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A simple protocol for attenuating the auto-fluorescence of cyanobacteria for optimized fluorescence in situ hybridization (FISH) imaging

Perrine Zeller [a,1], Olivier Ploux [b,c], Annick Méjean [b,*]

[a] INSERM U1193, Physiopathogénèse et Traitement des Maladies du Foie, Hôpital Paul Brousse, bâtiment Lavoisier, 14 avenue Paul Vaillant Couturier, 94807 Villejuif Cedex, France
[b] Laboratoire des Energies de Demain, LIED, UMR 8236 CNRS, Université Paris Diderot-Paris 7, 75013 Paris, France
[c] Chimie ParisTech, 75005 Paris, France

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ABSTRACT

Cyanobacteria contain pigments, which generate auto-fluorescence that interferes with fluorescence in situ hybridization (FISH) imaging of cyanobacteria. We describe simple chemical treatments using $CuSO_4$ or H_2O_2 that significantly reduce the auto-fluorescence of *Microcystis* strains. These protocols were successfully applied in FISH experiments using 16S rRNA specific probes and filamentous cyanobacteria.

1. Introduction

Cyanobacteria are ubiquitous photosynthetic microorganisms that produce a wide range of secondary metabolites, including toxins for animals and humans, the cyanotoxins (van Apeldoorn et al., 2007). The presence of toxic cyanobacteria in the environment therefore represents a health risk. Hence, the detection of such toxic cyanobacteria is an important challenge. The catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) technique is a powerful imaging strategy that has been used in several studies to detect unicellular cyanobacteria (Abed et al., 2002; Biegala et al., 2002; Biegala et al., 2003; Biegala and Raimbault, 2008; Dziallas et al., 2011; Gan et al., 2010; Metcalf et al., 2009; Ramm et al., 2012). However, most toxic cyanobacteria are filamentous and possess a thick cell wall that prevents the penetration, into the cells, of large probes linked to horseradish peroxidase (HRP) used in CARD-FISH experiments. Therefore, in these cases, it would be more appropriate to use the non-amplified FISH technique using probes linked to a small fluorophore. However, cyanobacteria contain pigments (chlorophyll a and b, phycocyanin, phycoerythrin, and carotenoids) that produce auto-fluorescence, leading to high background levels, when using epi-fluorescence microscopy. This auto-fluorescence may severely interfere when using the non-amplified FISH technology or even when detecting low copy number mRNA.

Reduction of cyanobacterial cell auto-fluorescence by pigment extraction has been previously proposed (Hosoi-Tanabe and Sako, 2005). We propose here two chemical treatments that are able to reduce the auto-fluorescence of cyanobacteria, and demonstrate their application in FISH experiments.

2. Materials and methods

2.1. Materials

Chemicals were purchased from SigmaAldrich. Milli-Q water was used to prepare all solutions.

2.2. Strains and culture conditions

Microcystis aeruginosa PCC 7806, *Microcystis* sp. PCC 7005, *Microcystis* sp. PCC 7820, *Microcystis* sp. PCC 7813, and *Oscillatoria* sp. PCC 6506 were obtained from the Pasteur Culture Collection of Cyanobacteria (PCC). All strains were grown in BG11 medium under constant light illumination at 20 °C (Osram Universal White fluorescent tube, 30 μmol m^{-2} s^{-1} of photons as measured with a LICOR LI-185B quantum radiometer–photometer equipped with a LI-190SB quantum

Abbreviations: Cy3, cyanine 3; FAM, fluorescein amidite; FISH, fluorescence in situ hybridization; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; TSA-FISH, tyramide signal amplification fluorescence in situ hybridization.

* Corresponding author.
E-mail address: annick.mejean@univ-paris-diderot.fr (A. Méjean).
[1] Present address.

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sensor). Cultures were routinely checked and regularly sub-cultured (Rippka, 1988).

2.3. Cell fixation

Cyanobacterial cells (approximately 10^6 cells in a total volume of 10 mL) were fixed with a filtered 1% (*w*/*v*) solution of paraformaldehyde in 10 mM sodium phosphate, 150 mM NaCl, pH 7.2, for 15 min at room temperature, and then collected by filtration on a Whatman Cyclopore track etched membranes (polyester clear, 0.2 μm pore size, 47 mm diameter). The filter was rinsed with water and the cells were subsequently dehydrated by soaking the filter in absolute molecular grade EtOH at room temperature, for 10 min. Filters were dried and stored at −80 °C until further analysis.

2.4. Cell permeabilization

Filters were thawed and quickly dipped into a warm 0.4% (*w*/*v*) agarose (analytical grade, Promega) solution in water, and dried at 37 °C for 10 min. The filters were then treated with 1 mL (per filter) of a 10 mg/mL solution of lysozyme (47 000 U/mg, Sigma-Aldrich) in 50 mM EDTA, 100 mM Tris–HCl, pH 7.7, at 37 °C for 1 h, and subsequently rinsed three times with water and then dipped into absolute ethanol for 10 min, and dried at 37 °C. Filters were then treated with 1 mL of a 60 U/mL solution of acromopeptidase (3 200 U/mg, Sigma-Aldrich) in 10 mM NaCl, 100 mM Tris–HCl, pH 7.5, at 37 °C for 30 min, and subsequently rinsed three times with water and then dipped into absolute ethanol for 10 min, and dried at 37 °C.

2.5. Chemical treatments to reduce the auto-fluorescence of cyanobacterial cells

All treatments were performed on immobilized cells after permeabilization, and after hybridization for FISH experiments, and just before mounting. Filters were incubated in sterile aqueous solution of $CuSO_4$ (from 0.5 to 20 mg/mL), or in sterile aqueous solution of H_2O_2 (from 1 to 20% *v*/*v*) for 10 min at room temperature. The filters were then soaked in absolute EtOH at room temperature for 10 min, and dried. The filters were then cut in equal portions (1/12) and directly mounted on glass plates using AF1 (Citifluor).

2.6. FISH experiments

After cell permeabilization, filters were cut in equal portions (1/12) and hybridizations were performed as already described in 50% formamide for 30 min at 37 °C (Biegala et al., 2002), using 0.5 ng/μL of probe EUB 338 (5′-GCTGCCTCCCGTAGGAGT-3′) labeled at its 5′-end with fluorescein (6-FAM, Thermo Electron Corporation, for standard FISH) or with HRP (Thermo Electron Corporation, for TSA-FISH). Filters were then rinsed according to Biegala et al., 2002. For standard FISH experiments, filters were treated with H_2O_2 or $CuSO_4$ (as described above), or not treated (control experiment), before mounting. For TSA-FISH experiments, amplification was performed using the TSA Plus Fluorescein Evaluation Kit (Perkin Elmer), and then the filters were treated with H_2O_2 or $CuSO_4$ (as described above), or not treated (control experiment), before mounting.

2.7. Microscopy and data acquisition

Images were acquired using an epi-fluorescence Eclipse Ci-S microscope (Nikon) equipped with a Ci-FL illuminator (Nikon), with an objective (×40 NA 0.75 N Plan Fluor W.D. 0.72 mm, Nikon), with two sets of filters (FITC set: excitation 470 ± 20 nm, emission 525 ± 25 nm; Cy3 set: excitation 545 ± 12 nm, emission 605 ± 35 nm), and with a digital camera (Retiga 2000R). For each filter portion (immobilized cells on 1/12 filter) at least four fields containing from 100 to 2000 cells were acquired at both wavelengths, and the acquired data were averaged. Fluorescence intensities were quantified using the Daime software (Daims et al., 2006). Statistical analyses were performed using the Prism software (GraphPad).

3. Results and discussion

Using epi-fluorescence microscopy we have quantified the auto-fluorescence of the unicellular *M. aeruginosa* PCC 7806, in varying conditions (Fig. 1). We have used two sets of filters that correspond to the excitation/emission wavelengths of the usual fluorophores used in FISH experiments: fluorescein isothiocyanate (FITC) and Cyanine 3 (Cy3). The cells were fixed on membrane filters, treated with different conditions and observed under epi-fluorescence microscopy. Heating the immobilized *M. aeruginosa* PCC 7806 cells at 70 °C for 60 min (which should degrade chlorophylls) did not lead to any significant auto-fluorescence reduction (data not shown), contrary to what was observed with *Synechococcus* strains (Scanlan et al., 1997). We then tested the effect of chemical treatments using H_2O_2 or $CuSO_4$ solutions on immobilized and permeabilized cells prior to mounting (Fig. 1). Previous experiments suggested that H_2O_2 could disrupt cyanobacterial photosystem II (Mikula et al., 2012). Fig. 1A shows the effect of increasing H_2O_2 concentration on the auto-fluorescence of *M. aeruginosa* PCC 7806, and statistical analysis of the data (ANOVA and Dunnett's test)

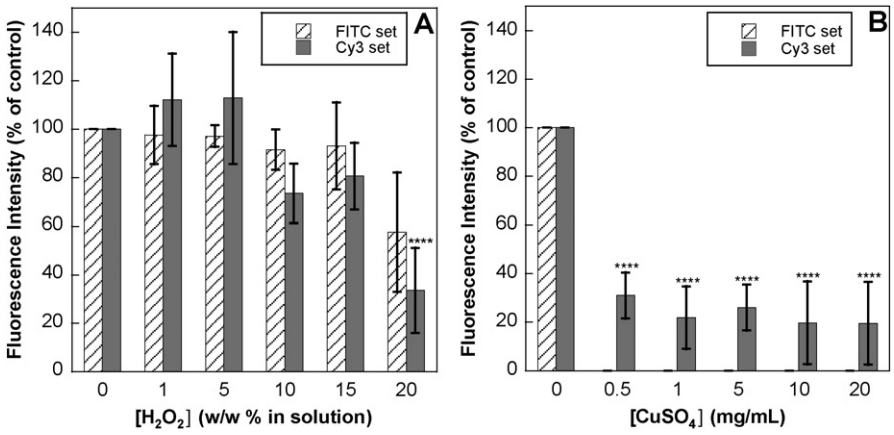

Fig. 1. Effect of H_2O_2 (A) and $CuSO_4$ (B) treatment on the auto-fluorescence intensity of *Microcystis aeruginosa* PCC 7806 cells measured using the FITC set (hatched bars) at and the Cy3 set (heavy gray bars) of filters. Untreated cells were used as the control experiment. Each data point is the average of three independent measurements corresponding to three filters. Bars represent the relative mean fluorescence intensity measured after 5 s acquisition, and error bars represent the standard deviation. Dunett post-test p-values relative to control (no chemical treatment): ****p-value < 0.0001. The intensities measured using the Cy3 set of filters, when the cells were treated with $CuSO_4$, were too low to be quantified.