APPLIED MICROBIOLOGY, June 1971, p. 1040-1045

Vol. 21, No. 6
Printed in U.S.A.

Microorganisms in Unamended Soil as Observed by Various Forms of Microscopy and Staining[1]

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Received for publication 23 March 1971

A light-diffraction microscope was modified to allow sequential viewing of the microorganisms in a soil smear by transmitted, reflected, and reflected-polarized incandescent light and by reflected ultraviolet light. Observations were also made by conventional incandescent and ultraviolet transmitted-light microscopy. All results for the various forms of bright-field microscopy with stained and unstained soils were in agreement, but they differed from the results obtained for two types of ultraviolet-fluorescence microscopy. The latter proved to be nonspecific for in situ soil microorganisms. Capsule-like areas were noted surrounding many of the resident microbial cells of soil when viewed by the various forms of bright-field microscopy. These areas could not be stained or removed by a variety of treatments, but they apparently often did take up stain after in situ soil growth had been initiated. It was concluded that these areas are not capsules but may represent a structural component of nonmultiplying microbial cells in soil.

Several microscopic methods are available for viewing the total in situ microbial flora of soil. These include bright-field viewing (6, 23) and color infrared photography (5) of nonstained soil, bright-field microscopy of stained soil preparations (1, 7, 8, 11, 12, 21, 23, 26, 31, 32, 35), and ultraviolet-fluorescence microscopy of soil stained with unconjugated (3, 4, 9, 10, 15–17, 24, 25, 30, 33, 36) and conjugated (20) fluorochromes. It has been commonly accepted that these methods selectively demonstrate microorganisms in soil preparations, and that they are in agreement with each other as to the types and numbers of microorganisms observed. The present study examines the validity of this conclusion for certain of these methods. Also, information is presented on a capsule-like area surrounding many of the soil microorganisms, as observed in situ by various techniques of bright-field microscopy but which is not detected by ultraviolet fluorescence microscopy.

MATERIALS AND METHODS

Suspensions of non-air-dried soil were prepared by shaking 1 to 2 g of soil in 25 ml of distilled water for 10 min and by subjecting 1 g of soil in 25 ml of distilled water to 1 min of sonic treatment at 25 to 50% of maximum power in a Biosonic II oscillator. In both instances, the larger soil particles were allowed to settle for a short period before smears were prepared from the supernatant fluids.

For Pronase treatment of soil, 0.1 g of non-air-dried sieved soil was mixed with 3 ml of 0.003 M potassium phosphate buffer (*p*H 7.4) containing 3 mg of Pronase (Calbiochem, 45,000 proteolytic units per g) in a 25-ml Erlenmeyer flask with a rubber stopper. The flask was incubated stationary at 38 C but was shaken by hand before each sampling for preparing smears. For toluene treatment of soil, 2 g of soil plus 25 ml of distilled water and 1 ml of toluene were shaken for 4.5 hr at 30 C. Samples for smears were removed at 30 min and 4.5 hr. Lysozyme-ethylenediaminetetraacetic acid (EDTA) treatment of soil was as described by Lichstein and Oginsky (18). Eight grams of soil in 40 ml of distilled water was sonically treated for 2 min at 30% of maximum power and then allowed to settle for 10 min. The supernatant fluid was used for the trials, and the lysozyme (K and K Laboratories, Inc., Plainview, N.Y.) levels ranged from 0.25 to 9.0 mg per ml.

The Anthony, Tyler, and Leifson capsule stains were those described in the *Manual of Microbiological Methods* (27). Dark field was not required for the Strugger (30) acridine orange fluorescence procedure, nor was it necessary to disperse the aqueous suspension of stained soil in immersion oil.

To obtain high resolution and color correction for all three primary colors, a Zeiss 100 × apochromatic objective with iris diaphragm, allowing numerical apertures of from 0.8 to 1.32, was used for all micros-

[1] This research was authorized for publication as paper no. 3939 in the journal series of the Pennsylvania Agricultural Experiment Station on 11 March 1971.

copy. Usually, its NA was set at 1.32, and the condenser NA was 1.4. Bright-field microscopy of stained soil smears usually utilized a conventional transmitted-light microscope with the above apochromatic objective, but comparisons were also made with the following modified microscope.

The reflected-light microscope described by Casida (6) was further modified to allow alternative viewings of microorganisms by transmitted incandescent light, reflected incandescent light diffraction with and without polarization, and reflected ultraviolet light. These modifications utilized a Leitz HBO 200 ultraviolet source (normally used with a Leitz Ortholux microscope), with connected switching arrangement to allow entry of incandescent light from the side but with the 6-v incandescent source removed. The 12-v incandescent source attached to the rear of the base of Casida's reflected-light microscope was detached, and the above ultraviolet source was added and aligned. The 12-v Zeiss incandescent source was then aligned where the 6-v Leitz source had been. The latter 6-v source plus the Leitz condenser was mounted above the gliding stage of the reflected-light microscope to allow transmitted-light microscopy. The aperture and field diaphragms were not stopped down, except in the case of reflected incandescent light diffraction microscopy where the aperture diaphragm was stopped down completely, and the field diaphragm was partially stopped down when decreased flare or increased resolution was desired. The NA of the objective diaphragm was reduced only when excessive flare was encountered. Mineral fragments were detected by reflected polarized incandescent light. A polarizer was mounted in the head of the microscope, and an analyzer was laid over the stopped-down aperture diaphragm. Rotation of the analyzer did not extinguish vision for the mineral fragments.

RESULTS

Stained preparations. Smears of unamended soil were stained with phenolic Rose Bengal (2) and observed by bright-field transmitted-light microscopy at 1,000- to 1,250-fold magnification. The soil materials appeared red or they were almost colorless due to lack of affinity for the stain. The soil bacteria were easily discerned as darker-red to purple-red coccoid and coccoid-rod cells, frequently surrounded by a distinct unstained capsule-like area. The latter was more easily observed in those areas of the preparation in which a slight excess of stain had caused negative staining

Preparations negatively stained with Anthony's capsule stain (and with the Tyler modification) clearly demonstrated wine-red bacteria surrounded by a colorless capsule-like area (Fig. 1). The soil materials were a purple to purple-red. Additional stains were evaluated and are as follows: (i) buffalo black 1% aqueous solution, 1 min; (ii) Brilliant Green 1% aqueous solution, 1 min; (iii) isatin 1% in ethanol, 1 min; (iv) Ziehl's carbolfuchsin, 30 sec; (v) Gram's safranin, 1 min, followed by Anthony's stain; (vi) Lugol's iodine, 1 min, followed by Anthony's stain; (vii) phenolic Rose Bengal, followed by Anthony's stain; (viii) Anthony's crystal violet, 2 min, wash and dry, and then Ziehl's carbolfuchsin, 30 sec (also with stain sequence reversed); (ix) ninhydrin (0.25%) in 90% acetone, 1 min, and then evaporated at room temperature, same but followed by Anthony's stain, same but followed by Gram's safranin, 30 sec; (x) Gram stain; (xi) BBL modified Leifson's capsule stain with borax methylene blue counter stain; (xii) Alcian Blue capsule stain (19); (xiii) erythrosin B in place of Rose Bengal in phenolic Rose Bengal stain; (xiv) aniline blue, water-soluble (12); (xv) iodine vapors, 35 min. In all instances, the bacteria were apparent as some shade of red and were surrounded by a colorless capsule-like area; the soil materials took on the colors of the specific stains applied. Unstained soil was also observed with the apochromatic objective, and the bacteria again presented a reddish hue within the colorless capsule-like area.

As noted above, the capsule-like area did not stain with the Anthony, Leifson, or McKinney capsule (or cyst) stains. To test the Anthony procedure, vegetative cells and cysts of *Azotobacter chroococcum* were mixed with soil, and smears were prepared. Examination of these smears revealed staining of the cysts as described by Socolofsky and Wyss (28). A similar trial with McKinney's (19) Alcian Blue stain for *A. chroococcum* and *A. vinelandii* added to soil showed red-stained cells surrounded by blue capsules.

Attempts were made to remove or alter the capsule-like areas by pretreatment of the soil smears before applying Anthony's stain. After treatment, the smears were washed and blotted dry before staining. Significant changes in the capsule-like area did not occur with any of the following treatments: (i) 1 min at room temperature for 95% ethanol, 90% acetone, formaldehyde, ethyl acetate, glacial acetic acid, pyridine, and chloroform; (ii) 1 min at room temperature for 1% aqueous solutions of EDTA, citric acid, oxalic acid, urea, $FeCl_3$, $K_2Cr_2O_7$, and $AgNO_3$; (iii) various combinations of tris(hydroxymethyl)-aminomethane buffer, sucrose, lysozyme, and EDTA; (iv) Chlorox, 20 min at room temperature; (v) 6 N NaOH, 20 min at room temperature; (vi) 1 N H_2SO_4, 30 sec at room temperature; (vii) 6 N HCl, steamed for 20 min; (viii) 5% phenol, steamed for 20 min; (ix) Pronase, during 22 hr; (x) toluene, during 4.5 hr; (xi) Biosonic II sonic oscillator, 1 min at maximum power. The toluene treatment apparently first dislodged cells

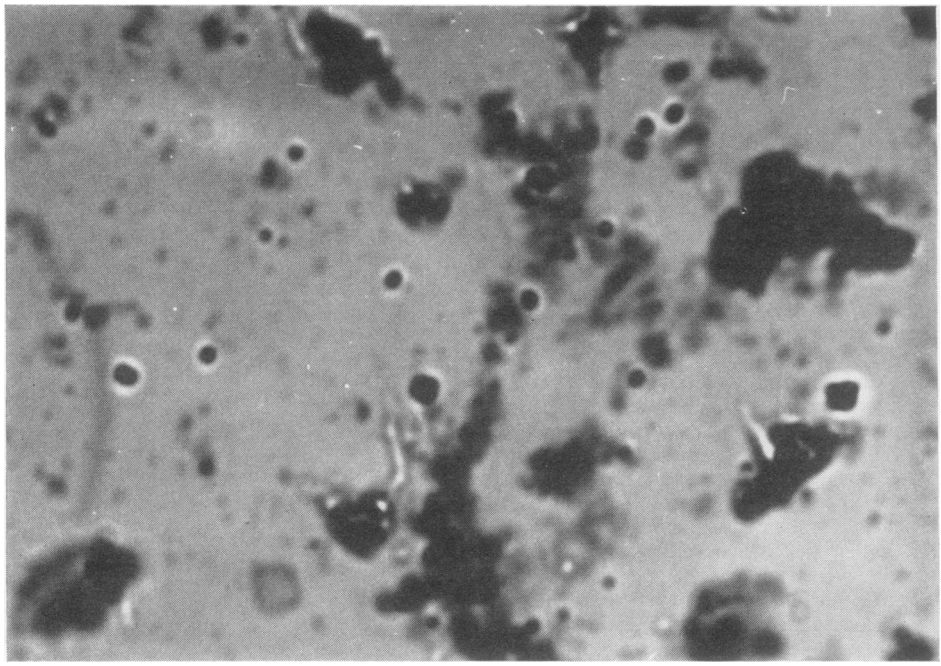

FIG. 1. *Soil stained with Anthony capsule stain. A clear capsule-like area surrounds many of the cells.*

to give slightly increased cell numbers and then caused some lysis of the cells.

In contrast to these findings, the capsule-like areas surrounding some of the indigenous soil organisms seemed to disappear, or become markedly reduced in size, by shaking 2 g of soil for 22 hr at 30 C in 50 ml of sterile Heart Infusion Broth (Difco) in a 250-ml Erlenmeyer flask. During this incubation, large purple-staining rods with and without endospores became apparent, as did smaller reddish-purple to purple-staining coccoid and rod-shaped cells lacking the capsule-like area. Soil bacteria which had not initiated growth were still present in high numbers, with their wine-red cells and capsule-like areas, but they appeared smaller than the multiplying soil organisms. However, their total diameters for cell plus capsule-like area of approximately 1.0 to 1.4 μm (the cell itself measuring approximately 0.5 to 0.8 μm) were approximately equal to the widths of many of the multiplying cells lacking the capsule-like area.

Bright-field microscopy of unstained preparations. Alternation between the transmitted- and reflected-light sources on Casida's reflected-light microscope (6) allowed a comparison of individual microbial cells as observed under both conditions of microscopy. By reflected light with the aperture diaphragm closed, the coccoid and coccoid-rod cells diffracted light as described by Casida, but the capsule-like areas were not visible. These areas often could be detected, however, by partially stopping down the field diaphragm in the head of the microscope. An objective numerical aperture of 1.32 on the apochromatic oil objective was used for these comparisons, and polarized light in conjunction with the reflected light microscope was used as an additional means for detecting artifact mineral fragments in the preparations. Thus, all forms of bright-field microscopy examined were in agreement concerning the bacterial cells observed and the capsule-like areas surrounding the cells.

Ultraviolet fluorescence microscopy. Addition of reflected ultraviolet light and transmitted incandescent light to Casida's reflected-light microscope allowed a comparison of the various forms of bright-field microscopy with the unconjugated fluorescein isothiocyanate (FITC) fluorescence methods of Pital et al. (24) and Babiuk and Paul (3) and the acridine orange fluorescence procedure of Strugger (30). Observation of soil by the unconjugated FITC procedure revealed large, fluorescing rod and coccoid bodies which appeared similar to the published photographs of Pital et al. However, these objects were not visible by either transmitted- or reflected-light bright-field microscopy. Reversing the order, FITC-stained in situ soil bacteria detected by the bright-field microscopy techniques did not fluoresce under ultraviolet light

Strugger's acridine orange fluorescence proce-

dure for in situ soil bacteria, with both transmitted and reflected ultraviolet light, also did not agree with the results of reflected- and transmitted-light bright-field microscopy. Only a small percentage of the objects fluorescing under ultraviolet light demonstrated a morphology by the bright-field microscopy procedures which could be attributed to soil bacteria. Some of the objects which had fluoresced blue-green under ultraviolet light appeared under bright-field microscopy as highly irregular forms with jagged edges, whereas other objects were amorphous. In several instances, the objects observed by fluorescence microscopy actually were portions of larger unidentifiable structures. Sometimes, part of a soil protozoan cell fluoresced blue-green; in other instances, the entire protozoan cell fluoresced. What appeared under ultraviolet light to be chains of strongly fluorescing large bacterial rods proved by bright-field microscopy to be partially degraded actinomycete or fungal hyphae with many nonfluorescing soil bacteria adhering to the hyphal surface.

The total numbers of fluorescing objects with acridine orange were one to several orders of magnitude less than the microbial cells observed by the various forms of bright-field microscopy. Also, at least a part of the soil material did not fluoresce in any manner. Regardless of these results, however, autoclaving of the soil before staining prevented the occurrence of the blue-green fluorescence. Also, the fluorescence characteristics described by Strugger and others did occur for a *Sarcina* strain newly isolated from food when it was mixed with soil previous to staining with acridine orange. These added cells were easily detected in the soil by both bright-field and fluorescence microscopy, with some of the cells fluorescing blue-green and the rest fluorescing orange-red

DISCUSSION

The methods of bright-field microscopy evaluated in this study are in agreement for stained and unstained preparations of unamended soil. They reveal coccoid and coccoid-rod cells approximately 0.5 to 0.8 μm in width, which appear as various intensities of a reddish hue regardless of whether stains are present or the particular stains used. Most of the cells were surrounded by a clear non-staining capsule-like area having a total width of approximately 1.0 to 1.4 μm. It would appear that the cells do not stain or take up only small amounts of stain and that they naturally exhibit a reddish hue, which is more apparent when viewed with an apochromatic objective corrected for red. The latter observation could explain the red image for bacteria in unstained soil recorded by Aero Ektachrome Infrared photography in conjunction with an apochromatic objective (5), if this type of photography should tend to intensify the red color.

The capsule-like area was visible in unstained and stained soil with all forms of bright-field microscopy tested, although it was more easily detected with apochromatic objectives and negative staining. It was not observed during fluorescence microscopy. Phase-contrast microscopy was not used because of its inherently lower resolution and its characteristic formation of halos of light surrounding the cells. Although not mentioned or discussed in their studies, other workers, using bright-field microscopy of stained soil, have published photographs showing this capsule-like area, e.g., Conn (8).

Casida (6) stated that the outer boundary of cells residing in soil was not detected by his light diffraction microscope but that it was visible when growth occurred in the soil and the light diffraction capability of the cells was lost. The present study notes that this outer cell boundary can be seen in light diffraction microscopy when the field diaphragm is partially stopped down to reduce flare and the objective diaphragm is not stopped down (which decreases resolution). For both stained and unstained soil, alternating observations of cells with this method and with transmitted-light bright-field microscopy revealed that the above outer cell boundary is the same as the capsule-like area. These observations and observations in the present study that coccoid and coccoid-rod cells which have initiated growth in soil apparently totally stain (including the capsule-like area) and that, even though the cells may elongate during growth initiation, their widths often are equal to the widths of the capsule-like areas in nonmultiplying cells indicate a possible major cytological difference in the resident cells as they occur dormant in unamended soil and as they are forced by soil amendment or root growth (6) into a type of growth more closely resembling that of laboratory-grown cultures.

The capsule-like area observed for resident cells in soil apparently is not a capsule, at least as occurs for laboratory-grown cultures. It was not removed or stained by any of the methods tried, which included capsule stains. Also, encapsulated cells of laboratory cultures examined separately and after addition to soil did not yield light-diffraction colors (6) as do resident soil organisms. Lastly, as a laboratory type of growth is initiated in soil, this area of the cell does not disappear but often becomes stainable and indistinguishable from the rest of the cell. It should not

be concluded from the above that resident organisms in soil do not possess capsules; there could be a thin capsule layer which was not detected in these studies.

The microscopy, appearance, and growth behavior of resident soil organisms somewhat resemble those of a cyst and its germination. However, the lack of cyst staining by the procedure of Socolofsky and Wyss (28) and the failure to remove chemically the outer boundary of the cell would predict that, if they are cysts, they must be protected in some manner or they are structurally different from the *Azotobacter* cyst. The latter might be predicted by the fact that laboratory-grown *Azotobacter* cysts do not yield light diffraction colors with Casida's microscope. Nevertheless, having the characteristics of a cyst-like stage associated with much of the resident soil microflora, if this should actually be the case, would be of considerable interest. The cells would be desiccation-, phage-, and radiation-resistant (14, 29, 34) and would have almost no endogenous activity, but they would, nevertheless, be able to utilize exogenous substrates without delay and at a lower respiration rate than encountered for vegetative cells (22, 29).

No ready explanation is available for the results obtained by both procedures of ultraviolet fluorescence microscopy. Obviously, they do not agree with any of the methods of bright-field microscopy with which they were compared. The fluorochromes do not seem to differentiate the more dormant resident soil bacteria, but they do fluoresce with various other types of soil microbial life, such as protozoa, and with various inanimate soil materials. The latter would be possible if certain soil materials should be coated with proteinaceous material, for which FITC has an affinity, or nucleic acids, which can fluoresce blue-green with acridine orange. The fact that partially degraded actinomycete and fungal hyphae fluoresced as if they were chains of bacterial rods would tend to support the latter concept.

Addition of a laboratory culture of a *Sarcina* strain to soil before acridine orange staining presented no problem as regards bright-field-microscopy cell detection or ultraviolet-fluorescence vital staining. Thus, the Strugger effect occurred for laboratory cultures, even when superimposed on soil. The validity of this vital staining characteristic, however, has been questioned (13) and, therefore, the lack of blue-green fluorescing objects for autoclaved soil in the present study or for "sterilized" soil (30) may not be a valid criterion for the efficacy of acridine orange as a fluorescence vital stain for soil.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant GB-14487 and contract NGR 39-009-180 with the National Aeronautics and Space Administration. I thank C. M. Hunt for able technical assistance and L. J. McElroy for helpful comments and assistance in combining microscope components.

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