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Monitoring viral DNA release with capillary electrophoresis

Svetlana M. Krylova,[a] Dmitry Rozenberg,[a] James W. Coulton[b] and Sergey N. Krylov*[a]

[a] *Department of Chemistry, York University, Toronto, Ontario, Canada M3J 1P3. E-mail: skrylov@yorku.ca*

[b] *Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada H3A 2B4*

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Viral DNA injection into host cells is one of the primary mechanisms of viral propagation. Drug development that targets viral propagation requires fast and sensitive methods for monitoring the release of viral DNA *in vitro*. Here we demonstrate the use of capillary electrophoresis (CE) for monitoring DNA release from virus particles. As a model for this study, we used T5 bacteriophages that infect the bacterium *Escherichia coli* K-12 by binding to the outer membrane FhuA receptor and then injecting DNA. DNA release from the T5 phages *in vitro* was induced by either elevated temperature or by interaction with the purified FhuA receptor. After DNA release, the viral samples were stained with the high affinity fluorescent dye YOYO-1, injected into the capillary and subjected to electrophoresis. YOYO-1-stained DNA generated a well-defined peak, allowing reliable detection of viral DNA from as few as 10^5 viral particles. The staining to track T5 phage DNA release exemplifies the great versatility that CE offers in studying viral systems. This CE-based method can be used to study molecular mechanisms of viral infections and to evaluate anti-viral drug candidates.

Introduction

T5 phages, members of the *Siphoviridae* family, depend on their host *Escherichia coli* to initiate the process of DNA release from the phage. For DNA release to occur, T5 phages must bind irreversibly to the outer membrane ferrichrome receptor FhuA of *E. coli*, by virtue of the T5 phage protein pb5 located on the distal end of the T5 tail. Upon T5 binding of FhuA, the T5 double stranded DNA is transferred linearly through the porin-like receptor.[1] The intriguing aspect of the T5 phage–*E. coli* system is that the release of the T5 double stranded DNA (121 kb) can be induced *in vitro* without the presence of *E. coli*, as long as the isolated FhuA receptor is maintained in an environment that mimics the bacterial membrane.[2] The limiting step in DNA ejection from T5 is the interaction of the T5 phage with FhuA. In the presence of nanomolar concentrations of FhuA,[2] the actual process of DNA release takes place within a few seconds. FhuA (78.9 kDa) belongs to a family of high-affinity transporters of the outer membrane of Gram-negative bacteria that aid in the transport of nutrients present at low concentrations in the environment.[3] FhuA also acts as a receptor for the bacteriophages T1, T5, and Φ80.[4]

Inhibiting viral DNA release is one of the methods by which viral infection can be controlled. The development of therapeutic agents targeting this essential requirement in the infectious pathway requires analytical methods for fast and sensitive control of DNA release from virus particles. The study of intact viral particles was examined previously using flow cytometry.[5,6] Brussaard *et al.* illustrated the potential to detect and discriminate between several virus families, including the *Siphoviridae* family, using flow cytometry with the aid of highly fluorescent nucleic acid intercalating dyes.[5] Dominique *et al.* illustrated the efficiency of flow cytometry over the other methods. Transmission electron microscopy and electrostatic force microscopy were used to enumerate sea-water viruses.[6] Flow cytometry is a valuable tool for distinguishing qualitatively and quantitatively between the various virus particles with the aid of fluorescent dyes and epifluorescence microscopy. The main drawback of flow cytometry is the inability to differentiate between infectious and non-infectious particles due to the limitations imposed on separating the viral components (*i.e.*, protein and nucleic acid).[5]

More recently, Kenndler's group studied in great detail the labelling properties of the human rhinovirus serotypes 2 and 14 with an RNA fluorescent dye, RiboGreen.[7] These authors used capillary electrophoresis (CE) to assess the quality of RiboGreen fluorescence with various incubation periods and to monitor RNA release from the human rhinoviruses (HRVs). In our work, a similar approach was applied to study the release of DNA from a different type of virus, T5 bacteriophage. In contrast to Kenndler's work, however, we chose not to label DNA intra-virally, so that DNA release could be induced in a more native environment. DNA was labelled after it was released from viral particles. Labelling of DNA after it was ejected ensured that extraneous factors such as structural modifications to the phage by the dye and temperature-caused degradation of the dye were prevented. Our work, along with that of Kenndler, shows that CE can be a highly versatile technique for studying viruses using various labelling methods.

Experimental

Materials

The T5 bacteriophages were obtained from the American Type Culture Collection (Manassas, VA). Deoxyribonuclease I from bovine pancreas and all buffer reagents were obtained from Sigma–Aldrich (Oakville, ON). The ferrichrome receptor FhuA was isolated and purified as described below. Fused-silica capillaries were purchased from Polymicro (Phoenix, AZ). YOYO-1 was obtained from Molecular Probes Inc. (Eugene, OR). All solutions were prepared using Milli-Q-quality deionized water and filtered through a 0.22 μm filter (Millipore, Nepean, ON).

Preparation of T5 phages and YOYO-1

The freeze-dried viral samples (1×10^9 phages/pellet) were initially diluted in 400 μL of deionized water. Centrifugation at

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1800 × *g* for 3 min was carried out to allow for the separation of the viral particles from the impurities, which were decanted. The viral particles present in the supernatant were used throughout the experiments. Viral particles were stored in a refrigerator at 4 °C.

YOYO-1 stock solution (1×10^{-3} M) was initially diluted 7.5 times in DMSO to a concentration of 1.33×10^{-4} M. The working concentration of YOYO-1 in all the samples was 6.3×10^{-6} M. To prevent photo bleaching of YOYO-1, its solutions were kept in the dark.

Isolation and purification of FhuA

FhuA, over-expressed by *Escherichia coli*, was extracted from cells by 1% lauryl dimethylamine oxide (LDAO) and purified on tandem columns using immobilized metal affinity chromatography: Ni-NTA Superflow (QIAGEN) and POROS MC-20. Preparations were assessed for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by silver staining; only one band was observed for up to 750 ng of FhuA. These protein preparations are crystallization grade.[8] The stock solution of 1.79 mg mL^{-1} FhuA was prepared in 50 mM ammonium acetate at pH 8.0 supplemented with 0.5% dimethyldodecylamine oxide (DDAO) (AD buffer) and kept at 4 °C for at least 8 months without a significant decrease in the receptor's activity.

Temperature-induced DNA release

To study temperature-induced DNA release, the T5 phage samples (20 μL) were heated for 3 min at either 65 °C or 75 °C or 85 °C in the Mastercycler Personal PCR (Eppendorf, Hamburg, Germany) followed by a 5-min incubation at 25 °C. The released DNA was stained with YOYO-1 by adding 1 μL of 1.33×10^{-4} M YOYO-1 in DMSO and incubating at room temperature for 3 min. The solution was then centrifuged at 1800 × *g* for 1 min to remove any precipitates from the viral preparation. The solution was decanted and subjected to CE analysis. The release of DNA was followed by the appearance of a new peak in the temperature-treated samples compared to the non-treated samples.

The nature of the new peak was confirmed by treating the DNA released, YOYO-1 stained sample with DNase I. DNase I cleaves dsDNA into a complex mixture of mononucleotides and oligonucleotides,[9] which hinders the intercalation of YOYO-1 with dsDNA. Therefore, if the new peak corresponds to DNA, it should disappear upon sample treatment with DNase. One μL of 4.0 μg μL^{-1} DNase in DNase buffer (90 mM Tris-HCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.4) was added to 9 μL of the heated viral sample and the resulting mixture was incubated at room temperature for 5 min prior to CE analysis.

FhuA-induced DNA release

The FhuA receptor requires an environment that mimics the outer bacterial membrane. Such an environment can be achieved by introducing a detergent into the buffer. In this work we used AD buffer,[8] which is 50 mM ammonium acetate at pH 8.0 supplemented with 0.5% DDAO detergent. To study FhuA-induced DNA release, 10 μL of varying concentrations of FhuA in AD buffer was added to 10 μL of the T5 phage sample to give final concentrations of FhuA of 85 μg mL^{-1}, 170 μg mL^{-1}, and 425 μg mL^{-1}. The mixture was incubated at room temperature for 5 min. To stain the released DNA with YOYO-1, 1 μL of 1.33×10^{-4} M YOYO-1 was added to 20 μL of the FhuA-treated phage sample and the mixture was incubated at room temperature for 3 min. The solution was then centrifuged at 5000 rpm (1800 × *g*) for 1 min in order to remove precipitates from the viral preparation. Finally, the YOYO-1-stained sample was subjected to CE. Release of DNA was followed by the appearance of a new peak that did not exist in the control sample devoid of FhuA. The nature of the peak was confirmed by degrading DNA with DNase I using a procedure similar to that described in the previous section.

Capillary electrophoresis

CE analyses were performed with a P/ACE MDQ instrument (Beckman–Coulter, Missisauga, ON). The running buffer was 25 mM sodium tetraborate at pH 8.3 supplemented with 10 mM SDS. SDS improves the separation the of DNA peak from the "background" peaks, most likely by solubilizing viral proteins and preventing their absorption to the capillary wall.[10] Separation was carried out in a positive polarity mode (positive electrode at the injection end) with an electric field of 400 V cm^{-1}. The capillary used had an inner diameter of 75 μm, outer diameter of 150 μm, a total length of 50 cm and the length to the detector of 39.8 cm. Samples were injected by a pressure pulse of 0.5 psi (3.45 kPa) × 5.0 s; the corresponding length and volume of the injected plug being 7.0 mm and 31.5 nL, respectively. The sample storage temperature was maintained at 4 °C and the capillary cartridge temperature was held at 25 °C.

Results and discussion

T5 phage preparation and YOYO-1 labeling

Examination of the various staining protocols used to label viruses for CE,[7] flow cytometry,[5] or microscopic analysis,[11] reveals extensive effort and time required for each sample preparation. In our work with T5 phages, we were able to avoid numerous microbiological techniques required to prepare the viral samples by using a commercial source of T5 phages. Capillary electrophoresis allowed rapid detection of commercially prepared T5 phages, even when the number of virus particles was as low as 10^5. Most importantly, the labeling protocol was minimized to less than 5 min with sufficient fluorescent intensity, a drastic difference from the time-consuming methods employed in other studies.

To label DNA released from virus particles, we used a DNA-intercalating fluorescent dye, YOYO-1, which increases its quantum yield approximately 100–1000 fold upon intercalating with double-stranded DNA (dsDNA).[12] YOYO-1 has been used successfully by staining sea-water viral samples to identify and enumerate specific strains of bacteria.[11] The ability of YOYO-1 to penetrate the viral protein capsid was also examined using the M13 bacteriophage, demonstrating that varying incubation temperature (6, 24 and 38 °C) had no effect on the fluorescent signal generated from the phage.[13] In our work, YOYO-1 was added to the suspension of T5 phages after the virus was subjected to either temperature or FhuA receptor induced DNA release. We adopted a different approach from the intra-viral labeling that many groups have chosen to use for viral staining for several reasons. YOYO-1 labeling following DNA release eliminates any extraneous environmental factors that could hinder the process of DNA release. Secondly, the stability of YOYO-1 is known to be both temperature- and light-sensitive.[12] As a consequence, intra-viral labeling prior to the heating experiments would not be feasible. Also, the time saved on labeling the samples post DNA release was very significant.

Temperature-induced DNA release

The thermodynamic properties of viral infection have been studied previously using differential scanning calorimetry and temperature scanning rotational viscosimetry to monitor dsDNA output from the viral capsids of several phages (S_d, DDVI).[14] Okun *et al.* studied the conformational changes in

several strains of human rhinoviruses upon exposure to elevated temperatures using CE.[15]

In our experiments, we attempted to use a simplified, qualitative approach to assess whether heat induced DNA release had occurred. For qualitative comparisons, a baseline control was established for the unheated, unstained T5 phage sample (Fig. 1, trace 1). Although the exact nature of the fluorescent species with a migration time of 4–7 min (Fig. 1, trace 1) was uncertain, there was consistent, indirect evidence that the fluorescent signal could be a result of some form of fluorescent contaminant as it was present in all sample preparations of T5. When the virus mixture was incubated for 3 min at elevated temperatures and then stained with YOYO-1 according to the protocol described in the experimental section, no significant changes in electropherograms were observed from the control sample of T5 phages (Fig. 1, trace 2) with temperatures below 75 °C (not shown). When the temperature exceeded 75 °C, a new peak with a migration time of approximately 10 min was observed in the electropherogram (Fig. 1, trace 3). There was no significant difference in electropherograms obtained from samples pre-heated at 75 and 85 °C. To ensure greater conformational stability of the released dsDNA, we chose to perform DNA release by heating the sample at 75 °C; 85 °C is closer to the dsDNA melting temperature.

We ascribed the appearance of the new peak to T5 dsDNA stained with YOYO-1, which was confirmed by peak disappearance (Fig. 1, trace 4) upon treating the reaction mixture with DNase I. DNase I digests dsDNA into mononucleotides and oligonucleotides,[9] hindering the intercalating YOYO-1 dye with the dsDNA. A minimum ratio of 5 : 1 DNA base pairs to dye molecules is required for effective fluorescence and an avoidance of dye precipitation.[12]

It turns out that the electrophoretic mobility of YOYO-1 (not shown) is very similar to that of the T5 phages (Fig. 1, traces 2–4). Thus, the nature of the larger and broader peak at around 12 min is speculative. The peak could be composed of three factors: (i) complex formed between YOYO-1 and dsDNA within the T5 phage, (ii) intrinsic fluorescence stemming from YOYO-1, and (iii) interaction of YOYO-1 with the T5 phage protein capsid. Further investigation on the nature of the peak was not carried out primarily because the main objective of the temperature experiment was a qualitative monitoring of DNA release in order to establish an approximate migration time for the FhuA induced DNA release.

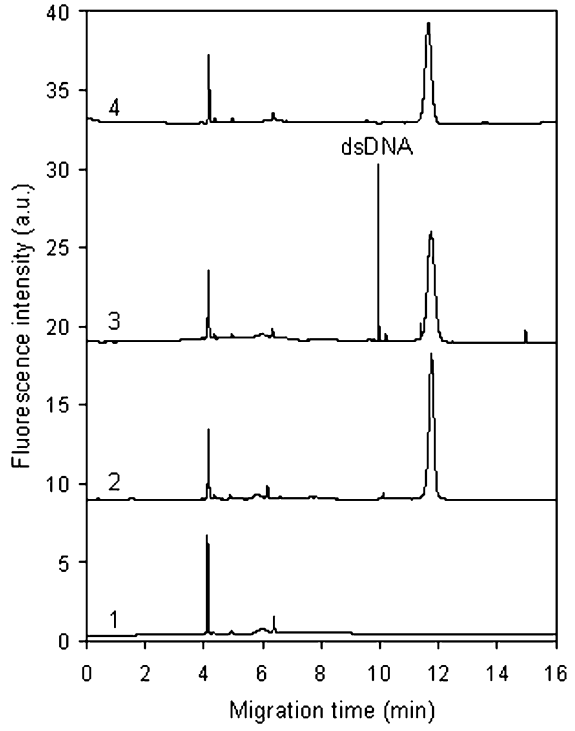

Fig. 1 Temperature-induced DNA release from T5 phages studied with CE-LIF. T5 phages in water (trace 1), T5 phages in water/YOYO-1 (trace 2), T5 phages in water heated for 3 min at 75 °C and then stained with YOYO-1 (trace 3), sample used for trace 4 after DNA decomposition with DNase I. The running buffer was 25 mM sodium tetraborate at pH 8.3 supplemented with 10 mM SDS.

FhuA-induced DNA release

Results similar to temperature-induced DNA release were obtained when DNA release was induced by the FhuA receptor. Using spectrofluorimetry, Boulanger *et al.* demonstrated that the interaction of 30–220 nM concentrations of FhuA with T5 phage resulted in a release of 90 ± 8% of the phage DNA.[2] A similar approach to DNA release was taken in the present study by using a solution of the receptor in AD buffer. The control established that the presence of AD buffer on its own did not induce DNA release from the T5 phages (Fig. 2, trace 2). Release of the T5 dsDNA was induced by the presence of a million-fold excess of FhuA relative to the number of T5 phages (Fig. 2, trace 3). From the experimental results, it was apparent that the three excess FhuA concentrations gave similar results in terms of the amount of DNA released. Thus, the moderate concentration of 170 μg mL^{-1} of FhuA was chosen for practicality purposes. Two peaks appeared upon FhuA-induced DNA release, both of which were assumed to correspond to viral DNA. To confirm the nature of the DNA peaks, we treated the reaction mixture with DNase I and observed the disappearance of the peaks (Fig. 2, trace 4) in a similar manner to the temperature-induced DNA release (Fig. 1, trace 4). Also, the migration time for free DNA was identical for both sets of experiments, temperature-induced and FhuA-induced, being close to 10 min. This is another strong indicator that DNA release had in fact taken place.

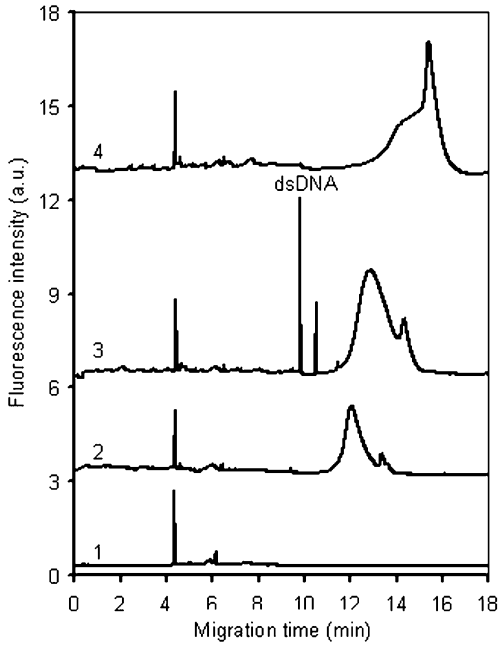

Fig. 2 FhuA-induced DNA release from T5 phages studied with CE-LIF. T5 phages in water/AD buffer (trace 1), T5 phages in water/AD buffer/YOYO-1 (trace 2), T5 phages in water/AD buffer incubated with 170 μg mL^{-1} of FhuA and then stained with YOYO-1 (trace 3), sample used to obtain trace 3 after DNA decomposition with DNase I (trace 4). The running buffer used 25 mM sodium tetraborate at pH 8.3 supplemented with 10 mM SDS.

The electropherogram (Fig. 2, trace 1) demonstrates the presence of the fluorescent contaminants with a migration time of 4–7 minutes, closely resembling that of the samples in the temperature experiments (Fig. 1). The presence of AD buffer, essential to mimic the hydrophobic environment required by FhuA, reduces the resolution of the peak believed to correspond to the T5 phages and the intrinsic fluorescence of YOYO-1 with a migration time of 12–13 min (Fig. 2, trace 2). Attempts were made to improve the resolution of the peak corresponding to the T5 phages by reducing the proportion of AD buffer used in the samples. However, a smaller proportion of AD buffer resulted in the inactivity of FhuA or a great decrease in the reproducibility of FhuA-induced DNA release. Also, the addition of DNase (Fig. 2, trace 4) had a drastic effect on the migration time and structure of the prominent peak corresponding to T5 phages in the presence of AD buffer, which was not apparent in the temperature induced sample (Fig. 1, trace 4). This indicates that the T5 phage sample in AD buffer is sensitive to minor environmental alterations.

Conclusions

DNA release, an essential step in the infectious process, may be monitored simply and rapidly with the use of LIF-CE. Our CE-based method illustrates the rapidity with which viral systems can be studied. Time-consuming viral propagation procedures can be avoided as commercially available viral samples can be detected, even when the number of virus particles is as low as 10^5. Also, laborious staining protocols with lengthy incubation times (24–48 h) are not necessary, as our experiments showed, with a slight sacrifice in fluorescent intensity. Most importantly, many microbiological techniques can be avoided in studying viruses with the use of CE since many systems can be studied *in vitro* with commercially isolated system components. The reduction in biochemical procedures allows for an increase in data reproducibility. We plan to undertake a quantitative approach in the future, when a suitable competitive inhibitor of FhuA binding to the virus is available. One possibility that is under consideration is the development of DNA aptamers as prospective competitive inhibitors to the virus. Thus, LIF-CE has a great deal of potential for the identification of infectious viruses and the development of therapeutic agents that can act as inhibitors for the evolutionary process of DNA release.

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