Affinity of Cellular Constituents of Two Bacteria for Fluorescent Brighteners

R. W. WEAVER* AND L. ZIBILSKE

Department of Soil and Crop Sciences, Texas A & M University,
College Station, Texas 77843

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Two fluorescent brighteners were used to stain an isolate of Bacillus cereus var. mycoides and a soil pseudomonad. The stained organisms were fractionated by two procedures to determine which cellular constituents were reacting with the brighteners. Both fractionation procedures provided evidence that the brighteners were adsorbed to proteins within the cells. Microscopy examination of ghost cells of the bacillus showed that cell walls were not being stained. Spheroplasts of the bacillus and the pseudomonad were stained by the brighteners.

The study of soil microorganisms is facilitated by the use of fluorescent dyes (9). Darken (2, 3) reported that derivatives of 2,2'-stilbene disulfonic acid stained bacteria and fungi, and fluoresced a brilliant blue when irradiated with ultraviolet light. Anderson and Westmoreland (1) used the same material for making direct counts of fungi in soil.

The staining affinity of the fluorescent brighteners must be known to extend their usefulness in studying organisms in soil. Harrington and Raper (6) determined that the brighteners have an affinity for cellulose. Because bacteria contain little or no cellulose (8), some other cellular component of bacteria must have an affinity for the brightener. Darken (3) stated that the brighteners have an affinity for cellulose and protein, but no supportive evidence was provided. Gull et al. (4, 5) observed that cell walls and protoplasts of fungi stained. If the brightener has an affinity for fungal protein the cytoplasmic membrane may have prevented it from entering the protoplast. The purpose of this paper is to report on the staining affinity of two fluorescent brighteners using a gram-negative and a gram-positive bacterium.

MATERIALS AND METHODS

The fluorescent brighteners used in these studies were Calcofluor M or New 4,4'-bis(4-anilino-6-diaethanolamine-s-triazine-2-ylamino)-2,2'-stilbene disulfonic acid and Calcofluor White M or New 4,4'-bis(4-diethanolamine-s-triazin-2-ylamino)-2,2'-stilbene disulfonic acid. Both were supplied by American Cyanamid Co., Bound Brook, N.J.

Stock staining solutions of Calcofluor White MR New were prepared by suspending 0.15 g of the brightener in 100 ml of distilled water and passing the suspensions through a membrane filter (Millipore Corp., 0.22 μm pore diameter) to remove undissolved particles. The stock staining solution of Calcofluor White M or New was prepared by adding 0.15 g of the brightener to 100 ml of distilled water and adjusting the pH of the suspension to 7.5 by the addition of dilute 0.05 N NaOH to accomplish dissolution of the brightener. Stock solutions were stored in the dark to prevent photodecomposition.

Bacillus cereus var. mycoides and a Pseudomonas species were used to determine the affinity of the brighteners. They were grown in 50 ml of nutrient broth (Difco) in 125-ml Erlenmeyer flasks at room temperature on a rotary shaker for approximately 14 h. The cells were in the early stationary growth phase when harvested by centrifugation. Physiological saline (0.85% NaCl) was used to dilute the cells to an optical density of 0.5; measured at 640 nm by a Turner model 350 colorimeter. Staining was accomplished by mixing one part stock staining solution with four parts of the cell suspension and incubating the mixture in the dark at approximately 21 C for 30 to 45 min. Excess stain was removed by centrifugation and washing three times with saline.

Observations and photomicrographs were made with a Reichert trinocular research microscope equipped with a mercury vapor ultraviolet light source. Kodak High Speed TRI-X film was used for photomicrography with exposure times of 15 s when cells were irradiated with ultraviolet light and 1 s when cells were irradiated with visible light.

To determine the cellular constituents that were responsible for the retention of the brightener, stained cells were fractionated and the fluorescence of each fraction was measured with a Turner model 110 fluorometer. Cells were fractionated according to the procedure of Roberts et al. (7) and by sonic treatment and chemical fractionation.

A Quigley-Rochester sonic dismembrator equipped with a 1.27-cm diameter titanium tip and an ice bath was used to disrupt stained bacteria suspended in
Fig. 1. (A) Stained, whole cells of B. cereus var. Mycoides, phase contrast-white light illumination. 630×. (B) Same field, ultraviolet illumination. (Note: cells which have lost protoplasm fail to fluoresce when illuminated with ultraviolet light.)
saline. Three 10-min sonic treatment periods, to avoid excessive heating of the cells, were used to disrupt the cells. The disrupted cells were centrifuged to separate the cell walls from the soluble constituents. Cell walls were washed once with saline and suspended in 20 ml of saline. The supernatant fluid from the first centrifugation was mixed with 10 ml of saturated ammonium sulfate (7.6 M) to precipitate protein. The mixture was incubated for 30 min at 5°C. The protein was removed by centrifugation and resuspended in 30 ml of saline.

Fluorescence of the fractions was quantitated with a fluorometer fitted with a primary filter having a transmission peak at 360 nm, and a secondary filter which passed wave lengths longer than 415 nm. Serial 10-fold dilutions of the fractions were made so that all fractions could be read on the fluorometer scale. A standard curve was prepared for the brightener (see Fig. 5) and the readings of cell fractions were extrapolated to yield readings for equivalent log dilutions.

Microscopy evidence for staining affinity was obtained by observing spheroplasts of stained Pseudomonas cells and ghosts of stained bacillus cells. Spheroplasts of the pseudomonad were produced by the procedure of Weiss and Fraser (10). Preparation of ghosts of bacillus cells was accomplished by fixing cells on a glass slide, covering with a cover slip, and pressing on the cover slip with a blunt pair of forceps to rupture the cells.

RESULTS AND DISCUSSION

Affinity of the stains to cellular constituents of Bacillus cereus var. mycoides and the Pseudomonas sp. was determined by microscopy examination and chemical fractionation of stained cells. The two brighteners exhibited comparable staining affinity, therefore only the results obtained in using Calcofluor White MR New is presented.

Microscopy examination of stained cells of both organisms revealed that they fluoresced intensely when irradiated with ultraviolet light (Fig. 1, 2). The entire cells appeared to be stained. Observation of stained Bacillus cells ruptured between the glass slide and cover slip showed, with phase and ultraviolet microscopy, that the cell walls did not fluoresce (Fig. 1). Additional evidence that the protoplasmic constituents of the cells contained the brightener was obtained by staining and observing Bacillus cells that were allowed to age at room temperature for several hours in saline. The degree of contrast of the cells, observed with phase microscopy, varied among the cells and paralleled their degree of fluorescence (Fig. 3).

Microscopy evidence of staining affinity in the pseudomonad was obtained by observing spheroplasts prepared from stained cells. The spheroplasts fluoresced when irradiated with ultraviolet light proving that the protoplasm of the cells was retaining the stain (Fig. 4). This does not preclude the possibility that the cell walls were also stained.

Results of cell fractionation of Bacillus pro-

![Fig. 2. (A) Stained whole cells of Pseudomonas, phase contrast-white light illumination. 1,000 x. (B) Same field, ultraviolet illumination.](image-url)
vide strong evidence that cellular protein constituents reacted with most of the brightener. Both of the fractionation procedures used lead to this conclusion (Table 1, 2). Because unreacted brightener is soluble in each chemical reagent used, co-precipitation of the brightener in the whole cells (Table 1 and 2) were probably similar even though the recorded fluorescence was very different. This was due to the nonlinear nature of brightener concentration and fluorescence (Fig. 5). The whole cells used in both fractionations fluoresced brilliantly when observed with the ultraviolet light microscope.

**Table 1. Fluorescence of cellular fractions of stained B. cereus var. mycoides**

| Fraction                      | Relative fluorescence |
|-------------------------------|-----------------------|
| Whole cells\(^a\)             | 20.0                  |
| Kreb’s cycle intermediates   | 0.0                   |
| Lipids and alcohol-soluble proteins | 0.0                |
| Nucleic acids and protein contaminants | 6.0                |
| Residual proteins and cell walls | 17.5               |

\(^a\) Roberts et al. (7) fractionation procedure.

**Table 2. Fluorescence of cellular fractions of stained B. cereus var. mycoides**

| Fraction                      | Relative fluorescence |
|-------------------------------|-----------------------|
| Whole cells\(^b\)             | 79.0                  |
| Crude cell walls              | 12.0                  |
| Purified cell walls\(^c\)     | 9.0                   |
| Cytoplasmic constituents      | 30.0                  |
| Crude protein                 | 28.5                  |

\(^b\) Sonic treatment and chemical fractionation.

\(^c\) Treated with C\(_2\)H\(_4\)OH to remove lipids and alcohol-soluble proteins.
Protein of the pseudomonad was also responsible for adsorption of most of the brightener (Table 3, 4). It is possible that the nucleic acids may have adsorbed some of the brightener (Table 3). Proteins associated with the cell walls were responsible for some of the fluorescence (Table 4), but not enough to make them visible by microscopy examination.

Staining of bacteria with fluorescent brighteners was due to the affinity of cellular proteins for the brighteners. These results provide an explanation for the results of Anderson and Westmoreland (1) that phenol aniline blue stained more bacteria in soil than fluorescent brighteners. Our results suggest that the brighteners would be selective in staining and would not stain lysed bacteria containing no protoplasm. Further studies are needed to determine whether the brighteners would be useful in counting viable soil bacteria and in studying the release of bacterial protoplasm in soil. The latter would be useful in evaluating the importance of biomass in the cycling of soil nitrogen.

**LITERATURE CITED**

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**TABLE 3. Fluorescence of Pseudomonas fractions**

| Fraction                        | Relative fluorescence |
|---------------------------------|-----------------------|
| Whole cells*                     | 77.0                  |
| Kreb's cycle intermediates      | 10.0                  |
| Lipids and alcohol-soluble proteins | 9.0                  |
| Nucleic acids and protein contaminants | 10.5                 |
| Residual proteins and cell walls | 64.0                  |

* Roberts et al. (7) procedure.
* Autofluorescence of unstained cells was negligible.

**TABLE 4. Fluorescence of cellular fractions of stained Pseudomonas**

| Fraction                        | Relative fluorescence |
|---------------------------------|-----------------------|
| Whole cells*                     | 55.0                  |
| Crude cell walls                 | 8.5                   |
| Purified cell walls             | 0.0                   |
| Cytoplasmic constituents        | 29.0                  |
| Crude protein ([NH₄]₂SO₄ precipitated) | 25.0                |

* Sonic treatment and chemical fractionation.
* Autofluorescence of unstained cells was negligible.

Crude cell walls treated with C₂H₅O₂ to remove lipids and alcohol-soluble proteins.

Fig. 4. (A) Sphaeroplasts prepared from stained Pseudomonas cells, phase contrast-white light illumination. 1,000X. (B) Same field, ultraviolet illumination. Note that cells retained fluorescence after cell walls have been removed.

Fig. 5. Fluorescence exhaustion curve of Calcofluor White MR New prepared by making serial log dilutions of 0.15% (wt/vol) stock staining solutions.

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