Nitrogen Dioxide Fixation in Bacterial Chromatin Studies

CHARLES ELLER and WILLIAM H. BECKERT

Biology Department, St. John’s University, Jamaica, New York

Received for publication 17 July 1965

Abstract

Eller, Charles (St. John’s University, Jamaica, N.Y.), and William H. Beckert. Nitrogen dioxide fixation in bacterial chromatin studies. J. Bacteriol. 90:1710-1712. 1965.—Satisfactory chromatin staining was obtained with two Bacillus species when nitrogen dioxide was used for fixation in conjunction with either the acid Giemsa or Feulgen staining methods. An impression method was employed for the transfer of bacilli to a new environment as well as for smears. Advantages of nitrogen dioxide fixation are discussed.

Numerous fixatives have been used for bacteria (Cassel and Hutchinson, 1955; Robinow, 1956a, b), including all the well known botanical fixatives. Kunster (1887) was one of the first to use osmium tetroxide (osmic acid) for bacteria, and numerous other workers have employed it as the fixative of choice in bacterial nuclear studies. As indicated by Robinow (1956a), osmic acid has the advantage of permitting the chromatin to be brightly stained. However, because this chemical is highly toxic and expensive, it was desired to find another gaseous fixative which would yield equivalent results. Olney (1953) used nitrogen dioxide for the fixation of algae, fungi, and higher cells and found it superior to osmic acid fumes. This communication reports the use of nitrogen dioxide with the Robinow (1944) acid Giemsa technique and the Feulgen reaction for bacterial chromatin.

Materials and Methods

Organisms. Bacillus cereus and B. megaterium, both from the St. John’s University Laboratory Collection, were employed for chromatin studies. Culture procedure. Brain Heart Infusion broth (Difco) was used to obtain cells in suspension. After 24 to 48 hr of incubation at room temperature, the cells were spread by means of a sterile cotton swab onto the agar surface of a petri dish containing a modification of the LB medium of Bertani (1951). The modified medium contained:

1% tryptone (Difco), 0.5% yeast extract (Difco), 0.1% dextrose, 0.5% NaCl, and 1.5% agar (Difco) dissolved in distilled water. The autoclaved medium had a pH of 6.7. To produce chromatin condensation, cells grown for 1.5 to 3 hr of incubation at 37 C were transferred from this standard medium to a similar medium containing 3% NaCl. The transfer of cells was accomplished by cutting out an agar block from the standard medium and inverting it onto the high salt medium. The block was then flicked off to leave an imprint of the cells on the new medium. At various subsequent incubation intervals (37 C), agar blocks from both regular and high-salt media were cut out, fixed, and inverted onto an acid-cleaned glass slide (side by side). After the blocks were flicked off, two impression smears remained. Any differences noted after the completion of the staining process could then be attributed to the cultural environment.

Robinow (1944) acid Giemsa chromatin staining method. Agar squares cut out from plate cultures of desired age were placed face side up onto a glass slide. The slide with the adhering blocks was inverted and placed across the opening on the bottom of a plain 125-mm Schleicher desiccator which contained approximately 0.25 inch of concentrated nitric acid, to which a 0.25-inch length of copper wire (no. 18 American wire gauge, 0.040 inch in diameter) had just been added. The cells were fixed for 20 to 40 sec in the red-brown nitrogen dioxide gas; time is not critical. Concentrated nitric acid gives mainly nitrogen dioxide with copper (Partington, 1939):

\[ \text{Cu} + 4\text{HNO}_3 = \text{Cu(NO}_3)_2 + 2\text{NO}_2 + 2\text{H}_2\text{O} \]

After fixation, a second slide was touched so that an imprint of the fixed bacterial cells was obtained. Hydrolysis was carried out at 60 C in 1 N HCl in a Coplin jar. For B. megaterium, the optimal hy-
drolysis time was 6 min; for B. cereus, 10 min. Excess acid was removed by thorough rinsing in four changes of distilled water. Staining was at 37 C for 30 min in Giemsa staining solution prepared by adding 100 drops of Giemsa stain (Gradwohl Laboratories, St. Louis, Mo.) to 50 ml of distilled water. After rinsing in distilled water, the impression smears were mounted in water. Number 1 cover slips were used. Excess water was removed by touching paper toweling to opposite sides of the

Fig. 1 to 6. Photomicrographs of chromatin in Bacillus cells fixed with nitrogen dioxide. (1) B. cereus 3-hr culture on LB medium with 0.5% NaCl stained with HCl Giemsa showing normal nuclear configurations. × 2,000. (2) B. megaterium 3-hr culture on LB medium with 0.5% NaCl stained with HCl Giemsa showing normal nuclear morphology. × 2,000. (3) B. cereus 5-min culture on LB medium with 3% NaCl stained with HCl Giemsa showing condensed chromatin bodies. × 2,500. (4) B. megaterium 5-min culture on LB medium with 3% NaCl stained with HCl Giemsa showing condensed chromatin bodies. × 2,500. (5) B. cereus 23-hr culture on LB medium with 0.5% NaCl stained with HCl Giemsa showing various chromatin forms (spirals, horseshoe shapes, short axial filaments, chromatin encirclement of lipid bodies, etc.). × 2,500. (6) B. megaterium 5-min culture on LB medium with 3% NaCl stained by the Feulgen method showing distinct Feulgen-positive condensed chromatin bodies. × 1,400.
cover slips. Sealing was performed by applying liquid paraffin wax or a molten birthday cake candle along the sides. 

Van Iterson and Robinow (1961) Feulgen method. This method was also used in conjunction with nitrogen dioxide fixation to selectively stain bacterial chromatin. The 3% NaCl medium was used to obtain cells with condensed chromatin so that a more intense staining would occur (Robinow, 1960).

Microscopy and photography. Stained preparations were examined with a binocular Beck Kassel microscope equipped with a 90 X (oil) apochromatic Leitz objective lens (N A, 1.30), aplanatic substage condenser (N A, 1.4), and a built-in base light source with variable power transformer. For photography, a 35-mm roll-type camera was attached to the microscope vertically. Figures 1, 2, 5, and 6 were taken with Kodak 35-mm Panatomic-X film, whereas Kodak 35-mm High Contrast copy film was used for Fig. 3 and 4.

RESULTS

Acid Giemsa, 0.5% salt. The large bacilli of B. megaterium and B. cereus in the exponential phase of growth contained 2 to 4 brightly stained chromatin structures of various shapes (Fig. 1, 2). As the bacterial culture aged, the chromatin condensed. The most common nuclear form in older cultures (23 hr) was a short, thin axial filament (Fig. 5). However, the chromatin also appeared in other forms, including granular, angular, spiral, horseshoe, and circular (around lipid bodies) shapes. These irregular nuclear shapes can be regarded as due to distortion and compression by cell inclusions.

Acid Giemsa, 3% salt. When the cells of either species were imprinted onto 3% NaCl medium, their chromatin was condensed within a few minutes (Fig. 3, 4).

Feulgen technique. B. megaterium cells grown for 3 hr on the 0.5% salt LB medium gave only a faint Feulgen reaction which appeared diffuse throughout the cell. When the cells were transferred to the 3% salt medium for 5 min (Fig. 6), the nuclei condensed and subsequent application of the Feulgen technique revealed distinct deep red Feulgen-positive chromatin bodies. Chromatin structures stained by the acid Giemsa method were somewhat larger, demonstrating that other material besides deoxyribonucleic acid is also involved in the Giemsa staining process. When the Feulgen procedure was performed without the hydrolysis step, the condensed chromatin, as would be expected, did not react with the Schiff's reagent and remained uncolored. These areas, which compared well with the positive Feulgen bodies, appeared as "vacuoles" in a non-specific stained cytoplasm.

Discussion

Results after fixation with nitrogen dioxide are comparable to those of numerous workers employing osmium tetroxide (DeLamater, 1951; Whitfield and Murray, 1956; Robinow, 1956a, b, 1960; Murray, 1960), regardless of age of cells, salt environments, or staining technique. Moreover, this fixative is much less toxic, cheaper, and requires only seconds of exposure. Finally, the same nitric acid can be used repeatedly, as long as the added copper wire produces vapors. In this investigation, as many as 15 0.25-inch pieces of wire could be added before the acid required replacement. It is realized that other oxides of nitrogen are evolved in small quantities when copper is added to nitric acid; however, with concentrated nitric acid, the primary gaseous product is nitrogen dioxide.

LITERATURE CITED


