STUDIES ON CELLULOSE FERMENTATION

II. AN ANAEROBIC CELLULOSE-DECOMPOSING ACTINOMYCETE, MICROMONOSPORA PROPIONICI, N. SP.

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During an investigation[1] of cellulose decomposition by termites, anaerobic shake tubes containing cellulose, proteose-peptone, and agar were inoculated with serial dilutions of the crushed alimentary tract of a worker termite (*Amitermes minimus*) from a laboratory colony. Several large clear spots which appeared in the cellulose after several weeks were found to contain numerous minute branching filaments, suggesting that the causal organism was an actinomycete. Since it appeared to differ from other cellulose-decomposing actinomycetes, some additional experiments were performed.

It was isolated in pure culture by inoculating it into shake tubes of cellulose or glucose with serial dilution and by subculturing similar series from a colony in the tube of highest dilution. A sparse formation of spores was observed in cultures grown on cellulose and proteose-peptone, but, if powdered dried grass or its aqueous extract was used in place of the proteose-peptone, an abundance of spherical spores developed. Their diameter averaged about 0.8 μ. They were borne singly on short side branches of the mycelium (figure 1) in a manner characteristic of the genus *Micromonospora*.[2]

The oxygen relationships of this strain of *Micromonospora* were studied by inoculating parallel series of aerobic and anaerobic dilution tubes containing cellulose, agar, and grass extract.

In the anaerobic series all air was displaced by bubbling oxygen-free nitrogen (95 per cent) and carbon dioxide (5 per cent) through the test tube before stoppering. In the aerobic series alveolar air (approximately 15 per cent O_2, 5 per cent CO_2, and 80 per cent N_2) was similarly bubbled. The tubes were then stoppered with a sterile rubber stopper, inverted several times, and quickly cooled in cold water to solidify the agar. This left a thin film of the agar medium lining the wall in the upper, gas-filled half of the tube. After two weeks of incubation cellulose digestion was evident in all parts of the anaerobic tubes, including the thin layer of cellulose agar in the upper half. In the aerobic series no colonies appeared in this thin agar layer exposed to the gas, nor were any colonies present in the upper 3 cm of solid agar in the lower part of the tubes. Colonies

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[2] The author is indebted to Dr. A. R. Colmer for the identification of this organism as a member of the genus *Micromonospora*, and also for suggestions on the manuscript.

appeared only in the depths of the aerobic tubes. These results indicate the anaerobic nature of the organism.

Growth is extremely slow in all media tested, two to four weeks being required for the development of visible colonies. This agrees with the slow growth of the aerobic forms isolated by Colmer and McCoy (1943). Temperatures of 30 to 40 C gave the most rapid development.

Glucose and cellulose are both suitable as a source of carbohydrate. Other sugars have not been tested. In addition to the carbohydrate, complex organic materials must be present. Extracts of liver, yeast, and dried grass support relatively rapid growth and these cannot be replaced by a mixture of pantothenic acid, thiamine, nicotinic acid, riboflavin, pyridoxine, biotin, and folic acid.

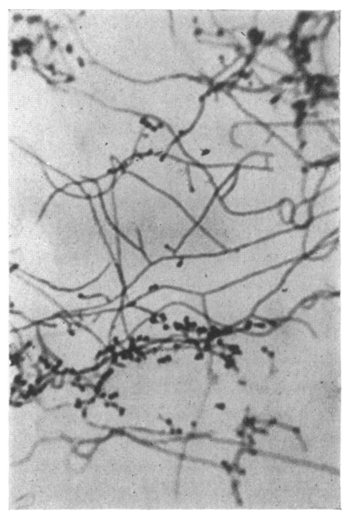

FIG. 1. PORTION OF A YOUNG COLONY OF MICROMONOSPORA SHOWING METHOD OF SPORE FORMATION (GRAM STAIN)

Colonies in cellulose agar are first visible as tiny clear areas in which the cellulose has been digested. As the size of the clear area increases, a white colony can be seen in the center. If deeply imbedded in cellulose, the colony enlarges equally in all directions, maintaining a spherical shape and completely digesting all cellulose in the region which it occupies. Older colonies differ macroscopically only in size from younger ones. If a colony develops adjacent to the glass, a difference in the interior of young and old colonies can usually be detected. Whereas colonies 1 mm or less in diameter display a uniform white opacity, the larger ones show this only in the outer part. The center is relatively transparent. Continued growth consists of an expansion of the outer white shell, which remains about 0.5 mm thick, with a corresponding enlargement of the central transparent region.

When a colony grows in the thin layer of agar lining the gas-filled part of the tube, it appears to consist of a white ring which gradually increases in diameter. A culture tube showing this is illustrated in figure 2.

If a thin section is prepared of an older colony imbedded in agar, it is found that it also consists of a white outer shell and a transparent center. When the section is examined microscopically, the extreme periphery of the colony is found to be composed of vegetative filaments which extend radially toward the undigested cellulose. They are separated from it, however, by a thin layer in which the cellulose is digested, but which contains no filaments, indicating that an extracellular cellulase is secreted. Inside the peripheral layer there is another layer or shell in which numerous spores are produced. Because of the scattering of light by the spores this is the part of the colony which shows the white opacity. The central transparent portion is relatively devoid of spores. Filaments are scarce, and in fresh mounts they are very indistinct. They fail to take the gram

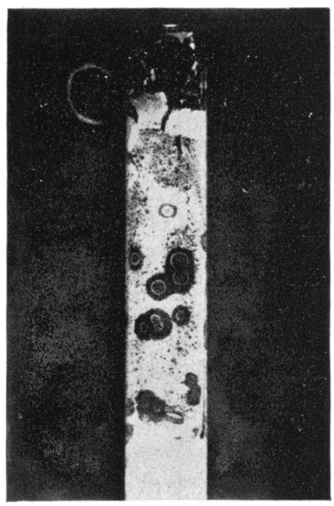

FIG. 2. COLONIES OF MICROMONOSPORA GROWING IN THIN CELLULOSE AGAR, ILLUSTRATING THE RINGLIKE POSITION OF THE SPORES

stain, in contrast to the young vigorous filaments on the periphery. The growing colony thus consists of a gradually expanding hollow shell, its outer surface composed of vegetative filaments, the adjacent inner portion containing numerous spores, and the center relatively devoid of protoplasm.

Two explanations for the disappearance of the spores and filaments in the central portion of the old colony may be suggested. They may be killed by the accumulation of metabolites and then undergo autolysis, or they may be resorbed and their substance transported to the peripheral portion where it is used in the synthesis of new cells. It has been noted that the outer, white shell of spores was still present in old tubes 3 years after they were inoculated and long after growth had ceased. These tubes contained maximal amounts of metabolic products. If accumulation of metabolites caused the disintegration of the central part of the growing colony, it would be expected that it would also cause the spores in the old tubes to disappear. This does not occur. Furthermore, in a growing colony the outer filaments and spores constitute the most active protoplasm. Consequently metabolic products would be as concentrated there as in any other region, including the center, yet the spores and filaments remain active. This shows that accumulation of metabolites cannot account for the absence of protoplasm in the center of the colony. It may be postulated that the various parts remain integrally related and that as materials are needed for growth in the peripheral portions they are drawn in part from the central region. A phenomenon of this sort is common in many of the higher fungi but is not usually encountered among lower forms.

On glucose the organism forms a solid rather than a hollow shell type of colony. The solubility of glucose and the insolubility of cellulose suggest an explanation for this difference. Glucose readily diffuses to the cells, and there is little stimulus to outward growth. On the other hand, digestion of cellulose adjacent to the colony increases the distance between the organism and the substrate, thereby reducing the efficiency with which the remaining cellulose may be utilized. Transfer of the protoplasm from the center to the periphery of the colony decreases its distance from the substrate and leads to more rapid utilization.

TABLE 1

Fermentation balances for Micromonospora

SUBSTRATE	PRODUCTS IN MILLIMOLS		
	CO_2	Acetic acid	Propionic acid
80 mg glucose	0.275	0.280	0.392
100 mg glucose	0.259	0.325	0.455
586 mg cellulose		0.87	1.73

Several cultures started with known amounts of glucose or cellulose have been analyzed for fermentation products, using methods already indicated (Hungate, 1944). Carbon dioxide and acids are produced. No hydrogen or neutral volatile products are formed. In one experiment the total acid produced (exclusive of CO_2) was found to be 0.794 milliequivalents. The volatile acid was 0.78 milliequivalents, indicating that the major part of the acids produced was volatile.

The Duclaux distillation of the volatile acid gave values intermediate between those for acetic acid and propionic acid. No formic acid was present. Acetic acid was identified as the sodium uranyl salt. In order to determine the nature of the other acids a fractional precipitation with silver nitrate was performed, and the silver contents of the first three fractions were determined. These were found to be 59.5, 58.6, and 58.5 per cent, respectively. This indicates the presence of propionic acid, silver content 59.63 per cent. No significant amounts of higher fatty acids were present.

From the values for the Duclaux distillation the relative amounts of acetic and propionic acids were determined by reference to the figures recorded by van Niel (1928). In several experiments the ratio of propionic to acetic acid was found to vary between 1.4 and 2.0. Fermentation data for three cultures are shown in table 1.

The fermentation products account for about 70 per cent of the carbon in the substrate. The amounts in which CO_2, acetic, and propionic acids are recovered indicate that the remaining materials have approximately the formula of a carbohydrate. The proportion of unidentified products is similar to that observed in the fermentation of glucose and cellulose by *Clostridium cellobioparus* (Hungate, 1944).

The ratios in which carbon dioxide, acetic, and propionic acids occur in the fermentation by *Micromonospora* are those commonly encountered in fermentations by the propionic acid bacteria (van Niel, 1928). Because of this feature it is appropriate to designate the present organism as *Micromonospora propionici*, n. sp. It is distinguished from other species of *Micromonospora* by its obligate anaerobic nature and its characteristic fermentation products. No colored pigment is formed.

In an old culture which was supplied with more cellulose than could be fermented, the extra cellulose disappeared after prolonged incubation. The culture medium showed significant copper reduction with Benedict's solution. Glucose was demostrated as the phenylosazone. No indications of cellobiose were observed and the reducing power of the culture was not increased by acid hydrolysis, indicating that the reduction was due to simple sugars. It is probable that glucose is the chief product of cellulose digestion by this organism. This demonstration of a cellulase in an old culture of *Micromonospora* is in agreement with the observation that an area of digested cellulose surrounds colonies growing in cellulose agar.

The significance of *M. propionici* to the termite from which it was isolated is of interest because in many wood-eating termites cellulose digestion depends on symbiotic microorganisms. The number of colonies developing in the original dilution series inoculated with the alimentary tract of a single *Amitermes* worker indicated that about 500 colony-producing units of *Micromonospora* were present in the gut. Although its occurrence in this number suggests that it may have been of some significance in the carbohydrate nutrition of the host, it does not appear probable that it is of major importance in this respect. Microscopic examination of a smear prepared directly from the gut of one of the termites failed to disclose any structures which could be identified as *Micromonospora*. The slow growth of *Micromonospora* in laboratory cultures also suggests that it would be of limited utility in the symbiotic digestion of cellulose.

M. propionici is not restricted to the termite gut. An anaerobic strain of *Micromonospora* indentical in appearance with it has been encountered and isolated from a culture of the protozoa from the rumen of cattle (Hungate, 1942). R. Meyer (1934) pictures a cellulose-digesting actinomycete which he observed in one of his anaerobic cultures. The morphology of the sporulating filaments shown in his photographs appears similar to that for *M. propionici*. The green color and the capacity for aerobic growth which he reported show that a different species was concerned.

DISCUSSION

An actinomycete exhibiting a characteristic propionic fermentation is of interest from the standpoint of the evolution of the group. It has been postulated on morphological grounds that the actinomycetes originated from the propionic acid bacteria (Stanier and van Niel, 1941). The latter show a tendency toward branching which finds a greater expression in the actinomycetes. *Micromonospora*, as one of the more primitive actinomycetes, might be expected to have a closer relationship to the propionic acid bacteria. The demonstration that it carries on a propionic acid fermentation provides physiological evidence supporting the hypothesis of an origin of the actinomycetes from the propionic acid bacteria.

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