied by Sigma. Purified 5S rRNA was provided at a concentration of 0.8 μg/μl and a tRNA mixture from *E. coli* MRE 600, containing 5S rRNA and tRNAs, were purchased from Boehringer Mannheim (Mannheim, Germany). This tRNA mixture was used as a standard low-M_r RNA mixture. Polydeoxyadenylic acids [$pd(A)_{25-30,\ 40-60}$] were obtained from J & W Scientific (Folsom, CA, USA).

Gel-filled capillaries, μPAGE-3 (3% T, 3% C PAGE[1]) and μPAGE-5 (5% T, 5% C PAGE), as well as open tubular, coated capillaries DB-1, were purchased from J & W Scientific.

[1] T = [g acrylamide + g N,N′-methylenebisacrylamide (Bis)]/100 ml solution; C = g Bis/T

2.2. Equipment

A Spectra Phoresis 500 CE (Spectra-Physics, Fremont, CA, USA) was used in negative polarity mode (anode at the detection side) for all separations. Temperature control was achieved with a peltier cooling system from Spectra Phoresis 500. Electrokinetic injections were always carried out and nucleic acids were detected at 260 nm using a UV detector.

2.3. Methods

RNA denaturation

A 20-μl volume of the standard low-M_r RNA mixture from *E. coli* (60 μg RNA/ml) was mixed with 40 μl formalin, 16 μl formaldehyde (37%) and 8 μl 40 m*M* Tris–HCl buffer, 1 m*M* EDTA (pH 5.0). The sample was heated for 15 min at 65°C, than cooled in ice and stored at 4°C.

Capillary gel electrophoresis

CGE was performed in gel-filled capillaries μPAGE-3 and μPAGE-5, 44 cm (36 cm effective length) × 75 μm I.D. The capillaries were conditioned daily at 25°C and 100 V/cm, negative polarity for 5 min. The applied voltage was increased stepwise to 250 V/cm over a period of 30 min. The current was stable at approximately 4–5 μA. Samples were introduced into capillaries by electromigration at −5 kV for 5 s. Separations were performed under constant voltage at 250 V/cm. The separation buffer solution contained 100 m*M* Tris–borate and 7 *M* urea at a pH 8.3.

Capillary electrophoresis using entangled polymer solutions

DB-1 open tubular capillaries, 70 cm (62 cm effective length) or 118 cm (110 cm effective length) × 100 μm I.D. with a coating of 0.1 μm dimethylpolysiloxane, were used without modification. 70 cm DB-1 capillaries were filled automatically with the appropriate polymer sieving buffer for 15 min (110 cm long capillaries required 25 min). Between runs, the capillaries were washed, in turn, with Milli-Q water and methanol twice (110 cm long capillaries three times) for 5 min. Column lifetime was determined to be in excess of 100 injections.

The first polymer sieving buffer, named "PCR product analysis buffer" from Bio-Rad, contained 267 m*M* Tris–borate, 7 *M* urea and polymer modifier (pH 8.3) and was "ready for use". The second polymer sieving buffer that was used contained 350 m*M* Tris–borate, 2 m*M* EDTA and 7 *M* urea (pH 8.6). A 10 ml volume of buffer was mixed with 0.5% (w/w) HPMC and heated for 10 min at 80°C in a closed vessel. The HPMC solution was stirred slowly at room temperature until it appeared homogeneous and transparent. The HPMC solution was used on the second day after preparation and was stable for at least one month at room temperature.

The samples were prepared at a concentration of 100 μg/ml in 40 m*M* Tris–borate–EDTA buffer (pH 5.0) and were introduced into the capillaries by electromigration. Before sample injection, the injection side of the capillary was washed with Milli-Q water twice for 0.1 min. All separations were performed at 20°C.

Conventional gel electrophoresis

Separation of low-M_r RNA was carried out under high power by denaturing PAGE [17]. Dried samples of low-M_r RNA (3 μg) were suspended in 4 μl of a loading solution [300 mg/ml sucrose, 460 mg/ml urea, 10 μl/ml 20% sodium dodecyl sulfate (SDS), 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol, all substances dissolved in TBE buffer] and loaded on polyacrylamide gels [gel size 550 × 170 × 0.4 mm, 10% acrylamide, acrylamide:N',N-methylenebisacrylamide 28.8:1 (w/w), 7 *M* urea, in TBE buffer: 100 m*M* Tris, 83 m*M* boric acid, 1 m*M* EDTA, pH 8.5] that were bound to a carrier glass plate and run in a high-power electrophoresis unit (2010 Macrophor electrophoresis unit and 2297 Macrodrive 5 power supply, LKB–Pharmacia, Bromma, Sweden). Gels were run at 60°C and at constant power that was increased stepwise from 40 to 160 W during the 3 h run. RNA bands were visualised by a modified ammoniac silver staining procedure [7]. Gel scans were performed using a transmission

densitometer (Elscript 400, Analysentechnik Hirschmann, Taufkirchen, Germany) at a wavelength of 546 nm. A detailed description of the conventional electrophoresis of low-M_r RNA has been given previously [7].

3. Results and discussion

3.1. Capillary gel electrophoresis

Contrary to work having been carried out on the separation of DNA, there have been no reports on the separation of RNA using CGE. Although given the differences between DNA and RNA molecules, we have based our first studies with RNA on the previous work carried out on the separation of single stranded deoxyoligonucleotides with lengths of 30–160 bases. Paulus et al. [10] reported good resolution of DNA oligonucleotides over a range of 30–160 bases with 2.5–4% T and 3.3% C polyacrylamide gel-filled capillaries. Therefore, we tested, as a first approach, two types of commercially available capillaries that have the reported concentration of polyacrylamide, μPAGE-3 with 3% T, 3% C and μPAGE-5 with 5% T, 5% C PAGE. Moreover, we were able to reproduce the results of the separation of the polydeoxyadenylic acids $pd(A)_{25-30}$ and $pd(A)_{40-60}$ using these capillaries. In comparison with μPAGE-5, shorter migration times were obtained using the μPAGE-3. This result was expected due to the larger mesh size on the 3% polyacrylamide gel allowing the linear polydeoxyadenylic acids to move faster through the gel pores.

However, the results obtained for ribonucleic acids were different from those obtained for the polydeoxyadenylic acids. The separation of $tRNA_{Phe}$, using a 5% cross-linked polyacrylamide gel, is shown in Fig. 1A. Good resolution of $tRNA_{Phe}$ (76 nucleotides) was obtained using the 5% T and 5% C gel-filled capillaries, comparable to resolutions obtained using conventional polyacrylamide gels (55 cm long) (Fig. 1B).

In comparison to 5% gel-filled capillaries, the separation for tRNA was improved using 3% gel-filled capillaries and the analysis time was decreased. The separation of a mixture of three low-M_r RNA standards ($tRNA_{Phe}$, $tRNA_{Tyr}$ and 5S rRNA) using a 3% gel-filled capillary is shown in Fig. 2. The first two peaks belong to the $tRNA_{Phe}$ and the two following ones to the $tRNA_{Tyr}$. The two peaks of $tRNA_{Tyr}$ are noticeably less sharp than the peaks of $tRNA_{Phe}$. The 5S rRNA is only detectable from the increase of the baseline. Additionally, a separation of the 5S rRNA could not be achieved and the increase of baseline suggests that a build-up of the 5S rRNA degradation products was occurring. 5S rRNA is a ribosomal component and might be more sensitive to the separation conditions than are cytoplasmic tRNAs. Alternatively, degradation of the gel is also possible explanation for the baseline increase. Lalande et al. [18] described a biomolecule deformation called "trapping form". In this case, both ends of the DNA molecule are very close to each other in the field direction. The electrophoretic forces acting on the molecule are cancelled, and the mobility is zero until a new, non-compact conformation is reached. Thereby, the resistance within the capillary is high, due to gel degradation.

Results from the separation of low-M_r RNA standards (Fig. 2) demonstrate that a separation of tRNAs, with good resolution using CGE, is only effective for small molecules, for example $tRNA_{Phe}$, belonging to the class 1 tRNA (72–79 nucleotides long [6]). The resolution of tRNAs decreased with increasing nucleotide length. On the contrary, a separation of the 5S rRNA could not be achieved using 3 or 5% gel-filled capillaries. Similar results were obtained for the separation of 5S rRNA using a different type of gel-filled capillary (eCap gel U 100P, Beckman, Fullerton, CA, USA) (data not shown). Probably, the mobility of low-M_r RNA molecules is different from that of DNA, and deformation of their structure, due to electric forces within the gel network, hinders a good resolution for molecules greater than 79 nucleotides. The limited column durability and the short lifetime are additional difficulties. Therefore, another CE separation method was explored.

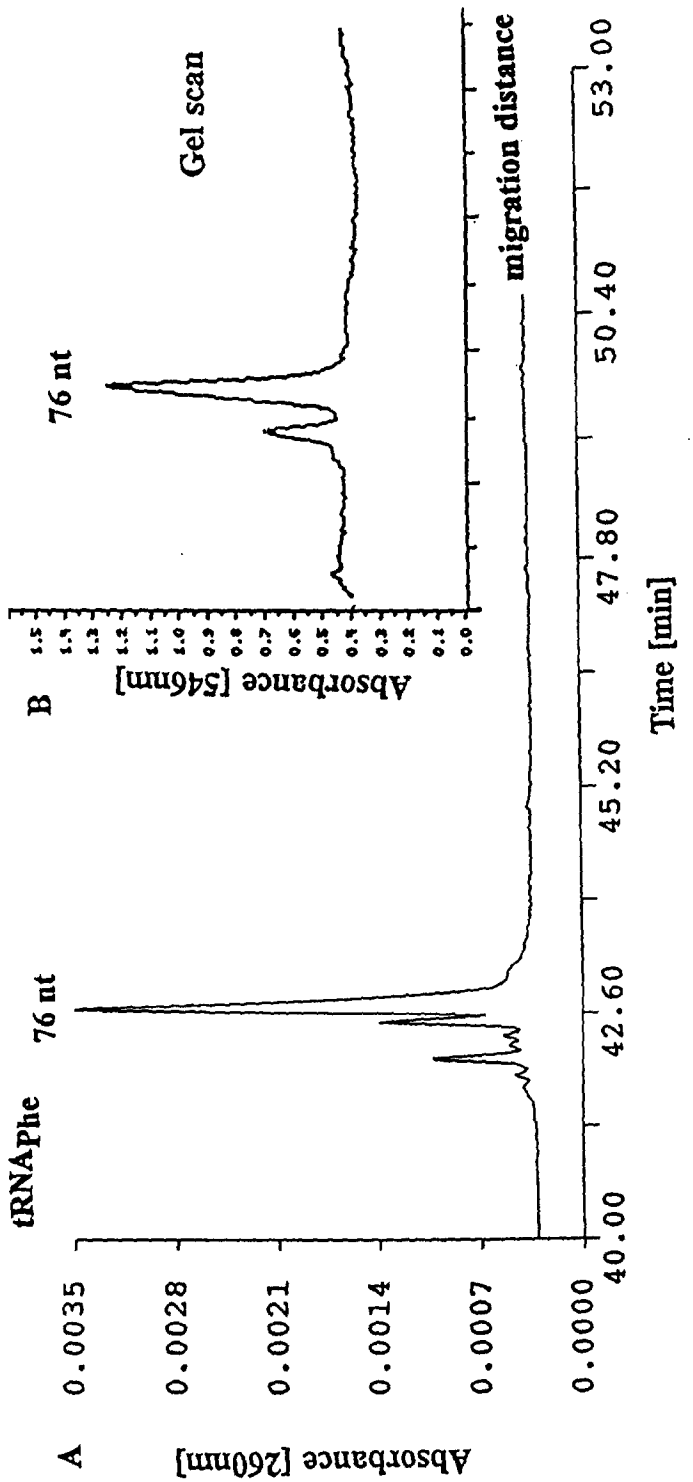

Fig. 1. (A) Gel-filled CE separation of $tRNA_{Phe}$ from *E. coli* MRE 600 using 5% cross-linked polyacrylamide gel. Buffer: 100 m*M* Tris–borate and 7 *M* urea, pH 8.3. Sample: 8 μg/ml. (B) Gel scan of $tRNA_{Phe}$ separated by conventional PAGE. nt = Nucleotides.

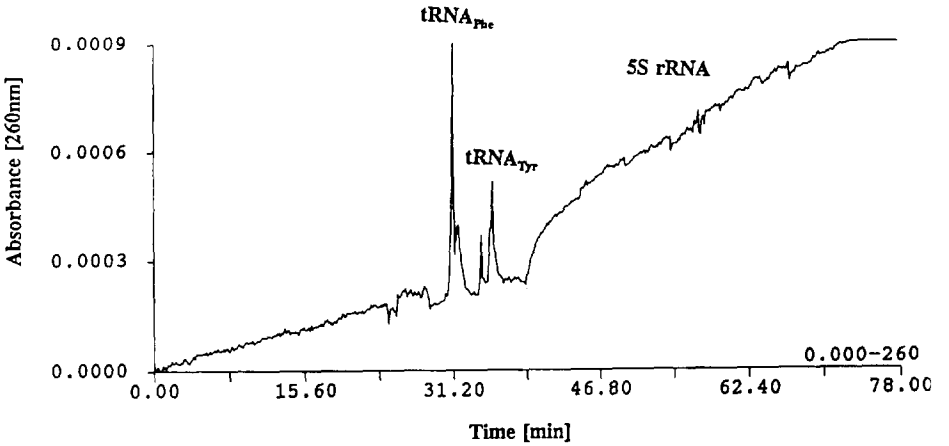

Fig. 2. Gel-filled CE separation of a mixture of three low-M_r RNA standards (tRNA$_{Phe}$, tRNA$_{Tyr}$ and 5S rRNA) from *E. coli* MRE 600 using 3% cross-linked polyacrylamide gel. Sample: 24 μg/ml.

3.2. Capillary electrophoresis using entangled polymer solutions with commercial buffer including sieving additives

Solutions of entangled polymers offer a good alternative to the CGE. This technique promises to combine the advantages of free-solution CE (automation, speed, reproducibility and accurate quantification) with the range of applications and resolving power of gel-based systems.

A dynamic, transient polymer network is formed in CE with entangled polymer solutions rather than a rigid, chemical, cross-linked gel network. These polymer solutions possess a viscosity without gel consistency. Many reports demonstrated separations of DNA restriction fragments and PCR products ranging in size from 72 to 1353 base pairs (bp), with resolutions comparable to CGE, using entangled polymer solutions [15,19–22].

Firstly, we tested, as sieving medium, a commercially available analysis buffer with sieving additives (Bio-Rad) for the separation of the low-M_r RNA molecules larger than 79 nucleotides that could not be separated by CGE. Fig. 3 shows the separation of tRNA$_{Tyr}$ (Fig. 3A) and of 5S rRNA (Fig. 3B). Both samples were separated as well by slab gel electrophoresis (gel scans, Fig. 3C and D). The 5S rRNA of *E. coli* easily separated into the characteristic two main fractions of 5S rRNA with lengths of 115 and 120 nucleotides. As demonstrated with 5S rRNA (Fig. 3B), separation of ribonucleic acids with, at least, 5 nucleotide differences are possible using this system. Moreover, we obtained high reproducibility of migration time of both separations with a relative standard deviation (R.S.D.) for 20 runs in the range 0.2–0.4%.

Fig. 4 presents the separations of a standard low-M_r RNA mixture from *E. coli* using DB-1 capillaries with a length of 70 cm (62 cm effective length) (Fig. 4A) and 118 cm (110 cm effective length) (Fig. 4B). The first six peaks (1–6) belong to the class 1 tRNAs (length of 74–79 nucleotides). The following five peaks (7–11), belong to the class 2 tRNAs (length of 83–90 nucleotides). The last peak (12) represents the 5S rRNA (120 nucleotides). The position of the 5S rRNA was determined by progressive addition of 5S rRNA to the sample. The separation profile obtained, using the 70 cm long capillary, represented a relatively poor resolution, although the three main groups of low-M_r RNA (class 1 and class 2 tRNA and 5S rRNA) could be clearly differentiated. However, no improvement was obtained through variation of running conditions (e.g., increased electric fields or temperature, longer washings and buffer filling of the capillary or use of DB-17 capillary). Finally, an increase in the length of the capillary from 70

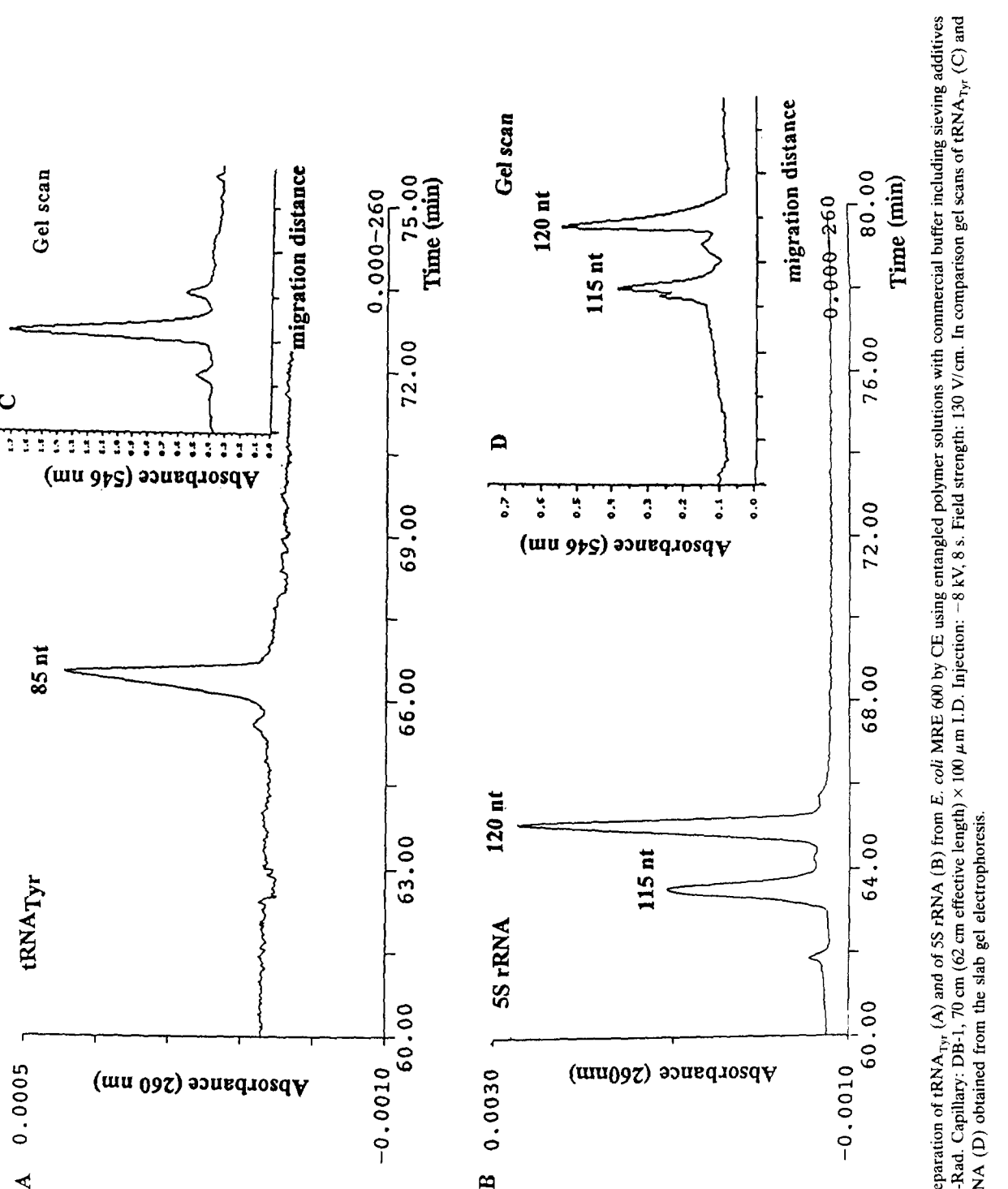

Fig. 3. Separation of $tRNA_{Tyr}$ (A) and of 5S rRNA (B) from *E. coli* MRE 600 by CE using entangled polymer solutions with commercial buffer including sieving additives from Bio-Rad. Capillary: DB-1, 70 cm (62 cm effective length) × 100 μm I.D. Injection: −8 kV, 8 s. Field strength: 130 V/cm. In comparison gel scans of $tRNA_{Tyr}$ (C) and of 5S rRNA (D) obtained from the slab gel electrophoresis.

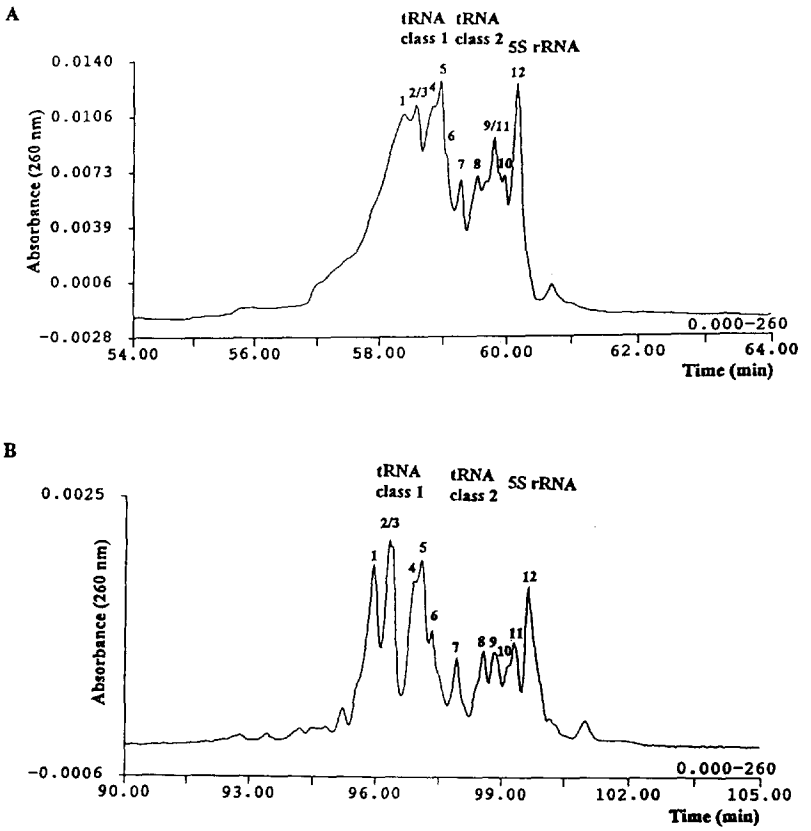

Fig. 4. Separation of standard low-M_r RNA mixture from *E. coli* MRE 600 by CE using entangled polymer solutions with commercial buffer including sieving additives from Bio-Rad. Capillary: DB-1, 70 cm (62 cm effective length) × 100 μm I.D. (A) and 118 cm (110 cm effective length) × 100 μm I.D. (B). Injection: −3 kV, 10 s. Field strength: 150 V/cm by the 70 cm capillary and 220 V/cm by the 118 cm capillary.

cm to 118 cm was observed to affect an improved resolution. The longer the capillary, the better the resolution. Thus, peaks number 1 and 2, as well as the groups of peaks 1–3 and 4–6, belonging to the class 1 tRNA, are separated from each other. A decrease of overlapping was obtained in the class 2 tRNA, the peaks 7–11 are nearly baseline separated.

In comparison to 70 cm capillaries, we observed an increase of migration time to at least 40 min using 118 cm capillaries. The separations were performed under low electric field strength (150 and 220 V/cm respectively). Although, retention times could be reduced by using higher electric fields, worse resolutions were also produced. Several reports have demonstrated the negative influence of high electric fields on the resolution of separation [23,24]. In comparison to standard gel scans of the same sample (Fig. 6B), a nearly satisfactory resolution was obtained using the 118 cm long capillary. However, the three groups of low-M_r RNA are separated better and differentiated easier using the slab PAGE.

As is shown in Fig. 4, tRNAs with small differences in length of 1–5 nucleotides (class 1 or class 2 tRNA) or with different secondary structures, were resolved by CE using entangled polymer solutions. The influence of the secondary structure in CE separations using polymer buffers was also demonstrated in the following experiment. The resolution of the low-M_r RNA mixture from *E. coli* was compared to the resolution of the same sample after denaturation

into single strands (ss). The low-M_r RNA mixture from *E. coli* was treated with formalin and formaldehyde for complete denaturation, and separated under the same conditions as the non-treated sample. Fig. 5 demonstrates the resolution of this separation. Only one broad peak was obtained instead of a resolved profile. A separation of components was not possible in the absence of secondary structures using this system. Inadequate resolution was obtained also by the separation of linear ss polydeoxyadenylic acids under the same running conditions. A minimum length difference of 10 nucleotide bases was necessary for a separation of two ss DNA oligonucleotides (data not shown).

Although, the separation by CE using entangled polymer solutions of 5S rRNA from *E. coli* appears to be size-dependent (Fig. 3B), a resolution of the low-M_r RNA mixture from *E. coli* also was obtained due to the secondary structure (Fig. 5). Probably, an interaction of the RNA molecules with the entangled polymer network exists also. An entanglement coupling interaction of biomolecules (as passing analyte), moving under the influence of an electric field, with polymer molecules in the buffer, has been reported previously [19,25].

In comparison to CGE, RNA molecules greater than 79 nucleotides can be separated by CE using entangled polymer solutions with commercial buffer including sieving additives from Bio-Rad. Low-M_r RNA profiles may be analysed, with satisfactory resolution, using this separation system. On the other hand, shorter migration times and better resolution are desired. Therefore, we employed additional polymers as sieving additives in the separation buffer.

3.3. Capillary electrophoresis using entangled polymer solutions with 0.5% HPMC as sieving additive

Zhu et al. [26] first demonstrated that electrophoresis buffers containing cellulose additives such as methyl or hydroxymethyl cellulose provide a molecular-sieving capability for the CE separation of nucleic acids (i.e. non-gel electrophoresis). Therefore, we tested separations using 70 cm long DB-1 capillaries and HPMC (0.5%, w/w) as a sieving agent in the buffer. The separation of the low-M_r RNA mixture from *E. coli* is shown in Fig. 6A. In comparison, the gel scans and the slab gel separation of the same sample are shown in Fig. 6B and C, respectively. The peak pattern obtained by the CE was different from the pattern of the conventional gel electrophoresis. Additionally, a larger separation distance was obtained between the different low-M_r RNA groups (class 1 and class 2 tRNA and 5S rRNA) using slab PAGE. High electric fields

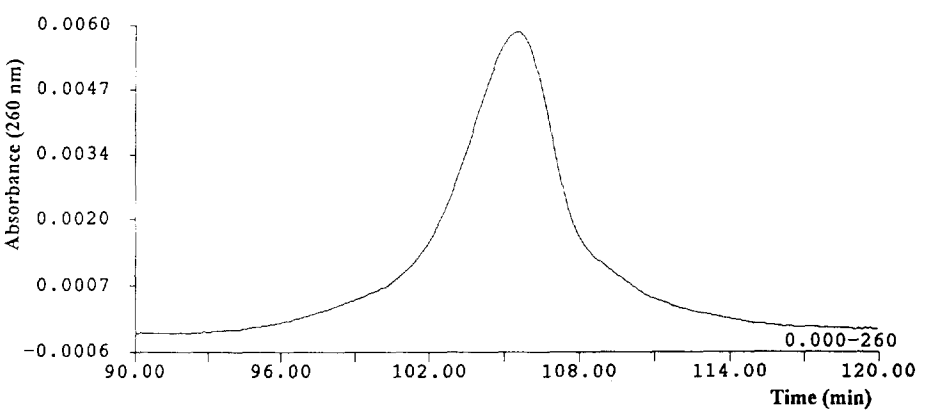

Fig. 5. Separation of denatured low-M_r RNA mixture from *E. coli* MRE 600 by CE using entangled polymer solutions with commercial buffer including sieving additives from Bio-Rad. Capillary: DB-1, 118 cm (110 cm effective length) × 100 μm I.D. Injection: −3 kV, 10 s. Field strength: 250 V/cm.

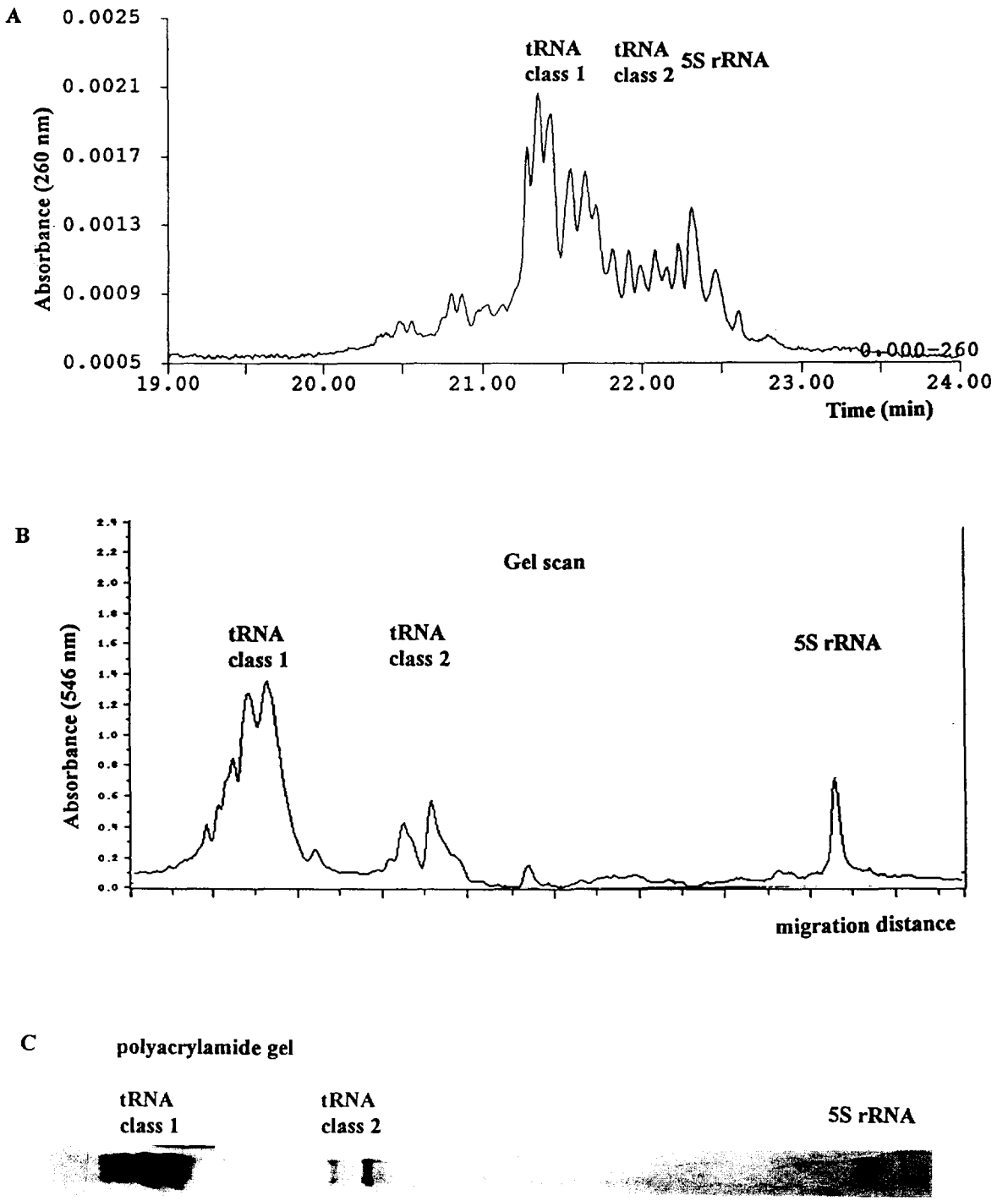

Fig. 6. (A) Separation of low-M_r RNA mixture from *E. coli* MRE 600 by CE using entangled polymer solutions with 0.5% HPMC. Capillary: DB-1, 70 cm (62 cm effective length) × 100 μm I.D., 0.1 μm thickness. Buffer: 350 m*M* Tris–borate, 2 m*M* EDTA, 7 *M* urea and 0.5% HPMC (pH 8.6). Injection: −10 kV, 15 s. Field strength: 570 V/cm. Gel scan (B) of the same sample from slab PAGE (C).

could be used (570 V/cm) by the CE using entangled polymer solutions with 0.5% HPMC, reducing the run time to 30 min and producing a very good resolution. This resolution was better than that attained by the CE using entangled polymer solutions with commercial buffers including sieving additives and 118 cm long capillaries (Fig. 4B) as well, as by the gel scans of the conventional slab gel electrophoresis (Fig. 6B). CE using entangled polymer solutions with 0.5% HPMC, therefore, becomes an attractive alternative to the slab gel electrophoresis. The shorter separation times and the potential for automation are additional advantages.

The use of 0.5% HPMC appeared to be more applicable for the separation of RNA than for separation of DNA fragments with small length differences. Comparing the separation of two double stranded (ds) DNA fragments (271 and 281 bp) under the same conditions, Schwartz et al. [20] observed that the separation of two components with a difference of 10 bp is only possible through intercalation of the ds DNA by the addition of ethidium bromide to the sample before electrophoresis. Probably the deformation of the DNA structure, by addition of ethidium bromide, leads to the separation due to interactions within the HPMC network. The deformation of the DNA was explained by the biased reptation theory wherein DNA, in solutions of entangled polymer, moves by repeated stretching, slippage, relaxation and re-extension [25]. Probably, ds DNA molecules, without addition of ethidium bromide, does not undergo all of these changes in conformation.

The different, previously reported model systems (e.g. Ogston model [27]; reptation model [28]; and biased reptation model [25]) for the moving of a passing analyte, particularly DNA, by CGE, as well as by CE using entangled polymer solutions may not be directly applicable for the CE separation of low-M_r RNA. The most important separation parameter appeared to be, besides mass and charge, the spatial structure, as well as the changes in conformation and the interaction with the appropriate separation network.

4. Conclusions

The separation of low-M_r RNA by CE using entangled polymer solutions, as well as by CGE, has shown that resolution of low-M_r RNA differs from the resolution of DNA molecules of comparable size. CE using entangled polymer solutions was reliable because of the improved resolution of low-M_r RNA molecules as well as the facility of handling. This CE method was more convenient and yielded more reproducible results than those obtained when gel-filled capillaries were used. The use of CE using entangled polymer solutions with HPMC as polymer additive was suitable for routine analysis of low-M_r RNA profiles (high resolution, reproducibility, system automation, high stability of the buffer, low cost of the capillaries). In this respect, the CE using entangled polymer solutions can represent a realistic alternative to the conventional slab gel electrophoresis. This approach can be of particular importance for future studies in molecular microbial ecology and taxonomy.

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