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Abundant Microorganism in Soil[1]

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ABSTRACT

CASIDA, L. E., JR. (The Pennsylvania State University, University Park). Abundant microorganism in soil. Appl. Microbiol. **13**:327–334. 1965.—The presence in soil of large numbers of a catalase-negative, microaerophilic, coccoid microorganism was demonstrated. Use of media of high nutrient value, without incorporation of inhibitors, and growth in the absence of antagonistic microorganisms were utilized to isolate this organism from soil dilutions greater than those providing growth by other means. The organism described does not grow on soil extract agars and is missed by conventional counting techniques for soil organisms. On the basis of morphological and growth characteristics, this organism appears to have at least some taxonomic relationships to the families Actinomycetaceae and Mycobacteriaceae. It is proposed that this organism makes up much of the coccoid microflora of soil as observed by light and ultraviolet fluorescence microscopy.

Various studies (Conn, 1918; Waksman, 1927; Thornton and Gray, 1934; Jones and Mollison, 1948; Russell, 1950; Skinner, Jones, and Mollison, 1952; Seifert, 1958) have revealed that the numbers of microorganisms in soil as obtained by plate counts do not agree with the total microbial numbers which have been observed microscopically in these same soils. In fact, estimates made by use of microscopic techniques have placed the total microbial population of soil at 50- to 100-fold or more than that observed by all plating and other laboratory culture methods. This difference has been explained in part (Russell, 1950; Skinner et al., 1952) by the assumption that many of the microorganisms observed microscopically were dead cells. However, this assumption may be questioned, because evaluation of the soil microflora by acridine orange ultraviolet fluorescence microscopy (Strugger, 1948; Seifert, 1958; Macaulay Institute for Soil Research, 1959) has shown a similar disparity in numbers for the living microflora of the soil. Also, calculations of respiration rates of soil bacteria and the numbers of bacteria needed to maintain the protozoal population feeding on them have tended to confirm the microbial counts for soil as obtained by microscopy (Russell, 1950).

Casida (1962) described a technique using acridine orange ultraviolet fluorescence microscopy combined with micromanipulation for isolating and growing individual microbial cells from soil. However, the microorganisms successfully isolated by this procedure were those which one might have expected by use of the more conventional soil plating techniques.

The object of the present study was to devise a method for isolation of the coccoid microflora of the soil, on the assumption that these microorganisms exist in numbers greater than those for all other soil microflora, and that these microorganisms comprise the differences between results of the cultural and microscopic methods for determining numbers of microorganisms in soil. The method selected was based on the possibility that these microorganisms might be nutritionally fastidious and might grow only poorly in the laboratory in competition with other soil microorganisms. Also, inhibitors of microbial growth were not utilized to lessen the possibility of induction of L-form growth of soil microorganisms.

MATERIALS AND METHODS

The soils used for this study were not dried, but were screened to remove stones, roots, and other debris. Soils B-1, B-2, H, W, A, and D were Hagerstown silty clay loams secured from various sites in Centre County, Pa. Soil C was a sandy loam obtained from the vicinity of Los Angeles, Calif. For all soils, vegetation, if present, and approximately the top 1 inch of soil were removed; the samples were taken from depths of approximately 1 to 3 inches, except for B-2 which was secured from a depth of 3 to 6 inches. Soil H was obtained from a fence row under sod, soil W from a sweet clover field, and soils B-1 and B-2 from an alfalfa-bluegrass field sampled in October and May, respectively. The latter soil samples were known to

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have received no manure or other fertilization for the previous 13 years, and livestock had not grazed the land during this period. Soil A was taken from a corn field in October and soil D from beneath shrubbery; soil C was fallow with no vegetation. Soils A, C, and D had *p*H values of 7.0, 6.1, and 7.7, respectively; *p*H values for the remaining soils are listed in Table 1.

Medium 1 contained (per liter): Heart Infusion Broth, 25.0 g; Casitone, 4.0 g; yeast extract, 5.0 g; bromocresol purple solution (1.6% in 50% ethyl alcohol), 10.0 ml; *p*H 7.4. Medium 2 was composed of (per liter): Heart Infusion Broth, 25 g; Casitone, 4.0 g; yeast extract, 5.0 g; dextrose, 5.0 g; $NaNO_3$, 1.0 g; *p*H 7.4. Medium 3 was Heart Infusion Broth containing 1.0 g per liter of $NaNO_3$. Soil extract agar contained (per liter): K_2HPO_4, 0.2 g; soil extract (Allen, 1957), 200.0 ml; tap water, 800.0 ml; agar, 15.0 g; *p*H 6.8 to 7.0. Brain Heart Infusion Broth, Heart Infusion Broth, Fluid Thioglycollate Medium, yeast extract, and Casitone were Difco products.

Anaerobic growth conditions were obtained by two procedures. Cultures were incubated in sealed desiccators under a mixture of 95% N_2 and 5% CO_2, after first flushing the system three times with this gas mixture. No attempt was made to remove traces of O_2 present in this gas mixture. When cultures were grown in screw-cap tubes (16 by 150 mm), the caps were tightened for incubation under air, but were loosened to allow gas diffusion for incubation under the gas mixture.

In the second procedure, a pyrogallol-carbonate seal was provided for test-tube cultures by trimming the cotton plug and pushing it inside the tube above the medium. An absorbent cotton plug then was inserted and moistened consecutively with three drops of 50% pyrogallic acid and fresh 10% Na_2CO_3 solutions. A rubber stopper then was inserted firmly.

Determinations of catalase activity were made on cells grown in both the presence and absence of O_2. Fresh 3% H_2O_2 was added to colonies on agar surfaces and, also, was mixed with cells on slides for microscopic observation. Colonies lacking catalase activity were further tested with H_2O_2 by macerating the colonies while observing at 14-fold magnification. In some instances, the latter procedure was necessary because of slow penetration of the peroxide into the colonies.

Organic acid products of glucose fermentations were checked by paper chromatography (Kennedy and Barker, 1951) and by thin-layer chromatography with silica gel G (Brinkman Instruments, Westbury, N.Y.). The latter procedure utilized solvent systems 12 (ethyl alcohol-NH_4OH-water, 20:1:4) and 23 (ethyl alcohol-chloroform-NH_4OH, 80:19:1); acid spots were detected by spraying the chromatograms with Schwartzman's (1960) reagent.

RESULTS

Preliminary microbial counts. Soils to be used for isolation were first plated by spreading samples of soil dilutions in sterile tap water on the surface of soil extract agar; samples were incubated at 20 C for 14 days. Colony counts were made by observing the surfaces of the plates with a stereo microscope at 14-fold magnification. The totals for all microbial colonies appearing on these plates are recorded in Table 1.

Preliminary counts by "dilution frequency" were also made on these soils. A 1-g portion of soil was blended in a sterile Waring Blendor micro-head with 100 ml of Heart Infusion Broth adjusted to *p*H 7.8 with KOH, for 1 min on, 1 min off, and 1 min on, in succession. The blended soil dilution was allowed to stand for approximately 30 sec to dissipate foam, and then a 1-ml sample was transferred to 99 ml of fresh Heart Infusion Broth (*p*H 7.8) and shaken. Further 10-fold dilutions of the soil suspension were carried out in a conventional manner in Heart Infusion Broth. For each 10-fold dilution from 10^{-5} to 10^{-10}, five 1-ml samples were dispensed aseptically in screw-cap tubes containing approximately 1 ml of slanted 1.5% agar in water. The lids were screwed tight, and the tubes were incubated for 4 days at 30 C and then observed for indications of growth. The numbers of tubes showing growth at each dilution indicate an approximate agreement with the soil extract agar plate counts; both are presented in Table 1.

Isolation and purification. Portions (1 g) of soil were dispensed and diluted in a manner similar to that for determination of numbers of soil microorganisms by "dilution frequency." Based on the results presented in Table 1 for total numbers of microorganisms in these soils, the 10^{-8} and 10^{-9} dilutions were selected for isolation attempts. For each dilution of each soil, fifty 1-ml samples were transferred to screw-cap tubes containing 1 ml of slanted 1.5% agar in water. The caps were screwed tight, and the tubes were incubated up to 4 weeks at 30 C, with periodic observations for growth. At intervals, all tubes,

TABLE 1. *Total microbial counts for soil samples*

Soil sample	Soil *p*H	Soil extract agar $\times 10^6$	Dilution frequency				
			10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
H	7.2	19*	5†	4	1	0	0
B-1	7.6	33	5	5	1	0	0
B-2	7.5	62	5	2	1	0	0
W	7.1	24	5	5	5	1	0

* Number of organisms per gram of soil $\times 10^6$ after incubation for 14 days at 20 C.

† Number of dilution tubes showing growth from a total of five tubes at each dilution incubated for 4 days at 30 C.

including those not showing growth, were streaked on the surfaces of slants of Heart Infusion Agar (*p*H 7.8) in screw-cap tubes; the caps were screwed tight, and the tubes incubated in air at 30 C. When growth occurred, it was streaked on plates of similar medium, and the plates were incubated in air at 30 C. The resulting small, white, opaque colonies were transferred to agar slants of similar medium for incubation in air at 30 C. The growth in these tubes was checked for catalase activity, and, if found to be catalase-positive, the tubes were discarded. All catalase-negative cultures were restreaked on a similar medium for purification.

The numbers of catalase-negative cultures recovered from each dilution for each soil are presented in Table 2. These isolations were carried out by four different individuals at various times over a period of 1 year. Isolations by other workers in this laboratory also have been made from soils A, C, and D.

A consistent growth appearance of the isolates was observed in the primary isolation tubes and on streak plates. In primary isolation tubes, initial growth occurred some time after the first week of incubation, and was evidenced by a white opaque button of cells at the butt of the slant under the liquid; the growth appeared ropey on swirling the tube. Usually, turbidity was not observed. The colonies on aerobic primary isolation streak plates were quite small (often less than 1 mm in diameter) and presented a "fried egg" appearance. Figure 1 shows these colonies as grown in air and under N_2-CO_2.

Isolates also were checked for cellular morphology with the Gram stain. The cells were Gram-variable, depending on the age of the culture, and appeared as coccoid cells or coccoid rods, often in short to long chains, and as highly pleomorphic swollen and elongated rods in the same culture.

Working stocks of these organisms were maintained in Brain Heart Infusion or Heart Infusion (*p*H 7.4) deep agar stabs or in deep broth cultures

TABLE 2. *Number of cultures isolated from soil samples**

Soil sample	Soil dilution	
	10^{-8}	10^{-9}
H	3	13
W	0†	4
B-1	13	8
B-2	4	2

* Results show the number of cultures isolated from 50 tubes at each dilution.

† See Table 1 for this soil sample.

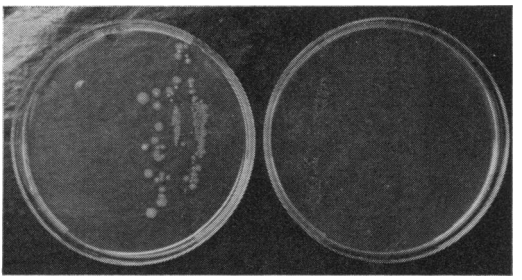

FIG. 1. *Colonies on Brain Heart Infusion agar. Plate at right incubated under air and at left under 95% N_2–5% CO_2.*

of similar media in screw-cap tubes with caps screwed tight. Primary stock cultures were grown in the above liquid media and then frozen. After initial isolation and purification, all cultures were incubated at 37 C under N_2-CO_2 or under a pyrogallol-carbonate seal. It will be noted that these growth conditions differ from those utilized in isolation of these microorganisms.

The isolates also were grown in Fluid Thioglycollate medium (containing sugar and indicator) without additional anaerobic precautions, but the cultures in this medium often died after a relatively short storage at refrigeration temperatures. Some difficulty also was experienced with cultures in Heart Infusion or Brain Heart Infusion broths or agars in that certain cultures died in these media rather soon after isolation. Proper conditions for maintenance of such strains have not been determined.

Macroscopic appearance of growth. Colonies grown on Heart Infusion or Brain Heart Infusion agars often presented a "fried egg" appearance and were of a larger size when grown under N_2-CO_2 than when grown in air (Fig. 1). Growth in Fluid Thioglycollate Medium or in deep agar shake tubes of Brain Heart or Heart Infusion agars incubated in air was continuous from a point approximately 1 to 10 mm below the surface of the medium to the bottom of the tube (Fig. 2). Submerged colonies in agar were biconvex discs. Deep stab cultures in agar medium incubated in air grew from just beneath or a few millimeters beneath the surface to the bottom of the stab and followed the line of the stab. Usually, growth was not observed at the surface of the agar, and there was no indication of gas formation within the agar either in these tubes or in Roux tubes.

Cellular morphology. Slants and petri plates of Brain Heart Infusion agar were inoculated by loop from broth inoculum (grown in a similar medium without agar), and were then incubated under air and N_2-CO_2 at 30 and 37 C. Cell mor-

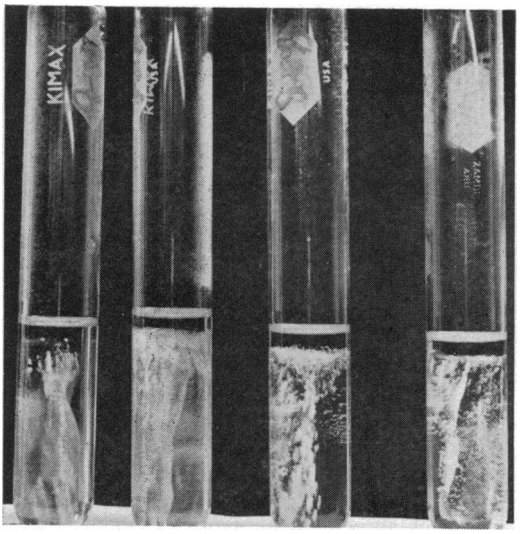

FIG. 2. *Growth in Fluid Thioglycollate Medium.*

phology was observed by Gram stain and by acridine orange ultraviolet fluorescence microscopy at 12, 36, 60, 88, and 108 hr of incubation. Typical strains also were observed at 2- to 4-hr intervals for the first 14 hr of incubation.

Observations of growth at these time intervals revealed a sequence of changes in the morphology and Gram reactions of these cells. The inoculum consisted mainly of gram-negative coccoid cells, singly, in pairs, and in chains. Some gram-positive cells of a similar size (approximately 0.5 μ or less in diameter) were interspersed in the gram-negative chains. At 10 hr of incubation, some swollen gram-positive cells (approximately 1.0 μ in diameter) were present, and by 12 hr all cells, regardless of size, were gram-positive.

At and beyond this time, some of the cells became markedly swollen and elongated to a length (approximately 3 to 7μ) several times that of the coccoid cells. This condition occurred with both aerobic and anaerobic growth, but persisted for a longer period of time in the presence of air. Coccoid cells were then produced within these swollen-elongated cells, as observed by acridine orange ultraviolet fluorescence microscopy, followed by disappearance of the wall of the mother cell. Figure 3 shows the appearance of these cells as viewed by dark-field microscopy. In some instances, the swollen-elongated cells produced one or two buds at one end of the cell, giving rise to branching as chains of the coccoid cells were produced (Fig. 4). The coccoid cells then divided so as to increase their numbers.

After 12 hr of incubation and at varying rates depending on the particular strain, the gram-positive coccoid cells and swollen-elongated cells became gram-negative in a stepwise fashion. A few coccoid cells in the chains became gram-negative, followed by most of the cells in the chains. These cells, as well as the swollen-elongated cells, individually lost their gram-positivity a little at a time, so that in a transition state the cells were largely gram-negative with one or two small areas of gram-positivity remaining within or on the cell (Fig. 5). This transition was also observed by fluorescence microscopy as a difference in fluorescence colors within the individual cell. Further division of some of the gram-negative cells yielded minute gram-negative cells barely visible at 1,800-fold magnification with a light microscope. Observations by acridine orange ultraviolet fluorescence microscopy (Casida, 1962) revealed that, at least by this method of observation, all cells were alive.

Attempts to secure passage of the small gram-negative coccoid cells through a 0.45-μ Millipore filter, followed by incubation in Brain Heart Infusion agar at 37 C under N_2-CO_2 or under conditions similar to those utilized for initial isolation from soil, were without success. Formation of heat-resistant spores did not occur in these cultures, as evidenced by spore stains and by lack of growth after pasteurization. Also, the cells were not acid-fast and did not exhibit motility.

Sugar fermentations. Sugar fermentation reactions were tested in Durham fermentation tubes containing medium 1 to which various sugars sterilized separately by filtration were added to a final concentration of 0.5%. Two drops of liquid inoculum grown in Brain Heart broth were added to each tube, and the tubes were sealed with pyrogallol-carbonate before incubation for 10 days at 37 C. Tubes to which cellobiose or cellulose (filter-paper strips) were added were incubated for 5 weeks.

No growth or only trace growth without lowered *p*H was observed for mannitol, glycerol, soluble starch, cellulose, ribose, arabinose, and xylose. Growth and decrease in *p*H to 4.7 to 5.0 without gas evolution were observed for glucose, maltose, galactose, and lactose. Results with raffinose and cellobiose were variable. Approximately half of the strains tested grew on raffinose and produced a lowered pH without gas formation. On cellobiose, 40% of the strains tested did not grow, 50% grew and decreased *p*H without gas formation, and 10% grew without *p*H change or gas formation.

It will be noted that in no instance was gas formation observed. Also, gas was not observed when glucose broth cultures were incubated under

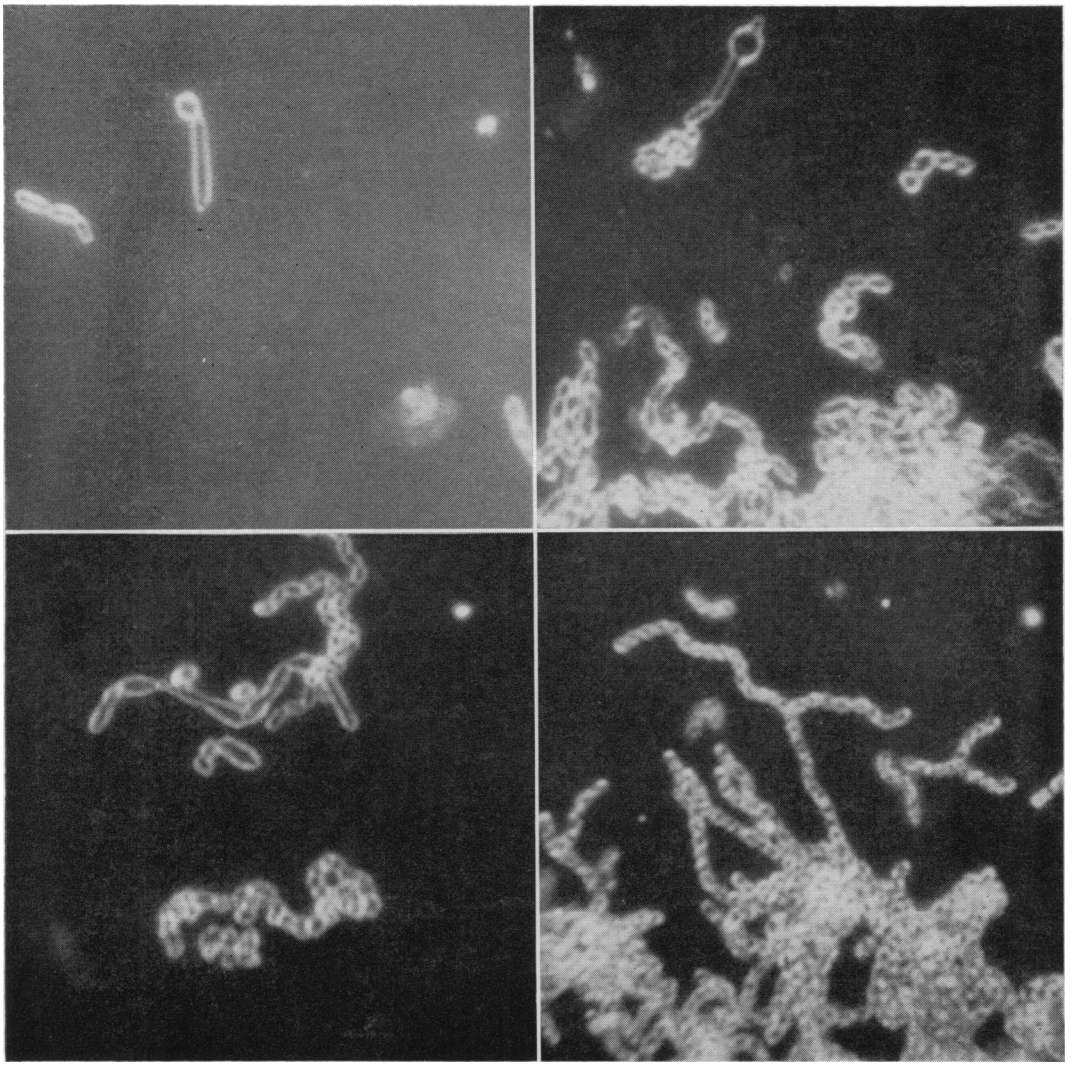

FIG. 3. *Cell morphology as viewed by dark-field microscopy.*

a 1:1 mixture of paraffin and Vaseline or incubated under N_2-CO_2.

Acid products of glucose fermentation were examined after 10 days of growth in medium 1 with 0.5% glucose and in nutrient broth and Heart Infusion Broth, each containing 0.33% glucose. Cultures in medium 1 were incubated at 37 C under pyrogallol-carbonate. The nutrient and Heart Infusion Broth cultures were incubated at 30 and 37 C under air and N_2-CO_2. Comparisons of paper and thin-layer chromatograms with chromatographed standards of formic, acetic, propionic, lactic, and butyric acids revealed that, at least by the methods of detection used, these acids had not been produced during growth of the organisms on glucose. Also, additional acid spots were not detected on these chromatograms.

Growth on various media. Brain Heart broth inoculum of four representative strains was surface-streaked and stabbed into agar slants of 10 media. The caps of the tubes were screwed tight, and the tubes were incubated for 15 days in air at 30 C. All strains grew in the stab but not on the surface of Tomato Juice Agar (Difco). One of the four strains grew in the stab of Sabouraud Maltose Agar (Difco). Growth did not occur in soil extract agar, with or without 0.2% glucose, or in the following Difco media: Potato Dextrose Agar; Malt Extract Agar; Sabouraud Dextrose

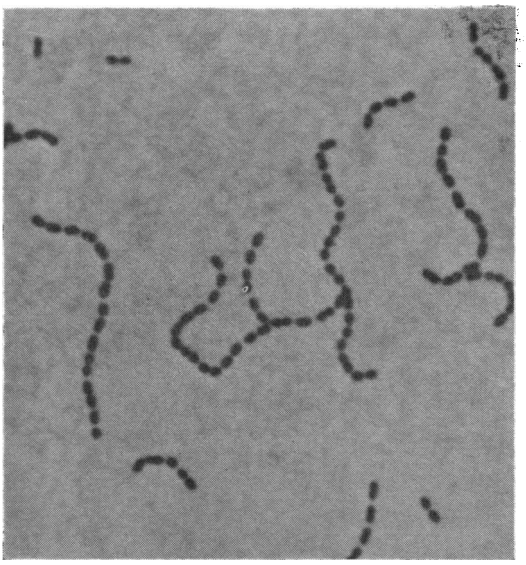

FIG. 4. *Branching chains of gram-positive coccoid cells.*

Agar; Wort Agar; Corn Meal Agar; Koser Citrate Medium containing 1.5% agar.

Hemolysis. Isolates were grown for 48 hr at 37 C on blood-agar under N_2-CO_2. Of the strains tested, 23% did not grow, and the rest presented no hemolysis or weak α-hemolysis.

Temperature limits for growth. Pyrogallol-carbonate sealed tubes of Brain Heart Infusion broth were incubated for 16 days at 10, 37, and 45 C. All cultures tested grew at 37 C, but results were variable at the other temperatures. At 10 C, 31% grew; growth occurred with 55% of the cultures at 45 C.

Litmus milk. Isolates were grown in air in litmus milk without additional nutrients for 10 to 14 days at 37 C. All strains tested produced an acid reaction, and 70% produced an acid curd without proteolysis. Slight reduction to reduction of the litmus occurred in 60% of the strains tested, but this reduction did not precede curd formation.

Nitrate reduction. Nitrate reduction was tested in media 2 and 3 in tubes incubated for 9 days

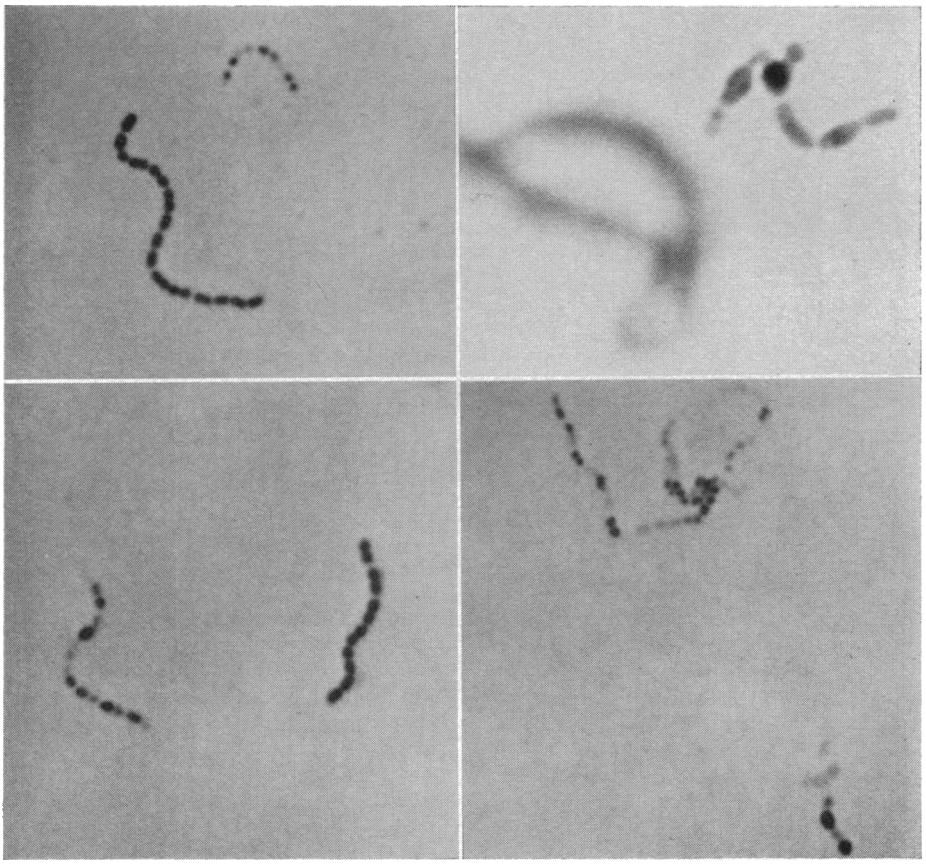

FIG. 5. *Transition of cells from gram-positive to gram-negative reaction. Black cells or areas within cells are gram-positive; gray areas are gram-negative.*

at 37 C under N_2-CO_2. Nitrite was determined by the α-naphthylamine-sulfanilic acid test, and negative determinations were further checked for residual nitrate by the addition of 20-mesh granular zinc to reduce the nitrate. None of the cultures tested was found to reduce nitrate.

Penicillin sensitivity. Two drops of broth inoculum were spread on the surface of Brain Heart Infusion agar in petri plates. Antibiotic assay discs (Schleicher and Schuell Co., Keene, N.H.) containing penicillin at 1, 5, and 20 units per ml were placed on the inoculated agar surface, and the plates were incubated at 37 C in air and under N_2-CO_2. Inhibition zones were determined at 24 hr for plates under N_2-CO_2 and at 40 hr or longer, depending on growth rates, for plates in air. Results under both incubation conditions were similar, with approximately 60% of the cultures sensitive to 5 units of penicillin per ml and the rest to 1 unit per ml.

DISCUSSION

A microorganism in soil has been demonstrated in numbers greater than those for all other soil microorganisms, at least as the latter may be counted by plating on soil extract agar or by conventional "dilution frequency" procedures. The high numbers of this microorganism were utilized to allow its isolation from soil by diluting the soil in broth medium to a point beyond which other soil microorganisms only rarely were present in the dilutions. Subdividing the dilutions into small portions for incubation assured that in most instances each individual cell did not have to compete with other members of the soil microbial population during growth. The isolated organism required media of high nutrient value for growth, and its lack of growth on soil extract agar explains its exclusion from counts of soil organisms performed with the latter medium.

Attempts to isolate this organism by direct plating of high dilutions of soil on agar media of high nutrient value were without success. Also, recovery of this microorganism from soil by the liquid culture technique was poor if the growth conditions for purified isolates were used. Thus, anaerobic incubation at 37 C was less successful than incubation at 30 C in air in tubes with caps screwed on tightly. No explanation is available for this observation unless a change in the organism occurs at the time of its separation from the soil.

The morphological cycle during growth of the isolates bears a resemblance to that of the genus *Mycococcus*, as described in *Bergey's Manual* and by Krasil'nikov (1958). However, *Mycococcus* is gram-positive, and the length of the rod-shaped cells ordinarily does not exceed twice the width. *Mycococcus* also differs from these isolates in that it grows well on ordinary culture media, it is aerobic, the gross appearance of the colonies is similar to that of the genus *Mycobacterium*, and the greater part of the species are chromogenic. In addition, Krasil'nikov (1958) states that, "In soil they are rarely encountered."

The genus *Actinomyces* also bears some resemblance to the organisms isolated from soil. This genus is anaerobic to microaerophilic, catalase-negative, requires media of high nutrient value, and produces colonies similar to those of the isolates. However, true mycelium is produced which fragments into irregular sizes but not necessarily into coccoid elements. As demonstrated by John Slack and Mary A. Gerencser (Medical Center, West Virginia University), certain of the isolates possess limited immunological relationship with *A. naeslundii* ATCC 12104. By immunofluorescence, conjugated antiserum yielding a 4+ reaction with this organism demonstrated a 1+ to 2+ reaction with 7 isolates, a limited reaction with 11 isolates, and no reaction with 7 isolates.

Thus, at present, the exact taxonomic position of the isolates is not known. Consideration of their cellular morphology might place them in the genus *Mycococcus*, but this classification would require a new subgroup encompassing the above described variations. The immunological relationship of the isolates to *A. naeslundii* cannot be explained at present.

The coccoid morphological phase of the isolates resembles the appearance of the major portion of the soil microflora as seen on direct microscopic observation of soil (*see* Jones and Mollison, 1948). If the isolates do comprise the major portion of the soil microflora, then the question must be raised as to the existence of soil nutrients to sustain a population of this size. No answer to this question is evident from the present studies, although it is possible that these organisms utilize the nutrients of humus or are parasites on some component of the soil micro- or macro- flora or fauna.

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LITERATURE CITED

ALLEN, O. N. 1957. Experiments in soil bacteriology. Burgess Publishing Co., Minneapolis.

CASIDA, L. E., JR. 1962. On the isolation and growth of individual microbial cells from soil. Can. J. Microbiol. **8**:115–119.

CONN, H. J. 1918. The microscopic study of bac-

teria and fungi in soil. N.Y. State Agr. Expt. Sta. (Geneva, N.Y.) Tech. Bull. 64.

JONES, P. C. T., AND J. E. MOLLISON. 1948. A technique for the quantitative estimation of soil micro-organisms. J. Gen. Microbiol. **2**:54–69.

KENNEDY, E. P., AND H. A. BARKER. 1951. Paper chromatography of volatile acids. Anal. Chem. **23**:1033–1034.

KRASIL'NIKOV, N. A. 1958. Soil microorganisms and higher plants. Academy of Science, USSR [Engl. transl. by Y. Halperin, Israel Program for Scientific Translations, 1961].

MACAULAY INSTITUTE FOR SOIL RESEARCH. 1959. Annual report. 1958–1959, p. 37.

RUSSELL, E. W. 1950. Soil conditions and plant growth, 8th ed., p. 147–148. Longmans, Green and Co., New York.

SCHWARTZMAN, G. 1960. Separation and identification of sodium salts of C_2 to C_5 volatile fatty acids by paper chromatography. J. Assoc. Offic. Agr. Chemists **43**:428–430.

SEIFERT, Y. 1958. The use of fluorescence microscopy in soil microbiology. Pochvovedenie **2**:50–54.

SKINNER, F. A., P. C. T. JONES, AND J. E. MOLLISON. 1952. A comparison of a direct- and a plate-counting technique for the quantitative estimation of soil micro-organisms. J. Gen. Microbiol. **6**:261–271.

STRUGGER, S. 1948. Fluorescence microscope examination of bacteria in soil. Can. J. Res. Sect. B **26**:188–193.

THORNTON, H. G., AND P. H. H. GRAY. 1934. The numbers of bacterial cells in field soils, as estimated by the ratio method. Proc. Roy. Soc. Ser. B **115**:522–543.

WAKSMAN, S. A. 1927. Principles of soil microbiology. The Williams & Wilkins Co., Baltimore.