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Fluorescent Staining Method for Bacterial Endospores

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Abstract

We have developed a quick, simple technique for the fluorescent staining of bacterial endospores. This technique was developed using *Bacillus subtilis* endospores. Our technique requires minimal sample heating so it can safely be carried out entirely in a glove-box. The endospores stain diagnostically as an oval with a standard fluorescent nucleotide stain, acridine orange.

Introduction

Bacterial endospores have been notoriously difficult to stain for fluorescent microscopy. The very morphology that makes them so stable in the environment makes them impenetrable to most traditional and all fluorescent staining regimens. Current world events have sparked an interest in the quick identification of bacterial endospores. We describe a fluorescent staining protocol to diagnostically label the endospores with acridine orange, an inexpensive and stable standard nucleotide labeling dye for fluorescent microscopy.

Acridine Orange (AO) is a popular fluorescent stain for nucleic acids in many cell types. AO is an intercalating dye typically used to differentiate between high molecular weight DNA, which fluoresces green, and low molecular weight RNA, which fluoresces red (1). AO also stains acid mucopolysaccharides and mucin, heavily glycosylated proteins (2, 3). Since AO is a stable and relatively inexpensive dye, we considered it to be an ideal candidate for endospore staining.

Materials and Methods

We grew spores according to the method outlined in Nicholson and Setlow (4). We inoculated 250ml Difco Sporulation Medium (DSM) with *B. subtilis* in a 1 liter flask. DSM consists of 2gm Bacto nutrient broth, 2.5ml 10% KCl, 2.5ml 1.2% $MgSO_4 \bullet 7H_2O$, 375μl 1M NaOH, and up to 250ml glass distilled water. After autoclaving and cooling, 250μl each of 1M $Ca(NO_3)_2$, 0.01M $MnCl_2$, and 1mM $FeSO_4$ were added sterilely. This was grown at 37°C with shaking at 300 rpm for 72 hr. The culture was centrifuged at 10,000g for 10 min at RT. Pellets were resuspended in 4°C sterile distilled water. Total volume of the resuspended pellets was increased to 200ml, and centrifuged again at 10,000g for 10 min at RT. Pellets were resuspended in 200ml 4°C water and stored at 4°C overnight to burst vegetative cells. The spore suspension was centrifuged, resuspended, and centrifuged again at 10,000g at RT using 4°C water. The final volume of the suspension was adjusted to 200 ml with 4°C water, and stored at 4°C. This suspension was diluted to 50% concentration with water to make the working spore solution. Spores were assessed for phase brightness on a Zeiss Axiophot using a Ph3 100x objective.

An aliquot of 10 μl working spore solution was applied to an ethanol cleaned glass slide. Using the pipette tip, the sample was smeared into a circle about 1.5cm in diameter. The slide was placed on a 42°C metal heating block to dry, for approximately 3 min. We used a heating block instead of open flame to accommodate researchers who might want to perform the procedure in a glove box. The results for flaming a sample slide or heating on a 42°C metal heating block were the same. The slide was placed in a Coplin jar containing 50 ml of ice cold MAA (3 volumes methanol, 1 volume acetic acid) for 5 minutes. This is a traditional treatment for plant

chromosomes to displace histones and other DNA protecting proteins, which prevent adequate stain penetration (5). The slide was then washed for 1 min in a Coplin jar with 50 ml ice cold 100% absolute ethanol. The slide was removed from the ethanol and allowed to dry on a 42°C metal heating block, approximately 3 minutes. We applied 10μl of 0.1μg/ml AO staining solution to the area of the spore smear and applied a coverslip. Slides were observed using a Zeiss Axiophot equipped with a BP480/20 excitation filter and LP520 emission filter. All images for this article were acquired using an API Deltavision DV4 deconvolution microscope system equipped with a BP490 excitation filter and LP520 emission filter. Images were deconvolved using API SoftWoRx v3.3.6.

Results and Discussion

The figure panels 1A, B, and C show the results from our treatment (heating, MAA and ethanol), and subsequent AO staining. There is no staining if any of these steps are omitted (data not shown). We varied the time for MAA treatment, but 5 minutes consistently yielded the best results. Increasing the endospore heating time did not alter the results. For the negative control, endospores were air dried onto the glass slide and stained with AO. Negative controls are shown in Figure 1D, 1E, and 1F.

An endospore consists of a complex outer layer of proteins called a spore coat. Inside this layer is the spore cortex, a thick, heavily crosslinked and complex peptidoglycan layer. Next is the membrane, surrounding the dried and stabilized cytoplasm and chromosome material (6). Note that the treated and stained endospores did not stain throughout, but rather in a characteristic green oval shape (Figure 1A and 1B). Vegetative cells stained entirely green, and were easily distinguished from the endospores (Figure 1G). AO is traditionally used as a nucleotide stain, but also has an affinity for acidic polysaccharide protein matrices (1). Bacterial endospores have such a matrix in a thick peptidoglycan cortex just

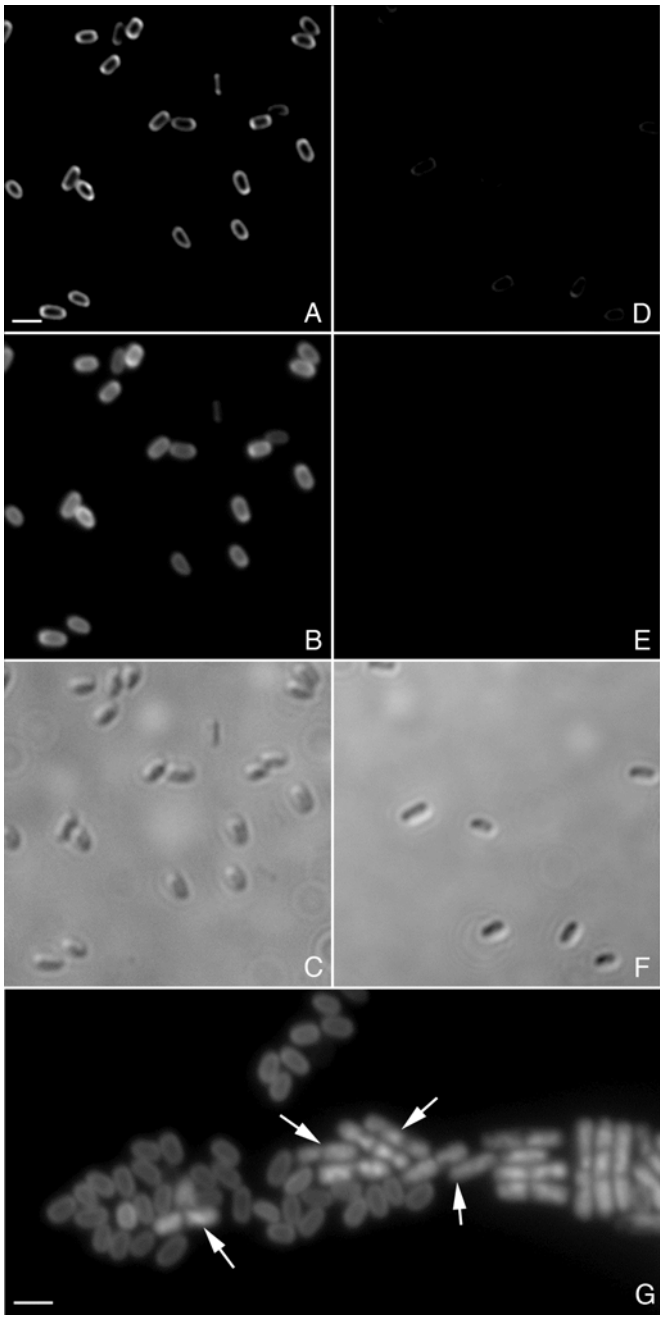

Figure 1. *Bacillus subtilis endospores all stained with AO.* ***A.*** *Deconvolved image of treated endospores.* ***B.*** *Wide-field image of treated endospores.* ***C.*** *DIC image of endospores shown in A and B.* ***D.*** *Deconvolved image of untreated endospores.* ***E.*** *Wide-field image of untreated endospores.* ***F.*** *DIC image of endospores shown in D and E.* ***G.*** *Wide-field image of mixed population of Bacillus subtilis endospores and vegetative cells. Arrows point to some of the vegetative cells. Scale bars = 1µm.*

below the outer protein spore coat (7). The combined action of heating the endospores and treatment with MAA and 100% ethanol has probably disrupted the outer spore coat enough to expose the peptidoglycan layer of the endospore, enabling staining with AO. The peptidoglycan layer is a highly crosslinked, acidic and aromatic structure that may have similar physical properties and attractions as double stranded DNA (8). It is interesting to note that other fluorescent nucleic acid stains, such as DAPI and SYTO also stain the treated endospores in the same oval pattern as AO (data not shown).

We set out to design a quick and easy method to detect endospores that can be performed in a glove box or isolation chamber. It is not acceptable to have an open flame in such a chamber. This protocol is an inexpensive and quick procedure that can be performed in a glove box. No open flame is required, unlike the traditional light microscopy staining method using malachite green (9, 10). While this works for several fluorescent nucleotide staining dyes, we chose AO because it is inexpensive in comparison to other, more specialized dyes such as SYTO, and very stable. This means the testing reagents can be stored in a portable testing facility under minimal storage conditions (dark and room temperature), resuspended in water when time to use, then give quick and easy to interpret results. This works well and predictably for *B. subtilis* endospores.

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