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Detection of Green Fluorescent Protein in a Single Bacterium by Capillary Electrophoresis with Laser-Induced Fluorescence

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Green fluorescence protein (GFP) is a common reporter used to monitor protein expression in single cells. However, autofluorescence from endogenous components can mask the signal from GFP, particularly at low expression levels in prokaryotes. We employ capillary electrophoresis with laser-induced fluorescence for the analysis of the expression of green fluorescent protein in a single bacterium. Capillary electrophoresis separates GFP from native cellular autofluorescent components, reducing the background signal and improving detection limits. Our system provides 100 ymol (60 copies) limits of detection for GFP. To demonstrate the performance of this instrument, we employ a model system of *Deinococcus radiodurans* that has been engineered to express GFP under the control of the *recA* promoter. We report resolution and detection of GFP and autofluorescent components in a single *D. radiodurans* bacterium. This paper presents the first example of expression of GFP in *D. radiodurans* and the first detection of GFP in a single bacterium by capillary electrophoresis.

Most proteins are present at low levels in bacteria; up to 80% of the *Escherichia coli* proteome is expressed at fewer than 1000 copies/cell.[1] Recent studies indicate that stochastic expression of low-abundance proteins has significant consequences for phenotypic variation within a clonal population.[2−5] Most current efforts to monitor protein expression in a single bacterium rely on the detection of fluorescent reporters by flow cytometry[6] and fluorescence microscopy.[7−9] In general, these methods are useful for studying highly expressed proteins; however, variations in the background fluorescence from autofluorescent cellular components determines the detection limits for the component of interest, preventing detection of low-abundance proteins.[10,11]

Several strategies have been employed to distinguish autofluorescence from fluorescent protein expression; these methods were recently reviewed by Billinton and Knight.[12] Optical resolution methods, including spectral discrimination and laser scanning microscopy, can induce photobleaching and require expensive instrumentation and data analysis tools. Xie and co-workers recently reported two fluorescence microscopy methods that achieve single-molecule detection of gene expression in individual *E. coli* cells by physical segregation of the fluorescent target and autofluorescent components. In one, β-galactosidase is used as a reporter, and extracellular fluorescent products are detected.[13] Single-molecule detection of yellow fluorescent protein is achieved by targeting the fluorescent protein to the cell membrane.[14] In both methods, intracellular autofluorescence prevents analysis of cytoplasmic fluorescent proteins. To our knowledge, no method has been reported that is capable of rapid resolution and detection of low-abundance intracellular fluorescent components.

Capillary electrophoresis is a well-established tool for studying the contents of single eukaryotic cells.[15] Green fluorescent protein (GFP) expression has been monitored by capillary electrophoresis in single kidney[16] and 4T1 cancer cells.[17] High-speed separation by capillary electrophoresis efficiently resolves fluorescent proteins from autofluorescent components; detection by ultrasensitive laser-induced fluorescence provides subzeptomole limits of detection and a dynamic range over 4 orders of magnitude.

The earlier studies on analysis of GFP expressed in single cells were performed on eukaryotic cells. This report considers the first example of the use of capillary electrophoresis for the analysis of GFP expressed in a bacterium; we study *Deinococcus radiodurans* in this paper. The mass of this prokaryote is 3 orders of magnitude smaller than the mass of a typical eukaryote cell, with a total protein content of roughly 30 amol,[18] and characterization of a

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single cell requires significant improvement in instrumental sensitivity. In this paper, we describe instrumentation capable of precise injection of a single bacterium by reducing the capillary inner diameter; this reduction also minimizes sample dilution during on-column cell lysis and proportionally reduces the Raman background signal. Etching the detection end of the capillary reduces the outer diameter, improving hydrodynamic focusing in the sheath flow cuvette and reducing the detection volume. With electrophoretic separation, enhanced green fluorescence protein (EGFP)[20] is resolved from cellular autofluorescent components and is detected at the yoctomole level. This technology is applicable to the analysis of any fluorescently tagged protein of interest in a single bacterium and has the potential to resolve post-translational modifications of GFP-tagged proteins.[19]

For this work, we constructed a strain of *D. radiodurans* that expresses EGFP under the control of the *recA* promoter. *Deinococcus* is the most radiation-resistant species known, capable of nonmutagenic recovery from hundreds of double-strand DNA breaks.[21] As a result, study of its DNA repair mechanism has attracted significant attention. RecA protein is involved in the induction of the SOS pathway[22] and has been implicated as key to the extraordinary DNA repair mechanism in this organism.[23−25] This DNA repair machinery tends to disrupt efforts to genetically engineer this organism; this is the first report of the successful expression of GFP in this organism.

EXPERIMENTAL SECTION

Reagents. Tryptone and yeast extract were purchased from Becton Dickinson (Franklin Lakes, NJ); phosphate-buffered saline (PBS) was purchased from Invitrogen (Carlsbad, CA); recombinant EGFP protein was purchased from Clontech (Mountain View, CA). Unless otherwise noted, all other reagents were acquired from Sigma-Aldrich (St. Louis, MO). All solutions were prepared in deionized water (Millipore, Bedford, MA), filtered with a Millipore 0.22-μm sterile vacuum filter, and centrifuged prior to use. The enzymatic cell lysis buffer, CelLytic B Plus, was freshly prepared each day according to manufacturer's protocol.

Bacterial Culture. All *D. radiodurans* strains were grown in rich liquid media (TGY (per liter): 5 g of tryptone, 1 g of glucose, 3 g of yeast extract) at 30 °C with mild shaking (251 rpm). Growth medium for the MaHa01 strain was supplemented with selective antibiotic (8 μg/mL kanamycin). The cells were harvested after overnight growth, and analyzed in the exponential growth phase (optical density, 0.5 at 600 nm). Prior to analysis, all cells were washed five times with PBS to remove the growth media. Cells were stored in PBS at room temperature during analysis.

MaHa01 Strain Construction. The MaHa01 strain of *D. radiodurans* expresses EGFP under the control of the *recA* promoter. Briefly, DNA sequences containing the aminoglycoside 3′-phosphotransferase gene for kanamyacin resistance and a multiple cloning site from pUC4K (Amersham Biosciences, Piscataway, NJ) were ligated into vector pHMR137 (kind gift from Dr. Mary Lidstrom). pHMR137 contained a pullulanase integration cassette and the necessary genes for selection and amplification in *E. coli*. A *D. radiodurans* ribosomal binding site (rbs) from pMTL23lacZrev (gift from Dr. Mary Lidstrom) was ligated in front of the EGFP gene in pEGFP (BD Biosciences, San Jose, CA). After amplification of rbs-EGFP in *E. coli*, the fragment was ligated with the pullulanase cassette in pUC4K-pHMR137. The vector was inserted into *D. radiodurans* strain 164.39 (kind gift from Dr. Mary Lidstrom) using the BioRad Gene Pulser II (Hercules, CA); insertion was confirmed by DNA sequencing. The *D. radiodurans recA* promoter was amplified by PCR from a section 600 bp long upstream of the open reading frame containing *recA*, using *D. radiodurans* genomic DNA as a template (ATCC, Manassas, VA). The *recA* promoter sequence was inserted into the pullulanase integration cassette and double-crossover transformation confirmed by antibiotic screening and PCR on the transformant's genomic DNA. Primer sequences are listed in the Supporting Information.

Cellular Homogenate. Cellular homogenate was prepared from each strain. Cells in the exponential growth phase were washed five times in PBS and resuspended in 1 mL of lysis buffer for 5 min at room temperature. The lysis buffer, CelLytic B Plus, contains a proprietary mixture of nonionic detergents, lysozyme, benzonase, and protease inhibitors. The suspension was centrifuged for 5 min at 21000*g* to remove debris, and the supernatant was collected for analysis.

Capillary Electrophoresis Instrumentation. All capillary electrophoresis separations were performed on a locally constructed instrument similar to those previously described.[26,27] Electrophoresis was driven by a high-voltage power supply (CZE1000R, Spellman, Hauppauge, NY). Fluorescence was excited with a 10-mW, 488-nm argon ion laser (2211-15SL, Uniphase, Milpitas, CA) focused through the sheath-flow cuvette with a 6.3×, 0.20 NA objective (Melles Griot, Rochester, NY). Fluorescence emission was collected by a 60×, 0.7 NA objective, filtered through a 500−540-nm band-pass filter (Omega Optical, Brattleboro, VT), imaged through an adjustable iris onto a GRIN-lens coupled fiber optic that was coupled to a single-photon counting module (SPCM-AQR-13FC, Perkin-Elmer). Data were acquired at 100 Hz by a PC running LabView software. Data were processed in Matlab; the data were first treated with a 7-point median filter and then convoluted with a 76-point Gaussian function with a 9-point standard deviation.

Capillary Etching. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) with 150-μm o.d, 10-μm i.d, and 30-cm length were used for all separations. Hydrofluoric acid etching was applied at the detection end of the capillary to reduce the outer diameter and improve hydrodynamic focusing in the sheath-

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flow cuvette.[28,29] The tip of the capillary was cut flat and 3 mm of the outer polyimide coating removed with a gentle flame. The capillary was continuously purged with hexane at 60 psi to protect the inner surfaces from HF. The capillary tip was suspended 1 mm above a fresh solution of 40% HF in a hood. After 90 min of vapor etching, the outer diameter of the capillary was reduced to 20−30 μm, while the inner diameter remained 10 μm. Immediately following etching, the capillary was submerged in a saturated sodium carbonate solution to neutralize residual HF. The capillary was purged for 30 min with absolute ethanol and then with water. The quality of the etching was verified with a microscope under 40× magnification.

Safety Considerations. Hydrofluoric acid is highly toxic and corrosive. Heavy-duty nitrile gloves, face shields, and lab aprons must be worn at all times. Latex gloves are not impermeable to HF. HF is incompatible with all glass, ceramic, and metal containers. Calcium gluconate gel must be available for first aid treatment, and all unprotected exposures should be treated as emergencies. HF should be neutralized with saturated sodium carbonate solution and disposed of according to local regulations.

Cell Lysis and Injection. Mammalian cell lysis typically employs a combination of surfactants and osmotic shock to release cellular contents. This protocol fails for many bacteria and plant cells, which have very robust cell walls. We employ a commercial lysis reagent (CelLytic B plus), which combines enzymatic and surfactant lysis components. The enzyme, lysozyme, digests peptidoglycans in the cell wall, which allows cell lysis from the action of a proprietary nonionic detergent. Benzonase is also included in the lysis reagent to free nucleic acid-bound proteins.

The injection apparatus used for single-bacterium injection has been described previously[30] and modified for this work. A vacuum hand pump (Nalge Nunc, Rochester, NY) was connected to the siphon valve to provide adequate siphon through the 10-μm-inner diameter capillary. Injection was monitored using an inverted fluorescence microscope with 40× objective and 10× eyepiece (IX-70 Olympus). On a Teflon-coated microwell slide, a 3-μL aliquot of enzymatic lysis buffer was placed in a well next to a 3-μL aliquot of cell suspension. First, a plug of lysis buffer was aspirated into the capillary with a siphon pulse (−10 mmHg, 3 s). The capillary tip was lowered using a micromanipulator to the center of the field of view. A bacterium of interest was identified and positioned below the capillary tip using red illumination to minimize photobleaching; bacterial fluorescence was verified using a FITC 460−490-nm band-pass filter cube with brief excitation by a mercury lamp. The cell was injected by a siphon pulse (−10 mmHg, 1 s); its injection was confirmed visually under the microscope. A second plug of lysis buffer was injected into the capillary (−10 mmHg, 3 s). The capillary tip was held in running buffer for 5 min; in this time, lysis buffer diffused past the bacterium, causing cell lysis and solubilizing intracellular proteins. After cell lysis, separation voltage was applied at 15 kV (500 V/cm).

Single-Cell Capillary Electrophoresis Separation. Electrophoresis buffer was 10 mM borate at pH 8.9. Between separations, the capillary was purged at 20 psi for 15 min with 0.1 M NaOH, water, and buffer. In blank runs, the cellular supernatant was injected instead of a bacterium; all other procedures remained the same. Electrokinetic injection (2 kV, 2 s) was used to introduce cellular homogenate for separation; all electrophoretic parameters remained the same.

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RESULTS AND DISCUSSION

Detection of EGFP in *D. radiodurans*. In this study, we created a plasmid construct expressing EGFP under the control of the *recA* promoter in *D. radiodurans* (Supporting Information Figure 1). The expression of EGFP has no significant effect on the growth or survival of the strain, as determined by growth curves and survival plating (data not shown).

To determine the limit of detection of the CE-LIF instrument, a standard curve was prepared by injecting EGFP solutions ranging from 10^{-10} to 10^{-7} M (Supporting Information Figure 2). The highest concentration sample generated an intense signal that began to saturate the photon counting photodiode. The manufacturer's calibration chart (Supporting Information Figure 3) was used to correct for this saturation, and the corrected calibration curve was reasonably linear. A plot of logarithm of peak area versus logarithm of concentration had a slope of 0.90 ± 0.08, $n = 4$, $r = 0.992$. Noise was estimated from the standard deviation in the smoothed baseline over a 1-min period. The detection limit (3σ) equaled 2×10^{-12} M injected, corresponding to the injection of ~100 ymol (60 copies). An average of ~10 photons was detected from each molecule of EGFP passing through the laser beam.

The probe volume of our optical system is roughly given by the volume of a sphere with a radius of 5 μm. At the detection limit, there is 1.0 molecule of EGFP present, on average. Of course, this calculation does not imply that we have achieved single-molecule detection; during the 10-ms signal integration period, roughly 20 molecules pass through the laser beam.

Cellular homogenates from wild-type and EGFP-expressing *D. radiodurans* (MaHa01) were prepared to identify components in the CE-LIF electropherogram. A blank injection of the cell lysis buffer was also performed to identify any fluorescent contribution from the buffer components. The electropherograms from wild-type and MaHa01 homogenate and from the buffer blank are shown in Figure 1 A and B, respectively. The buffer blank shows no significant fluorescent signal, Figure 1C, indicating that the peaks detected in the bacterial homogenate separations result from cellular components. The separation profile of the wild-type lysate shows two major peaks. Since the wild-type strain expresses no EGFP, these two peaks derive from autofluorescent components, which should be produced in both the wild-type and EGFP strains. The MaHa01 homogenate separation also shows a set of four peaks that do not appear in the wild-type separation, indicating that these peaks are related to expression of EGFP. As noted by Krylov, GFP undergoes intercellular proteolysis, producing several electrophoresis peaks from the product of a single gene.[17]

Single-Bacterium Analysis. Previous studies using Western blots have shown an average constitutive *recA* expression in *D. radiodurans* on the order of 10^4 copies/cell,[25] well within the detection range of our instrument. By using a 10-μm-i.d. capillary, injection of a single bacterium can be performed with rapid on-column enzymatic lysis and limited sample dilution. Single-bacterium injection is verified with the use of a fluorescence

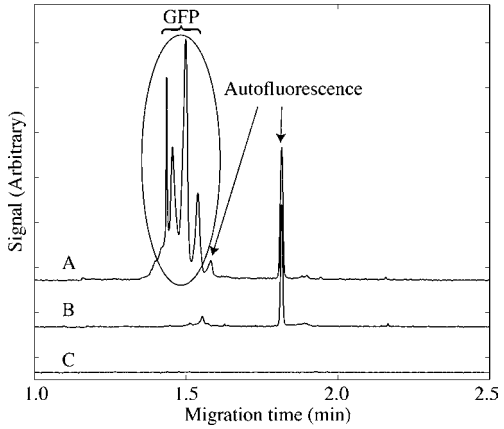

Figure 1. CZE separation of *D. radiodurans* cellular homogenate. Homogenates were prepared by enzymatic lysis of cells. Electrophoresis was performed with 10 mM borate (pH 8.9) at 500 V/cm. (A) Separation of MaHa01 homogenate. (B) Separation of wild-type homogenate. (C) Separation of lysis buffer. Autofluorescent cellular components are identified by comigration in the wild-type and MaHa01 separations; EGFP peaks are identified as components present in the MaHa01 cells but not the wild-type cells. Traces offset for clarity.

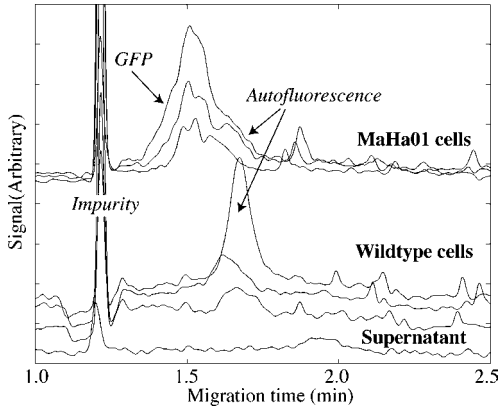

Figure 2. CZE separation of individual *D. radiodurans* cells. Separation conditions are the same as those described in Figure 1. The figure shows data generated from three individual MaHa01 cells, three wild-type cells, and the cellular supernatant. Traces are offset for clarity. For the lowest trace, a blank separation was performed by injecting cellular supernatant from the MaHa01 sample instead of a cell; all other conditions remained the same.

microscope; fluorescence of both wild-type and MaHa01 strains can be observed prior to injection.

Single-bacterium capillary electrophoresis was used to analyze wild-type and MaHa01 cells. For each analysis, a dilute suspension of cells was prepared in PBS; a cell of interest was identified by fluorescent microscopy and injected between plugs of enzymatic lysis buffer. Cellular components were separated with the application of high voltage and detected by laser-induced fluorescence. Figure 2 shows electropherograms from the analysis of three wild-type and three MaHa01 cells. To verify that fluorescent signal results from cellular components, a blank injection of cellular supernatant was also performed. The blank separation indicates that the first peak at 1.2 min results from a fluorescent impurity in the lysis buffer and that the other peaks result from cellular components. This impurity peak is barely detected in the homogenate analysis, which is dominated by the high-concentration cellular components, and may be a residual component from the growth medium.

Autofluorescence generates a peak at 1.7 min, which is partially resolved from the EGFP peak produced by the engineered cells, which is centered at 1.5 min. There appears to be little interference between the EGFP signal and autofluorescence, which allows detection of the EGFP on a very low background. The EGFP peak generated in single cells is broader than that produced by the homogenate, which reflects diffusional band broadening during the 5-min cell lysis step. As a result, we are not able to resolve the EGFP components from a single cell.

The EGFP peak from the three MaHa01 cells generated an average of $(1.8 \pm 1.0) \times 10^5$ photons, corresponding to $\sim 2 \times 10^4$ EGFP molecules/cell, which is remarkably consistent with the literature value for the constitutive expression of *recA* in *D. radiodurans*.[25] This result is interesting. We are expressing EGFP under the control of the *recA* promoter, but we are not expressing the fusion protein between RecA and EGFP. It is likely that translation from mRNA to protein will occur at different rates for RecA and EGFP. The similar number of expressed protein copies suggests that transcription from DNA to mRNA occurs at the same rate for the two genes and controls the overall expression level of the protein.

CONCLUSIONS

We have developed a method for resolving and detecting fluorescent proteins from cellular autofluorescent components in single bacteria by capillary electrophoresis with laser-induced fluorescence. Using this method, we have observed cell-to-cell variation in EGFP expression. This technology is amenable to the analysis of other strains of bacteria; by producing strains with translational fusions between a target protein and a fluorescent tag, quantitative analysis of protein expression in a single bacterium is possible. This separation-based method is especially useful for studying low-abundance proteins that would otherwise be masked by autofluorescence and could be used to study post-translational modification of these proteins. However, slow lysis results in diffusional broadening of components. It would be useful to use fast laser-based lysis in these experiments to allow very high resolution of components.[31]

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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