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Application of the Fluorescent-Antibody Technique to an Ecological Study of Bacteria in Soil

I. R. HILL AND T. R. G. GRAY
Hartley Botanical Laboratories, The University of Liverpool, Liverpool, England

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The fluorescent-antibody technique was used to identify cells and spores of *Bacillus subtilis* and cells of *B. circulans* from soil. From cells grown in three broth media of different nutrient status, i.e., a cold extracted soil medium (CSE), an unamended autoclaved soil extract (HSE), and nutrient broth (NB), antisera were produced with both quantitative and qualitative differences in antibody content. The specificities of antisera to two strains of each of the *Bacillus* species were determined. Antisera for *B. subtilis* O antigens were species-specific and showed no cross-reactions, whereas those for the *B. circulans* O antigens were strain-specific and in some cases showed cross-reactions with *B. alvei*. This cross-reaction was removed by absorption of the antiserum with *B. alvei* O antigen. Fluorescein isothiocyanate γ-globulin conjugates prepared from these antisera showed the same specificity reactions. A method for staining bacteria on soil particles was developed, by use of small staining troughs. By mounting stained soil particles on slides and irradiating them with transmitted and incident ultraviolet blue light, bacteria on both mineral and organic particles, taken directly from soil, could be observed. Fluorescent antibodies against cells grown in CSE gave brighter fluorescence of stained bacteria on soil particles than did fluorescent antibodies against cells grown in either HSE or NB. Colonies of both *Bacillus* species were generally small and localized. Spore antisera, though not rigorously tested for specificity, were used to identify spores of *B. subtilis* on soil particles. The uses and implications of the technique in soil bacteriology are discussed.

A major difficulty in interpreting experiments with soil bacteria in laboratory culture is knowing whether the conclusions drawn are applicable to bacteria in situ in the soil. Even though it may be shown that a particular species exists in a soil, cultural experiments cannot prove whether the organism is vegetative or dormant, whether it occurs as single cells or in colony form, or whether it is associated with any one type of particle, or microhabitat, in the soil. Direct observation techniques have provided useful evidence on these points, but until recently it has not been possible to identify the organisms observed. The ecological value of being able to do this has been stressed by Schmidt and Bankole (17), who used the fluorescent-antibody technique to identify the hyphae and spores of *Aspergillus flavus* in soil (17, 18, 19). They used *A. flavus* to inoculate sterile soil and soil preinoculated with known species of fungi, and buried slides in the soils. After incubation, the slides were removed and stained with a specific fluorescent antibody known to react with *A. flavus*. Hyphae of *A. flavus* stained well whereas most other fungi examined were either nonfluorescent or showed weak autofluorescence; only a few strains showed strong autofluorescence. Eren and Pramer (3), also working with artificially inoculated soils, prepared fluorescent antisera for *Arthrobotrys conoides* and used them to study this nematode-trapping fungus.

Others have used the fluorescent-antibody technique to identify colonies of soil bacteria on dilution plates. Unger and Wagner (21) showed that it was possible to prepare replicas of colonies from dilution plates, on circular glass slides, and to stain these replicas with fluorescent antisera. When the slides were irradiated with ultraviolet light, the complete colonies of the organism being studied fluoresced whereas other colonies did not. They used antisera for *Escherichia coli*, *Bacillus cereus* var. *mycoides*, and *Sarcina flava* in their experiments, but did not attempt to observe or identify bacteria directly on soil particles or on contact slides. Except for a photograph, taken by Paton (16), of an unnamed species of *Pseudomonas* in the rhizosphere, no attempts have been made to identify soil bacteria in situ.

The aims of this paper are: (i) to describe a

technique for the application of the fluorescent-antibody technique to the identification of *B. subtilis* and *B. circulans*; (ii) to describe a method for observing the colonization of soil particles by these organisms; (iii) to present some preliminary data on the occurence of the above *Bacillus* spp. in a pine forest soil, developing on a sand dune system at Freshfield, Lancashire (*5a*).

MATERIALS AND METHODS

Preparation of fluorescent antisera for B. subtilis and B. circulans. Cultures of *B. subtilis* (strain NCTC 6284 from the National Collection of Type Cultures, London, England, and a strain freshly isolated from the pine forest soil) and *B. circulans* (strain NCTC 7578 and a strain freshly isolated from the pine forest soil) were grown in an unamended autoclaved soil extract (HSE; 6), nutrient broth (NB), and CSE, a soil extract prepared by ball-milling soil for 1 week at 2 C. Somatic (O) antigens were prepared from all four strains and spore antigens were prepared from the *B. subtilis* forest soil isolate by use of the methods described by Norris and Wolf (15). The antigens were injected intravenously into rabbits as follows: day 1, 0.5 ml; day 4, 1.0 ml; day 7, 2.0 ml; day 10, 3.0 ml; day 13, 3.0 ml; day 16, 3.0 ml; day 19, 3.0 ml; day 22, 3.0 ml. On days 32, 33, 36, and 37, the rabbits were bled from the marginal ear vein. Secondary injections and bleedings were carried out after a 1-month rest period.

Antisera were prepared from the blood samples and were sterilized by membrane filtration. The agglutination titers of the antisera to the O antigens were determined by testing them against homologous O antigens and whole-cell antigens, by use of Lamanna's (11) modification of Noble's (14) tube agglutination test. If these were satisfactory, appropriate antisera were bulked and their specificities were determined.

In the preparation of the γ-globulin fractions of these antisera, equal volumes of antiserum and 3.9 M ammonium sulfate were mixed at 2 C. The resulting precipitate was centrifuged, washed with 1.95 M ammonium sulfate, and dissolved in a minimal volume of distilled water. The solution was dialyzed (in $\frac{9}{16}$-inch diameter tubing) against 0.85% sodium chloride, at 2 C, until all the ammonium sulfate had been removed. The protein content of the γ-globulin solution was determined by the Folin-Ciocalteau method (12), and was adjusted with 0.85% sodium chloride and 0.5 M carbonate-bicarbonate buffer (*p*H 9.0) to give a sample containing 10 mg of protein per ml and 10% carbonate-bicarbonate buffer.

The sample was kept at 2 C, and crystalline fluorescein isothiocyanate (BBL) was added over a 1-hr period, with continuous stirring for 12 hr. Conjugates were prepared with the minimal dye-protein ratio necessary to produce optimal staining of homologous antigens; this ratio varied between 1:40 and 1:100 for different γ-globulin preparations. The conjugated sample was then passed through a Sephadex [G-25 bead form; Pharmacia (G.B.) Ltd., London, England] column (25 by 2.5 cm), equilibrated with 0.01 M, *p*H 7.1 phosphate-buffered saline (4); and an hourly flow rate of 4.5 ml/cm was maintained. The conjugated protein passed rapidly through the column, and was diluted no more than twofold. This preparation was run into Bijou bottles in 5-ml volumes and stored at −20 C.

The specificity of the conjugates was determined by staining films of 24-hr-old cultures, grown on 1.2% agar media of CSE, HSE, and NB, with the use of controls suggested by Nairn (13). Fluorescence of organisms was assessed as follows: 4+, maximal fluorescence, brilliant yellow-green; 3+, bright yellow-green fluorescence; 2+, less bright but clearly yellow-green fluorescence; 1+, dull fluorescence; 0, no fluorescence. Whenever possible, all-glass apparatus was used to avoid fluorescence contaminants (10).

Technique for staining soil and observation of bacteria on soil particles. Freshly sampled soil was placed in a glass cylinder (1.5 by 1.45 cm), fitted with a nylon-weave base (20-μ twill weave). The conjugated γ-globulin was pipetted into a trough into which the glass cylinder was inserted (Fig. 1). For every 0.1 g of soil, 0.06 ml of conjugate was placed in the trough plus an extra 0.05 ml to occupy the dead space between the two vessels.

The soil container was lowered into the trough and left there for 40 min at 18 C, and was gently agitated at 10-min intervals. The container then was removed, allowed to drain, and placed in 0.01 M, *p*H 7.1 phosphate-buffered saline (one rinse and three 2-min washes) in troughs similar to that used for staining. Soil particles were carefully removed from the containers and placed on slides in a very small volume of water (to facilitate their distribution on the slide). When observation was delayed, the slides were stored

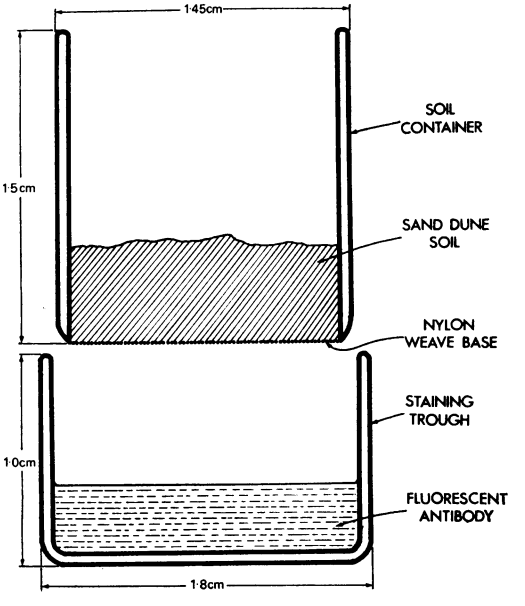

FIG. 1. *Apparatus for staining soil with fluorescent-antibody preparations.*

in moist chambers in the dark at 2 C. No fading or other detrimental changes occurred during 24 hr of storage.

Preparations were observed with a monocular Leitz Orthulux microscope equipped with a 10× eyepiece and dry objectives (× 10, 25, 40, and 70); the objectives were fitted with ultraviolet-absorbing Euphos glass and were corrected for use without a cover glass. Two HBO 200 mercury vapor lamps which could be used simultaneously were attached to the microscope, one providing transmitted illumination, and the other, incident illumination. All transmitted light was passed through a dry, dark-field condenser (D, 0.80), and the incident light was passed through a Leitz vertical illuminator. In this way, bacteria on both translucent and opaque soil particles could be examined. Schott BG 12 (3 mm) and OG 1 (2 mm) glasses were used as primary and barrier filters, respectively. Photographs were taken with a Leitz Orthomat camera, with Kodak high-speed Ektachrome, or Ilford HP 3, film.

RESULTS

Effect of growth medium on antigen production. When fluorescent antisera are to be used to identify organisms growing in soil, it would seem logical to obtain antisera against antigens derived from organisms grown as nearly as possible under soil conditions. To test this proposition, O antigens were prepared from cells grown in CSE, HSE, and NB. Antisera were produced and were tested, by use of the tube agglutination test, against O antigens and whole-cell antigens from organisms grown in each of these media (Table 1). Higher titers were obtained when the antiserum and the test antigen were derived from cultures grown in the same medium. Thus, *B. subtilis* NCTC 6284 antiserum agglutinated the homologous O antigen at a higher dilution when both had originated from cells grown in the same medium, i.e., both in CSE, HSE, or NB. The same phenomenon was observed with the antisera from all four strains tested. These differences in antisera titers were not entirely quantitative, since the agglutinating capacity of any antiserum could only be partly neutralized by absorption with antigens from cells grown in a different medium to the cells whose antigens had been used to produce the antiserum. Observations on cell growth in the three media showed that cells grown in CSE were more gram-variable and often more irregularly shaped and smaller in size than cells grown in HSE or NB.

Specificity of the antisera. Antisera to the O antigens of the four organisms were prepared. The tube agglutination method was used to test their specificity against O antigens, whole-cell antigens, and spore antigens prepared from the following organisms (the number of strains tested for each species is indicated): *B. subtilis*, 37; *B. circulans*, 39; *B. circulans-alvei* intermediates, 3; *B. circulans-brevis-laterosporus* intermediates, 6; *B. alvei*, 6; *B. cereus*, 7; *B. cereus* var. *mycoides*, 3; *B. licheniformis*, 10; *B. megaterium*, 7; *B. pumilus*, 8; *B. coagulans*, 4; *B. firmus*, 1; *B. lentus*, 3; *B. badius*, 1; *B. polymyxa*, 4; *B. macerans*, 4; *B. brevis*, 9; *B. laterosporus*, 3; *B. sphaericus*, 5; *B. pantothenicus*, 5; *B. pasteurii*, 1. Antisera were also tested against O antigens and whole-cell antigens from three soil isolates of each of the following genera: *Pseudomonas*, *Arthrobacter*, *Streptomyces*, *Achromobacter*, and *Flavobacterium*; two soil isolates of the following genera: *Staphylococcus*, *Micrococcus*, and *Nocardia*; and

TABLE 1. *Effect of medium used for growth of antigens, on agglutination titers of antisera*

Antiserum to O antigen of	Derived from cells grown in	Agglutination titer of antiserum and the homologous antigens[a]					
		Whole cells grown in CSE	Whole cells grown in HSE	Whole cells grown in NB	O antigen grown in CSE	O antigen grown in HSE	O antigen grown in NB
Bacillus subtilis NCTC 6284	CSE	*1:640*	1:320	1:160	*1:5,120*	1:1,280	1:1,280
	HSE	1:80	*1:640*	1:320	1:1,280	*1:5,120*	1:5,120
	NB	1:320	1:640	*1:640*	1:640	1:2,560	*1:5,120*
B. subtilis soil isolate	CSE	*1:640*	1:160	1:80	*1:5,120*	1:1,280	1:640
	HSE	1:160	*1:640*	1:320	1:640	*1:5,120*	1:2,560
	NB	1:160	1:640	*1:640*	1:640	1:2,560	*1:10,240*
B. circulans NCTC 7578	CSE	*1:640*	1:160	1:80	*1:5,120*	1:1,280	1:320
	HSE	1:80	*1:640*	1:320	1:320	*1:2,560*	1:1,280
	NB	1:160	1:640	*1:640*	1:640	1:5,120	*1:10,240*
B. circulans soil isolate	CSE	*1:640*	1:160	1:80	*1:10,240*	1:1,280	1:640
	HSE	1:160	*1:640*	1:320	1:640	*1:5,120*	1:1,280
	NB	1:80	1:320	*1:640*	1:640	1:2,560	*1:5,120*

[a] Titers obtained when antiserum and antigen were derived from cultures grown in the same medium are in italics.

one strain of each of the following organisms obtained from culture collections: *Escherichia coli*, *Proteus vulgaris*, *Mycobacterium phlei*, *Azotobacter chroococcum*, *Sarcina lutea*, *Rhizobium* sp., *Klebsiella* (*Aerobacter*) *aerogenes*, and *Agrobacterium tumefaciens*.

Table 2 records the results obtained for the *B. subtilis* antisera. Both antisera were completely specific for the O and whole-cell antigens of this species. There were no cross-reactions with any spore antigens prepared from *B. subtilis*, or with whole cells, O antigens, or spore antigens of any other *Bacillus* species or species of other genera tested. All the *B. subtilis* strains tested reacted positively with both antisera. Table 3 records the results obtained for the *B. circulans* antisera. The reactions of the antisera to the two strains were totally different. Although *B. circulans* NCTC 7578 antiserum agglutinated whole-cell and O antigens from most of the 10 *B. circulans* strains and the 3 *B. circulans-alvei* intermediate strains originating from culture collections, it agglutinated none of the antigens from the 19 *B. circulans* strains or the 6 *B. circulans-brevis-laterosporus* intermediate strains isolated from the forest soil. Conversely, the antiserum to the *B. circulans* soil isolate agglutinated the whole-cell and O antigens from all the 25 soil isolates of *B. circulans* and *B. circulans-brevis-laterosporus* intermediates, but would not react with any of the antigens from the culture collection strains. In addition, it cross-reacted with most of the whole-cell and O antigens of *B. alvei* culture collection and soil strains. These cross-reactions were removed by absorption of the antiserum with *B. alvei* O antigen, leaving an antiserum that was specific for all soil isolates of *B. circulans* and *B. circulans-brevis-laterosporus* intermediates examined.

Specificity of the conjugated γ-globulin preparations. Determination of the specificity of the conjugated γ-globulins, by use of the same test cultures, confirmed the antiserum agglutination results. Nevertheless, a number of confirmatory inhibition tests were carried out as suggested by Nairn (13). All bacteria that fluoresced when stained with conjugated, immune γ-globulin were also tested with conjugated, nonimmune γ-globulin. No reactions were observed. Inhibition tests were performed, the first involving the treatment of the antigen with unlabeled specific γ-globulin before staining with the conjugated, immune γ-globulin, and the second, the simultaneous treatment of the antigen with both these preparations. Either a reduction or complete inhibition of staining occurred in all such tests. Antigens were also stained with an immune conjugate that had been absorbed with its homologous antigen, resulting in complete inhibition of staining for all antigens tested. All the necessary controls were included in these tests. The intensity of the fluorescence of organisms was assessed. Bacteria stained with their homologous fluorescent antibody showed 4+ or 3+ fluorescence.

Autofluorescence of bacterial cells varied from

TABLE 2. *Agglutination reactions of the antisera for the somatic antigen (O) of Bacillus subtilis*

Origin	Antigen	No. of strains tested	No. of strains agglutinated with antiserum to O antigen from		Titer range of reactive strains	
			B. subtilis NCTC 6284	*B. subtilis* soil isolate	*B. subtilis* NCTC 6284	*B. subtilis* soil isolate
Culture collections[a]	*B. subtilis* O	21	21	21	1:80–1:5,120	1:40–1:2,560
	B. subtilis WC[b]	21	21	21	1:40–1:1,280	1:10–1:640
	B. subtilis spore	21	0	0	—	—
Forest soil	*B. subtilis* O	16	16	16	1:40–1:2,560	1:80–1:10,240
	B. subtilis WC	16	16	16	1:20–1:640	1:40–1:2,560
	B. subtilis spore	16	0	0	—	—
Culture collections and forest soil	Other *Bacillus* spp. O	119[c]	0	0	—	—
	Other *Bacillus* spp. WC	119[c]	0	0	—	—
	Other *Bacillus* spp. spore	119[c]	0	0	—	—
	Other genera O	29	0	0	—	—
	Other genera WC	29	0	0	—	—

[a] Strains were obtained from the National Collection of Type Cultures, the National Collection of Industrial Bacteria (Teddington, England), the National Collection of Plant Pathogenic Bacteria (Harpenden, England), and J. Wolf (Department of Agriculture, Univ. of Leeds, Leeds, England).

[b] Whole cell.

[c] Two strains of *B. cereus* showed strong autoagglutination.

TABLE 3. *Agglutination reactions of the antisera for the somatic antigen (O) of Bacillus circulans*

Origin	Antigen	No. of strains tested	No. of strains agglutinated with antiserum to O antigen from		Titer range of reactive strains	
			B. circulans NCTC 7578	*B. circulans* soil isolate	*B. circulans* NCTC 7578	*B. circulans* soil isolate
Culture collections	*B. circulans* O	10	9	0	1:10–1:10,240	—
	B. circulans WC[a]	10	7	0	1:10–1:1,280	—
	B. circulans spore	10	0	0	—	—
Forest soil	*B. circulans* O	19	0	19	—	1:80–1:10,240
	B. circulans WC	19	0	19	—	1:20–1:1,280
	B. circulans spore	19	0	0	—	—
Culture collections	*B. circulans-alvei* O	3	3	0	1:160–1:1,280	—
	B. circulans-alvei WC	3	3	0	1:40–1:640	—
	B. circulans-alvei spore	3	0	0	—	—
Forest soil	*B. circulans-brevis-laterosporus* O	6	0	6	—	1:80–1:2,560
	B. circulans-brevis laterosporus WC	6	0	6	—	1:40–1:640
	B. circulans-brevis-laterosporus spore	6	0	0	—	—
Culture collections	*B. alvei* O	4	0	3	—	1:160–1:640
	B. alvei WC	4	0	3	—	1:40–1:80
	B. alvei spore	4	0	0	—	—
Forest soil	*B. alvei* O	2	0	2	—	1:160–1:320
	B. alvei WC	2	0	2	—	1:40–1:80
	B. alvei spore	2	0	0	—	—
Culture collections and forest soil	Other *Bacillus* spp. O	112[b]	0	0	—	—
	Other *Bacillus* spp. WC	112[b]	0	0	—	—
	Other *Bacillus* spp. spore	112[b]	0	0	—	—
	Other genera O	29	0	0	—	—
	Other genera WC	29	0	0	—	—

[a] Whole cell.
[b] Two strains of *B. cereus* showed strong autoagglutination.

0 to 2+, but was clearly distinguishable from the fluorescence of stained cells. A number of fungi and actinomycetes, freshly isolated from the forest soil, were also observed for autofluorescence to examine the possibility of their interference with the correct identification of the bacilli. Autofluorescence of actinomycete hyphae and spores, as well as fungal spores, never exceeded 2+, and only a very small proportion of fungal hyphae exceeded 2+, such hyphae being clearly distinguishable from stained bacterial cells or spores. Sometimes autofluorescence colors other than green were seen, particularly amongst the actinomycetes. None of these fungal or actinomycete strains showed increased fluorescence when stained with the fluorescent antibodies.

The possibility that older *Bacillus* cells might fluoresce less brightly when stained with fluorescent antibodies was also tested. Stained cells from 21-day-old cultures showed the same, or only slightly less, specific fluorescence compared with cells from 24-hr-old cultures. Maximal fluorescence of pure culture preparations was obtained by use of a 1:4 dilution of the *B. subtilis* conjugates and a 1:2 dilution of the *B. circulans* conjugates. The greatest dilution of conjugate with maximal staining power was obtained when the stained cells were grown in the same medium as that used to produce the fluorescent antibodies.

The specificity of the spore antisera was not determined. However, the fluorescent antibodies for spores of the *B. subtilis* forest soil isolate stained spores of six strains of *B. subtilis* with 4+ fluorescence. They did not stain the vegetative cells of any of these strains, or the vegetative cells or spores of six strains of *B. circulans*.

Detection of Bacillus spp. on soil particles. Organic and mineral particles from the A_1 horizon of the Freshfield soil were stained with fluorescent antibodies for the O antigens of *B. subtilis* forest soil isolate, *B. subtilis* NCTC 6284, *B. circulans* forest soil isolate, and *B. circulans*

NCTC 7578, and for the spore antigen of *B. subtilis* forest soil isolate. Observation of soil stained with fluorescent antibody for the O antigen of *B. circulans* NCTC 7578 showed that no cells were stained, confirming the results of agglutination tests with the homologous antiserum. All of the other three preparations, for O antigens, produced stained cells, and, of the two *B. subtilis* strains used, the fluorescent antibody for the soil isolate produced slightly brighter staining. Thus, further staining of soil was with the O antigen fluorescent antibodies of the *B. circulans* and *B. subtilis* forest soil isolates. The antigens of these forest soil isolates were grown in CSE, because the brightest staining of the bacteria on soil particles was from fluorescent antibodies prepared from these antigens. A 1:2 dilution of the *B. subtilis* preparation and a 1:1 dilution of the *B. circulans* preparation gave optimal fluorescence.

Figures 2 and 3 show a quartz mineral particle and a fragment of decomposing root, respectively, stained with fluorescent antibody for *B. subtilis* O antigen. Very few *B. subtilis* cells were observed on mineral particles but, when present, were generally regular rods (1.0 to 2.0 μ by 0.3 to 0.5 μ) and were in colonies of 1 to 14 cells. Colonies on mineral particles were composed of an average of two to three cells. These cells were very similar to those of *B. subtilis* growing in culture, but were smaller (culture cells: 2.0 to 4.0 μ by 0.6 to 0.8 μ). Organic material, on which the majority of *B. subtilis* cells were observed, was most frequently colonized by large, and often curved, cells (4 to 11 μ by 0.8 to 1.1 μ) in colonies of 1 to 40 cells, with an average of 4 to 5 cells per colony. Thus, the cells on organic particles were larger than cells of *B. subtilis* grown on culture media in the laboratory. Colony sizes, however, may have been slightly underestimated, since a small number of cells were found, on slide surfaces, detached from soil particles, and others, again in very small numbers, were found in the used stain and washing salines. These cells may have become detached from particles or may have been originally present in the soil solution.

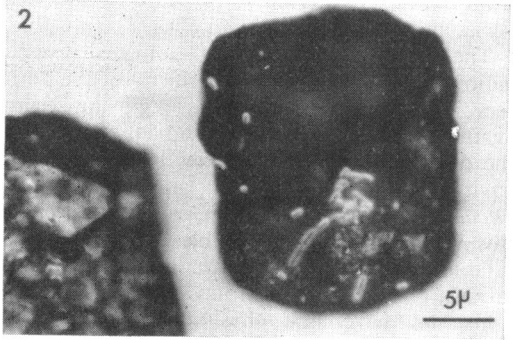

FIG. 2. *A_1 horizon forest soil stained with Bacillus subtilis (forest soil isolate) O antigen fluorescent antibody, showing the presence of a colony of B. subtilis cells on a small cryptocrystalline quartz particle coated with organic matter.*

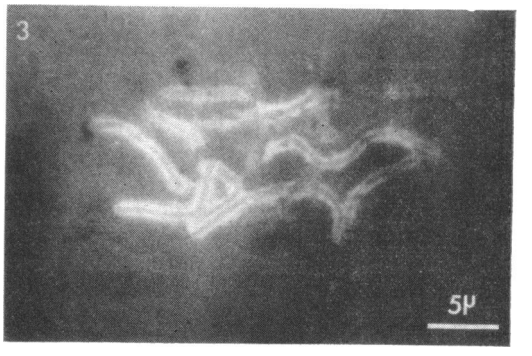

FIG. 3. *A_1 horizon forest soil stained with Bacillus subtilis (forest soil isolate) O antigen fluorescent antibody, showing a fragment of decomposing pine root colonized by cells of B. subtilis.*

Colonies of *B. circulans* detected in the same way were most frequently composed of 2 to 10 cells per colony, on both mineral and organic particles, though colonies of up to 25 cells were observed. The cells were either short rods or coccoid (*see* Fig. 4), were most frequently recorded on organic matter, and were quite unlike the long cells that are characteristic of this species when grown on artificial culture media.

The intensity of staining of cells on soil particles was usually slightly lower than that observed on

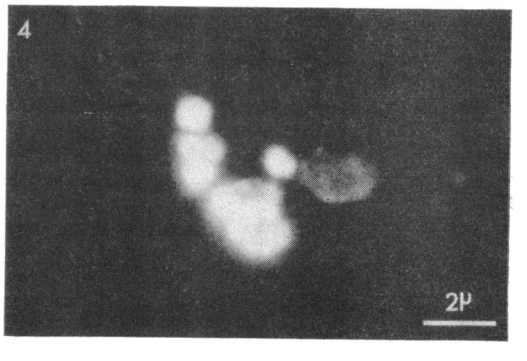

FIG. 4. *A_1 horizon forest soil stained with Bacillus circulans (forest soil isolate) O antigen fluorescent antibody, showing a colony of intensely staining coccoid cells on the surface of a loose aggregate of decomposing organic matter, bound together by nonfluorescent fungal hyphae.*

corresponding pure culture slide preparations. However, *B. circulans* cells on organic particles were frequently noted as being intensely stained and having a diffuse outline (Fig. 4). Biegeleisen (1), observing tissue imprints of dried meat stained with *B. anthracis* fluorescent antibody, reported cells fluorescing in a very similar manner and attributed this to the presence of a large quantity of capsular material.

Figure 5 shows part of the surface of a quartz mineral particle stained with undiluted fluorescent antibody for *B. subtilis* spore antigen. Several spores are visible. When soil was stained with a mixture of fluorescent antibodies for both *B. subtilis* O and spore antigens, free spores appeared to be present within colonies of cells. Both antisera, however, were conjugated to fluorescein isothiocyanate, making a distinction of spores and cells more difficult.

Autofluorescence and nonspecific staining of the soil particles seemed of little consequence in the soil examined. A few particles (mostly organic) autofluoresced bright apple-green or bright yellow, whereas some others took up the conjugated dye to fluoresce a dull green. However, the great majority of the particles were nonfluorescent or else autofluoresced a dull green. Particles fluorescing white, yellow, red, or blue were relatively rare, and often these colors were only present as patches or streaks on large particles. Attempts were made to eliminate soil particle fluorescence by use of the following range of potential fluorescence quenchers on stained soils (at concentrations of 1, 0.1, 0.01, and 0.001%): methylene blue, acid fuchsin, congo red, Lugol's iodine, basic fuchsin, phenol, Ziehl's carbol fuchsin, potassium permanganate, sodium hydroxide, and sodium pyrophosphate (also at 2%).

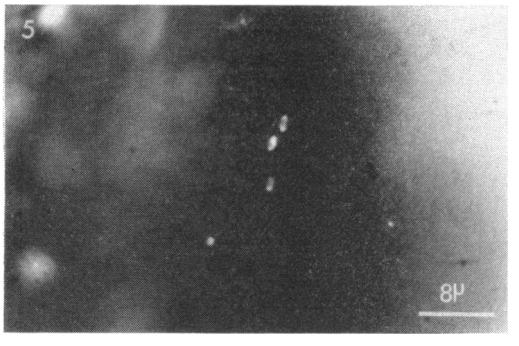

FIG. 5. *A_1 horizon forest soil stained with Bacillus subtilis spore antigen fluorescent antibody, showing the presence of spores on the surface of a mineral quartz particle, with oxidized iron present as inclusions and as a component of an external coating.*

In no case did a quencher reduce soil particle fluorescence, or improve the contrast between cells and soil particles.

DISCUSSION

This investigation has shown that several precautions may be necessary to aid the successful application of the fluorescent-antibody technique to the identification of bacteria in soils. It is evident from the data obtained on the changes in antigen production encountered by growing organisms on different media that the medium used to produce antigens for injection into rabbits should be carefully specified. A cold extracted soil medium (CSE) enabled antisera with higher titers (and possibly with a more relevant content of antibodies) to be produced, when tested against antigens from cultures grown in CSE, and when used as a fluorescent-antibody preparation to stain cells on soil particles. High titers have not always been regarded as advantageous when using fluorescent antisera, since it has been suggested that nonspecific reactions can occur if highly potent antisera are used. However, with the advent of high-purity crystalline dyes, it is possible that high-titer antisera conjugated with low dye-protein ratios may reduce nonspecific staining reactions (2, 5, 7). Also, when antiserum is scarce, high-titer antisera are useful because they can be diluted to give larger quantities of reactive serum. Although the changes in antigen production appeared to be to some extent quantitative, qualitative changes did occur, and these must always be carefully considered when preparing antigens for studies on soil organisms. Also, the specificity of antisera against capsulate and noncapsulate strains might be different. Organisms failing to produce a capsule under soil conditions might need to be stained with antisera from noncapsular strains of organisms, and vice versa, although this might not always be possible.

The use of the technique in ecological investigations is governed by the specificity of the antisera. Species-specific antisera, e.g., antiserum against *B. subtilis* O antigen, can be used to study the distribution of these species in different soil environments. Strain-specific antisera, e.g., *B. circulans* O antigen antiserum, cannot be used in this way but may be valuable in studying the establishment of competing strains in mixed populations, especially if the strains reacting with the antiserum have key physiological properties. Antisera against different strains could be conjugated with different-colored dyes, e.g., fluorescein isothiocyanate and rhodamine isothiocyanate. Finally, antisera that are specific for only one component of the cell or phase of growth can be of considerable importance; e.g., antisera against

spores of *B. subtilis* might be used to study the onset of dormancy in bacterial populations. Unfortunately, fluorescent antisera will not distinguish between living and dead cells or, if the cells are living, will give no indication of their physiological activity. To be certain that stained vegetative cells are active, information on the survival time of antigens in dead cells in soil is needed.

The method of observing the stained bacteria is also important. If one wishes to determine the growth pattern of an organism in soil, especially mycelial forms which grow from particle to particle, the contact slide method as used by Schmidt and Bankole (17) is useful. However, one cannot be sure whether the forms observed on contact slides are typical of those on soil particle surfaces. The factors limiting growth on the surface of a humus particle may be quite different from those affecting growth in a water film on a glass slide. For this reason, we have examined the growth of cells on individual particles taken directly from the soil. In the past, examination of soil particles has been partly impaired by the limitations of the illuminating system. With incident ultraviolet light, however, it is possible to see organisms not only on mineral particles, but also on opaque pieces of organic matter. This is important, since it has been shown that approximately 60% of all bacteria in this soil occur on organic particles, even though these particles provide only approximately 15% of the observable surface area of the soil particles (6*a*). In other soils, e.g., clays, with smaller particles, this observational problem may be less serious; however, difficulty in staining the soil without washing away the clay fraction may be encountered. Eren and Pramer (3) found that clay particles decreased the intensity of cell fluorescence and interfered physically with their observations of *Arthrobotrys conoides*.

Initial observations of *Bacillus* spp. in the soil have shown that, although *B. subtilis* retains the approximate size and shape of cells found in culture, cells of *B. circulans* become more variable, and possibly encapsulate on organic material, suggesting that the ecological roles of the two species differ. This conclusion is supported by Goodfellow (Ph.D. Thesis, Liverpool University, Liverpool, England), who studied soil isolates of these two species and showed that *B. subtilis* was much more biochemically active than *B. circulans*, as determined by laboratory tests. These tests showed that some isolates resembled *B. circulans*, whereas others were intermediate between it and *B. brevis* and *B. laterosporus*. The *B. circulans* strains found in this soil are also serologically related to *B. alvei*, further illustrating the difficulties experienced by Knight and Proom (9), Smith, Gordon, and Clark (20), Iyer and Bhat (8), and Goodfellow (PhD. Thesis) in delineating the soil isolates belonging to morphological group 2 of the genus *Bacillus* (20).

We have also shown that cells of *B. subtilis* are capable of forming spores under soil conditions, although the possibility cannot be excluded that the spores were formed during the earlier stages of preparation of the slides. Although pasteurization experiments have provided evidence for the presence of spores, they have not proved it, since it is possible that the presence of extraneous organic matter may affect the survival of the vegetative cells under these conditions. Now that the position of these spores can be determined in soil, more rational experiments on the factors affecting their formation and disappearance can be formulated. Further data on the occurrence of spores and vegetative cells of these bacteria under different environmental conditions will be published elsewhere.

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