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Differential Spore and Lipid Staining at Room Temperature by Use of Fluorescent Dye

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Several methods have been described for the rapid staining of bacterial spores at room temperature (Lechtman et al., J. Bacteriol. **89**:848). These involve a pretreatment which alters spore permeability, followed by staining, and observation with an ordinary light microscope. Subsequent to this work, it was found that the fluorescent dye auramine O possesses a stronger affinity for spore than for vegetative cell material. Thus, if ultraviolet illumination is available for fluorescence microscopy, a rapid and simple differential spore stain is possible.

For spore staining, a 0.1% aqueous solution of auramine O (Allied Chemical Corp., certification number NAu3) was used. Fresh solutions are recommended, since stored solutions gave a rapid quenching effect of fluorescence upon exposure to ultraviolet light. Smears with vegetative cells and endospores were prepared from 18-hr agar slant cultures of *Bacillus subtilis* var. *niger* (ATCC 9372) incubated at 37 C. Washed, free spores were obtained from older cultures. Staining was accomplished without pretreatment by exposing heat-fixed smears to 0.1% aqueous auramine O for 2 min at room temperature. Since both vegetative-cell material and spores had an affinity for auramine O, a differential step was necessary. Differentiation of spores from vegetative-cell material was accomplished by counterstaining with 0.25% aqueous safranin for 1 min, followed by a very brief water wash. Safranin replaced the auramine O in vegetative-cell material, but did not replace the auramine O adsorbed onto (or into) endospores or free spores. Figure 1a shows a smear stained with auramine O and safranin, as observed with an ordinary light microscope. The endospores appeared unstained; the vegetative cells, red. Figure 1b (bottom) shows the same field as seen with fluorescent microscopy. Endospores and free spores were brightly fluorescent, and the vegetative-cell material was essentially invisible.

It was suspected that auramine O might have a strong affinity for lipid, which could result in the masking of endospores in lipid-rich cells, or in the confusion of lipid granules for endospores. *B. subtilis* var. *niger* was grown for 18 hr on 1% glycerol-nutrient agar slants. These cells were demonstrated to be rich in sudanophilic granules. When such cells were stained with the new spore stain procedure, the lipid granules were only dimly fluorescent. A mixture of 0.1% aqueous auramine O and 3% phenol was prepared as used for the fluorescent acid-fast stain (*Society of American Bacteriologists, Manual of Microbiological Methods*, 1957, McGraw-Hill Book Co., Inc., New York, p. 19). After a 2-min staining with this reagent at room temperature, the lipid granules were brightly fluorescent. Thus, lipid granules would not be confused with spores when aqueous auramine O was used, and the phenolated auramine O resulted in very good lipid staining. In the latter instance, the best differentiation of lipid from cytoplasm was obtained when the time of safranin counterstaining was extended to 2 min.

For fluorescent microscopy, a Reichert, Biozet, microscope was used with an Osram HBO 200-w mercury vapor arc lamp as the source of ultraviolet light. A BG12 exciter filter and a OG1 barrier filter were used. For photography, Royal Pan X film (Eastman Kodak Co., Rochester, N.Y.) was used with a 2-min exposure. For visible-light photography, a built-in 6-v tungsten lamp was used, with a green (Wratten #58) filter. Photography with such a lighting system required 0.25-sec exposure on Royal Pan X film. An ultraviolet-transmitting 1.4 N.A. substage condenser was used for all work. Although a dark-field condenser could be used, it is not necessary when the proper exciter and barrier filters are used together.

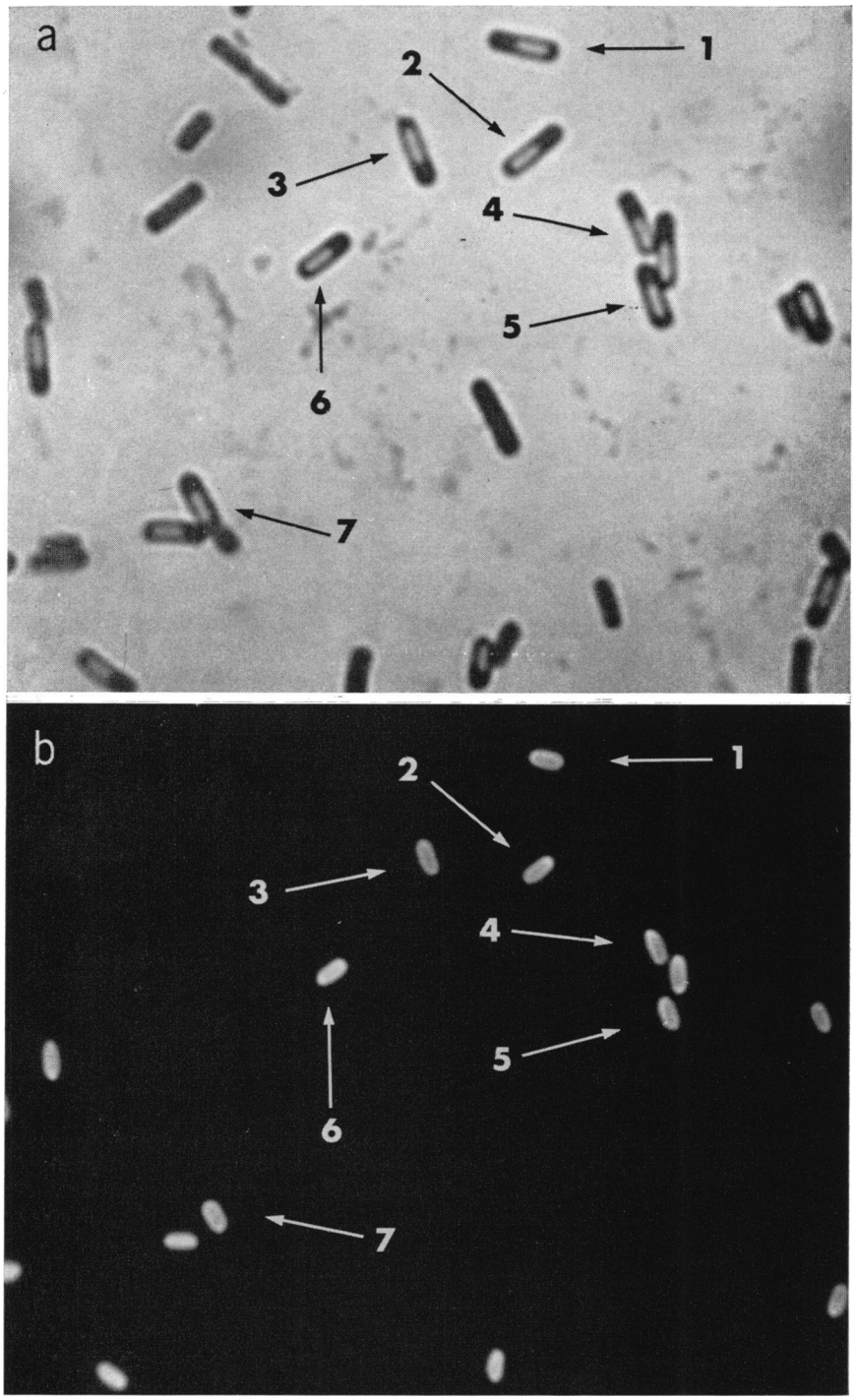

FIG. 1a. *Appearance of endospores of Bacillus subtilis var. niger after staining at room temperature with auramine O, and counterstaining with safranin. The endospores appeared to be unstained; the vegetative cells, red.*

FIG. 1b. *Same field shown in Fig. 1a as seen on exposure to ultraviolet light.* × *2,500.*