STUDIES ON SOME LAKE-MUD STRAINS OF MICROMONOSPORA

DAGNY ERIKSON

Department of Agricultural Bacteriology, University of Wisconsin, Madison

Received for publication July 3, 1940

1. INTRODUCTION

Representatives of the genus *Micromonospora* have so far been reported mainly from soil or, in the case of thermophiles, from composts. The type species, *M. chalceae*, described by Ørskov (1923) who erected the genus, was first isolated by Foulerton (1905) under the name *Streptothrix chalceae*. This remained the only known strain among the Actinomycetes bearing the generic name *Micromonospora* until Jensen (1930, 1932) described a large number of soil micro-organisms which corresponded with Ørskov's description: "A branched unicellular mycelium is formed, consisting of very delicate hyphae with short lateral branches, each of which bears a single terminal spore." Recently, Waksman, Umbreit, and Cordon (1939) observed thermophilic strains of *Micromonospora* in composts and concluded from a study of the literature that some of the thermophiles isolated from soil or manure by Tsiklinsky (1899), from composts by Miehe (1907), and from decomposing clover hay by Schütze (1908) should also come into this genus.

The N.C.T.C. strain no. 4582 (Lister Institute) isolated by Gibson from blood culture in a case of Banti's disease and described by the writer (1935), which was tentatively classed as an *Actinomyces* (Ørskov's Group I) because of a sparse development of aerial mycelium, should probably be regarded as a *Micromonospora* of slow growth with scant lateral spore formation, resembling *M. parva* Jensen. Another strain corresponding with *M. fusca* Jensen was observed by the writer (1940) in a

study of pathogenic Actinomycetes. This was isolated by Duncan from diseased human hair, but was probably a saprophyte.

The ten strains which constitute the material of the present study were, with one exception, obtained from bottom mud samples from Lake Mendota, Wisconsin, taken from a depth of 18 to 20 m. They were picked at random from dilution plates (medium—sodium caseinate agar) which had been poured in the course of another study. The one other strain (TL) was fished from a plate which had been inoculated with lake water taken at a depth of 30 m., from Trout Lake in Northern Wisconsin.

Numerous workers on the microflora of these lakes have pointed out the comparatively large percentage of chromogens to be found. Snow and Fred (1926) estimated that yellow and orange organisms amounted to 35 per cent, and pink and red to 11 per cent of the total bacterial population of water samples from Lake Mendota. The mean count for chromogens in the bottom mud was found by Henrici and McCoy (1938) to be 13.4 per cent at 0 cm., the count descending regularly to 18 cm., below which level the proportions became distinctly lower. When it is remembered that the characteristic radiating *Actinomyces* appearance can, because of its minute size (the typical *Micromonospora* colony is only 1 mm. in diameter) be detected microscopically only, and when it is recalled that these workers were concerned with the population question in general and not with pure culture studies, the possibility arises that some of the chromogens already listed may have been *Micromonospora* strains. The figures 51, 52, and 53 in the colored plate given by Snow and Fred might very well portray species of this genus.

The ease with which the small pink or orange, hard-textured, *Micromonospora* colonies of this study were picked from a small selection of plates (in some instances there were many colonies of the same strain) has suggested that these organisms may be of some importance in lacustrine ecology. Preliminary work at present under way in this department indicates that *Micromonospora*-like colonies may constitute an appreciable proportion of the chromogens in the lake microflora.

2. EXPERIMENTAL

(a) *Cultural and biochemical reactions*

Although considerable individual variation was found, the ten strains studied all agreed in cultural characteristics with the large species-group, *M. chalceae*, to which 60 out of the 67 soil micro-organisms described by Jensen belonged. The growth was dense and compact on solid media, the small discrete colonies increasing very little in diameter with age, the color varying from pale pink to deep orange and becoming bluish- to greenish-black

TABLE 1

Two days' growth at 37°C.

STRAIN	SWEDISH FILTER PAPER	HOLOCELLULOSE	LIGNIN	CHITIN
Me1	+ (colorless)	± (colorless)	+ (colorless, on wall)	+++ (colorless)
Me2	−	−	−	−
Me3	+ (colorless)	± (colorless)	+ (colorless)	++ (colorless)
Me4	+++ (pale pink)	± (colorless)	+ (colorless)	+++ (colorless to pale pink)
Me5	++ (colorless)	± (colorless)	±	++ (colorless)
Me6	++ (pink)	± (yellow to pink)	+ (colorless)	++ (pink)
Me7	+ (colorless)	−	+ (colorless)	+++ (colorless)
Me8	+ (colorless)	−	+ (yellow-brown)	+++ (colorless to pink)
Me9	+ (colorless)	−	+ (yellow)	++ (colorless to pink)
TL	+ (colorless to pale pink)	−	±	+ (yellow)

as the glistening superficial spore layer was produced; no typical soluble pigment was yielded on synthetic media; growth in liquid media was in the form of small colorless to pink or orange granules with no turbidity of the medium; considerable diastatic and proteolytic activity was displayed, all strains slowly liquefying gelatin, digesting milk (after previous coagulation in the case of three strains), and clearing sodium caseinate agar medium; most strains showed a marked ability to decompose cellulose.

In view of the origin of these strains, this ability to decompose cellulose seemed of particular interest. Jensen (1930) had noted that several of his strains of *Micromonospora*, "when incubated

for a sufficiently long time (35 to 90 days), are capable of bringing about a marked decomposition of cellulose—stronger than that caused by most other ray fungi," and this phenomenon, together with the fact that the addition of cellulose or lignin to the soil yielded a selective increase in Micromonosporae caused him to

TABLE 2

Six weeks' growth

STRAIN	SWEDISH FILTER PAPER	HOLOCELLULOSE	LIGNIN	CHITIN
Me1	+++ (greenish-black colonies along paper strip)	+++ (colorless to black)	+++ (grey-green colonies)	+++ (orange growth, black on surface)
Me2	± (colorless)	−	++ (brown)	+ (yellow)
Me3	+++ (pink below liquid, grey-brown above)	±	+++ (brown)	+++ (orange, black on surface)
Me4	+++ (pink below surf., purplish-brown above)	±	+++ (brown)	+++ (orange, brown on surface)
Me5	±	±	++ (colorless)	+++ (orange)
Me6	+++ (pink to black)	±	+++ (pink to brown)	+++ (yellow to orange)
Me7	+++ (paper disintegrated below liquid, orange to brown above)	±	+ (brown)	+++ (yellow)
Me8	+ (colorless)	−	++ (dirty brown)	+++ (orange, black on surface)
Me9	+++ (purplish-pink below liquid, black above)	−	+++ (colorless to brown on wall)	+++ (orange, black on top)
TL	+++ (pale pink to dirty green)	−	++ (colorless colonies on wall)	+++ (orange)

suggest "that they may play an important role in the decomposition of cellulosic material in the soil." The lake-mud strains, which had been shown to attack ordinary filter paper in a mineral salt solution, were accordingly sown in this synthetic solution ($NaNO_3$, 1.0 gm.; K_2HPO_4, 0.5 gm.; $MgSO_4$, 0.5 gm.; H_2O,

1000 ml.; pH 7.2 to 7.4) to which was added (a) Swedish filter paper O.K., (b) maple holocellulose, (c) spruce lignin, (d) shrimp chitin, as the sole source of carbon. The results are tabulated in table 1.

It will be seen that, with the exception of Me2, all the strains grew very readily on cellulose and chitin. In both cases the growth appeared actually on the solid substrate. Longer incubation resulted in better growth on lignin and increased pigment production on the other two media, but yielded good growth on holocellulose in the case of one strain only.

TABLE 3

	ALIPHATIC HYDROCARBONS AND DERIVATIVES				FATS AND STEROLS		
Strain	Paraffin oil (purified medicinal)	Paraffin wax	α-hepta-chloro-propane	Trichloro-acetic acid	Olive oil	Palmitic acid	Cholesterol
Me1	++	+++	−	−	+++	+++	++
Me2	++	++	+	−	±	+	±
Me3	++	+	+	−	−	+	−
Me4	++	+++	+++	±	++	+++	+++
Me5	−	+	++	−	−	++	−
Me6	++	++	+	−	+	++	+++
Me7	+	+	−	−	±	−	±
Me8	+	+	−	−	−	++	±
Me9	++	++	++	±	+++	+++	+++
TL	+	+	+++	−	−	+++	++

The abundant growth on chitin and slow but relatively very good growth on lignin suggested that these micro-organisms might be capable of utilizing other carbon compounds of a resistant nature. Culture experiments were therefore made with the same synthetic stock solution, to which was added a large variety of organic compounds in the proportion of 1:1,000. The aromatic substances were steamed; the others were autoclaved in the ordinary way. Except where the material added colored the medium, the pigment produced in the growth was of the same nature as that described in tables 1 and 2; in many instances the growth remained colorless. The presence or absence of growth is noted after six weeks' incubation (table 3).

In a few instances the control tubes, which lacked any source of carbon, showed a minimal amount of growth; this was probably due to the carrying over of traces of nutrients in the inoculum, since it became progressively less on re-inoculation into fresh controls. In all cases where a positive sign is shown in the tables the quantity of growth is considerably more than that yielded by these control tubes.

TABLE 4

	AROMATIC HYDROCARBONS AND DERIVATIVES CONTAINING NO OXYGEN			PHENOLIC COMPOUNDS				
Strain	Toluene	Naphthalene	Para-dichlorobensene	Phenol	Resorcinol	m-cresol	β-naphthol	Tyrosine
Me1	−	+++	+++	−	+	−	+	++
Me2	−	++	+	−	±	+	+	−
Me3	++	−	++	−	+	+	++	±
Me4	+	−	+++	−	−	−	−	+
Me5	+	−	±	++	++	+	++	+
Me6	−	+	−	−	++	−	−	−
Me7	++	+++	+++	++	++	+	−	+
Me8	++	+	−	−	+	−	++	−
Me9	++	++	+	+	++	++	−	++
TL	−	−	+	−	+	−	−	+++

TABLE 5

	NITRO- AND SULPHONIC-DERIVATIVES OF PHENOLS			COMPLEX CARBOHYDRATES AND GLUCOSIDES				
Strain	Picric acid	Trinitroresorcinol	Na β-naphthoquinone-4-sulphonate	Inulin	Pectin	Amygdalin	α-methyl glucoside	Gluconolactone
Me1	−	−	+	++(a)	−	++	+	−
Me2	−	−	−	+(a)	+++	++	++	+
Me3	++	−	−	−	−	+++	++	++
Me4	−	−	−	+	−	−	+	+
Me5	−	++	++	+(a)	++	++	+	+
Me6	++	−	+	±	+	+++	+	−
Me7	−	−	+	−	++	+	++	++
Me8	+	++	++	+(a)	−	+++	+	+
Me9	+	+	−	++(a)	+	+++	++	−
TL	−	++	+	−	−	+	−	−

The accepted view of the chemical constitution of lignin is that it is a condensation product derived from a simple phenolic substance. It is therefore of interest to note that these organisms can grow, in some cases very well, on phenols such as resorcinol, and also upon other aromatic compounds such as naphthalene and para-dichlorobenzene. In table 5 it will be seen that fairly good growth is obtained for most of the strains on a sulphonic derivative of a phenol such as sodium β-naphthoquinone-4-sulphonate.

In the tubes marked (a) in the inulin column a definite alkaline reaction was given with brom-thymol blue as indicator. A simi-

TABLE 6

Sugars and sugar alcohols

STRAIN	SORBOSE	MELEZITOSE	RHAMNOSE	HEPTOSE	ERYTHRITOL	QUERCITOL	DULCITOL	*d*-ARABITOL
Me1	++	++	+	+++	++	++	±	+
Me2	++	−	+	±	−	−	++	++
Me3	−	++	−	±	++	±	+	++
Me4	−	+++	+	+	+	+	++	++
Me5	−	++	±	++	+	−	+	−
Me6	±	+++	++	+	+	+	+	+
Me7	±	++	+	±	−	−	++	+
Me8	++	++	++	±	+	+	++	++
Me9	±	+++	+++	−	+	++	++	+
TL	++	+	+	+	++	±	+	+

lar reaction was given in a few instances with α-methyl glucoside, and (in table 6) with dulcitol. Although growth on all the ordinary sugars is good, acid is very slowly produced—a yellow coloration with brom-thymol blue being produced within 30 days only by glucose, maltose, sucrose, and, to a much lesser degree, lactose. As with all the other Actinomycetes, no gas is evolved. Table 6 illustrates the ability of these Micromonosporae to grow on some of the rarer sugars and sugar alcohols.

From a survey of tables 1 to 6 it is apparent that these organisms are capable of obtaining their carbon requirements from a surprisingly large variety of substances. The excellent and rapid growth on chitin (tables 1 and 2) suggested that they might also derive their nitrogen from this complex material. Ac-

cordingly cultures were made using a salt solution from which the $NaNO_3$ was omitted. Table 7 shows the growth on chitin as the sole source of both carbon and nitrogen, compared with that on a few other substances also containing both C and N. The letter (p) in the tyrosine column indicates the appearance of a soluble pink to red pigment, and the numeral after it represents the number of days before this pigment appeared. The period of incubation was 30 days.

Here the growth was as good on chitin as a provider of both N and C as it was when sodium nitrate was added to the solution. The attack was as rapid and pigments were produced in the

TABLE 7

Without sodium nitrate

STRAIN	CHITIN	CAFFEINE	TYROSINE	TRINITRO-RESORCINOL
Me1	+++	−	++(p 10)	+
Me2	++	−	−	±
Me3	+++	−	+	−
Me4	+++	−	+	−
Me5	+++	−	+	+
Me6	+++	−	+(p 5)	++
Me7	+++	−	+(p 15)	++
Me9	+++	+	++(p 5)	±
Me10	+++	+	+(p 5)	−
TL	+++	−	++(p 8)	−

growth in the same way as in table 1. The appearance of the soluble red pigment in the tyrosine media (and in no other) is interesting as indicating the precursor to melanin which has been demonstrated for many other varieties of Actinomycetes. The little growth on caffeine confirms the findings of Jensen who noted that this substance was a poor source of nitrogen for his Micromonosporae. It is noteworthy, however, that a fair to moderate growth was obtained on one of the phenolic derivatives —trinitroresorcinol—when serving as the source of both nitrogen and carbon.

(*b*) *Aerobiosis and pigment production*

From the descriptions of growth in table 2 it will be evident that there is a characteristic difference in pigmentation above and

below the surface of liquid cultures. Thus, Me4 produces pink colonies in the liquid and purplish-brown ones on the filter paper strip above the solution; on chitin it is orange below and dark brown above. This is typical of the growth in liquid media whenever the organisms have a chance to anchor themselves to some solid material near the surface. To demonstrate that this phenomenon is due to the increased access to air afforded by some mechanical support, parallel cultures were made with Czapek sucrose solution, using plain tubes in one set, tubes containing narrow glass slides in the second, and tubes containing strips of asbestos in the third. The three sets were incubated in an upright position for 30 days.

The first set contained the usual bottom growth of colorless to pink or orange granules; Me7, which is a particularly vigorous strain, showed some orange growth on the walls of the tube also. In the second and third sets growth—of a similar nature—was in some instances confined to the bottom of the liquid only; but in all cases where the organisms adhered to the supporting surface, whether slide or asbestos, the growth showed the characteristic dark pigmentation associated with abundant spore formation. This darkening of the pigment would also, of course, appear in time on the ordinary liquid cultures as the level of the medium was lowered by evaporation and the volume of the growth increased to meet it. Even more quickly it would appear in the case of strains like Me7, which grew up the walls of the tube. The same phenomenon generally takes place on all slope or plate cultures on solid media within 10 to 20 days.

As further evidence that pigmentation generally, and in particular the subsequent production of a dark insoluble pigment, is causally connected with free access to oxygen, the three following experiments may be cited:

1. All the strains were sown on a variety of solid and liquid media, including the test Czapek sucrose solution and Swedish filter paper strips half-immersed in the stock salt solution. The basket containing the tubes was then wrapped in several thicknesses of dark paper to exclude light, and the cultures incubated in the ordinary way. In all cases the dark pigment was produced as usual on the solid media and upper portions of filter

paper after 30 days' incubation, while the bottom growth in liquid media was pink or orange, thus showing that the presence or absence of light is not a factor.

2. Strains Me4 and Me9 were sown in tubes of Czapek's sucrose solution, through which an intermittent stream of sterile air was passed for six days. The control tubes were inoculated from the same parent cultures and placed in a container nearby (room temperature about 25°C.). When the two sets of tubes were examined at the end of the period of aeration, it was found that the controls showed only a little colorless bottom growth while the two aerated tubes yielded a very much more abundant growth of bright pink colonies. In the case of strain Me9, two or three of the colonies which had been thrown up on the wall of the tube above the liquid level by the force of the air stream were already showing a trace of a purplish-black pigmentation.

3. All the strains were sown on (a) Czapek's sucrose solution, (b) Swedish filter paper half-immersed in the stock salt solution, and (c) chitin floating in the same solution. They were then incubated in an oat anaerobic jar for one month (McClung, McCoy, and Fred, 1934). At the end of this period a very scant colorless growth was apparent in the sucrose and chitin cultures (Me1 and Me9 showed macroscopic clusters of colonies), while the filter paper had to a very slight extent been eaten into, in the case of strains Me7, Me8, and TL, by a minute colorless growth. Subcultures from these tubes to Czapek's sucrose agar slopes, incubated aerobically, resulted in the usual pigmented growth. At the end of two months' anaerobic incubation, all the strains showed a very definite increase in growth (still colorless) on both sucrose and chitin, while the filter paper was disintegrated at water level in the case of Me7 and perceptibly softened in the case of TL. Again, subcultures incubated aerobically produced the ordinary pigmented growth.

The capacity, thus demonstrated, of these Micromonosporae to grow under conditions of reduced oxygen tension is of considerable interest, even though the growth is poor and very slow, since it throws some light on their natural habits at the bottom of a lake where the supply of oxygen is very slight or

even non-existent for large portions of the year. These results are at variance with those of Jensen, who reported that his soil Micromonosporae were unable to grow at all under anaerobic conditions. This is probably due to the fact that the period of observation in his case was 14 days only. At the end of 14 days' anaerobic incubation of our lake-mud strains there was no perceptible growth.

(*c*) *Colonial morphology*

The mesophilic soil Micromonosporae have been described by Jensen as morphologically similar: producing an extensively branched mycelium composed of delicate non-septate hyphae bearing at the ends of lateral branches single highly refractile spores. Observations on the present lake-mud strains confirm these findings. In some instances the lateral branches may be so short as to give the appearance of the spores being given off in twos, threes, or clusters (fig. 3), but repeated examination of living slide cultures and stained preparations has not elicited a constant picture of single spores only, or spores in clusters only, for any given strain. The regular rule is single terminal spores (fig. 8), but any strain may show in the same field two, three, or more spores in close juxtaposition or in *Botrytis*-like clusters, as mentioned by Jensen. These findings therefore are not in accord with the classification made by Waksman (1940) on the basis of his observations on thermophilic Micromonosporae.

When slide cultures with Czapek's sucrose agar as the medium are employed, the mycelium is seen to develop rapidly at 37°C. with the production of one, two, or more germ-tubes from a spore (fig. 1). Sporulation usually takes place within the mycelium on the second day. The concentration of spore-bearing branches is greatest at the center of the minute colony (fig. 2), and very soon this area becomes so dense as to limit direct microscopical observation to the margins of the colony. Figure 4 shows a few of the much thicker, angularly branched hyphae which sometimes emerge from the margins of older colonies, but on the whole fragmentation of the hyphae—so characteristic of the Proactinomycetes—does not occur, and the young filaments

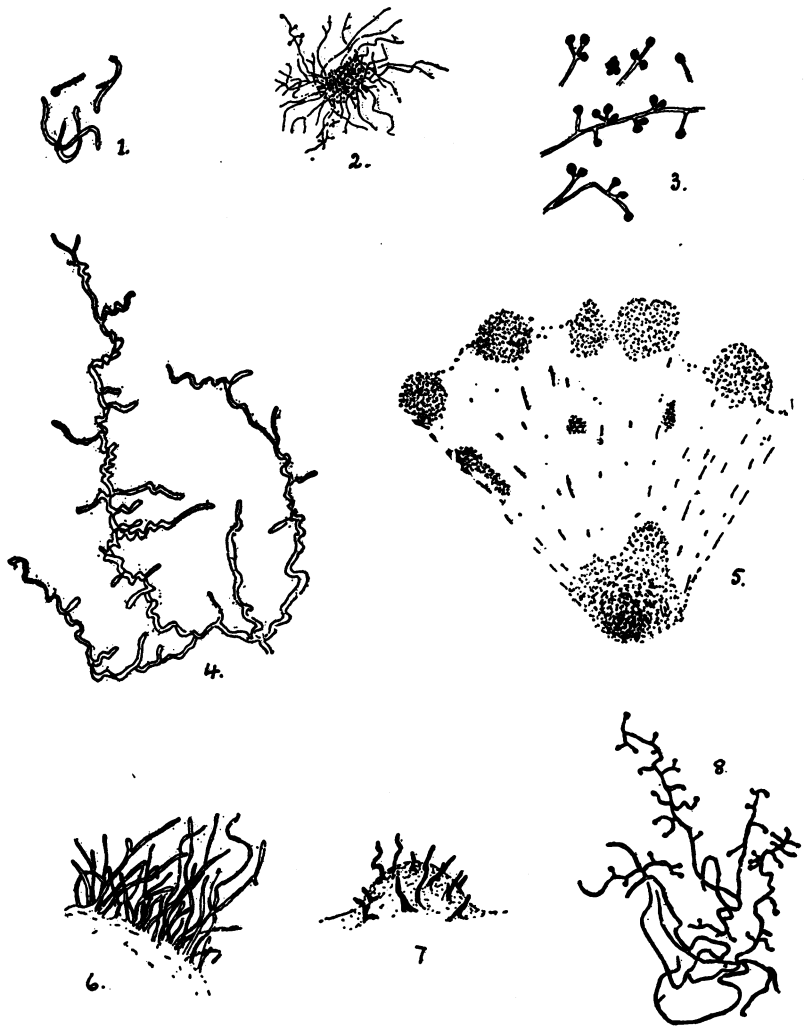


FIG. 1. Me1: Germination of spores, 18 hours 37°C., ×1250; living material, unstained, on Czapek sucrose agar.

FIG 2. Me7: Young mycelium sporulating at center, 2 days 37°C., ×500; living material, unstained, on Czapek sucrose agar.

FIG. 3. Me8: Highly refractile spores, borne in ones, twos, or threes, 8 days 25°C., ×1250; living material, unstained, same medium.

FIG. 4. Me8: Occasional much thicker, angularly branched hyphae at margin of colony, 15 days, 25°C., same medium, ×1250.

FIG. 5. Me4: Sector of rosette colony showing radial distribution of spore aggregates, 2 months 25°C., ×500; same.

FIG. 6. Me9: Marginal hyphae showing indications of dichotomous branching, 15 days, 25°C., ×1250; same.

FIG. 7. Me9: Aerial spikes on crest of colony, 15 days, 25°C., ×500; same.

FIG. 8. TL: Single spores on lateral branches, 7 days 37°C., ×1250; Gram-stained preparation from Czapek sucrose solution.

are always non-septate. Branching is in general monopodial, but an appearance of dichotomy is occasionally given by the extruded hyphae at the margin of a colony (fig. 6).

Aerial hyphae have been reported by Waksman, Umbreit, and Cordon (1938) for one thermophilic organism classed as a *Micromonospora*, but not by Jensen—with the exception of an occasional rudimentary growth—for the mesophilic varieties. These latter observations have been confirmed with the present strains, only one or two showing a sparse growth of aerial hyphae which never divide into spores (fig. 7). Such aerial hyphae have been noted only under unfavorable conditions, as when the medium is almost completely dried up and the colony aged. When the cultures are maintained under conditions which permit of the medium retaining considerable moisture over long periods of time, as in 12 oz. metal-capped, medicine bottles kept at room temperature (20 to 25°C.), colonies which are two to six months old frequently present a rosette appearance, as in figure 5. This diagram shows a sector of the original colony covered by a dense spore layer in the center, and smaller aggregates of apparently sessile spores radiating outwards. When such a rosette is transferred to a slide and examined with the highest dry objective, it is found that the intervening vegetative hyphae are almost completely disintegrated at the surface, although here and there a few well-defined branches can be seen bearing spores; it is only occasionally at the margins of the large clusters of very refractile spores that the short stalks which bear the individual spores can be discerned.

The difficulty of following microscopically all the stages in the development of such a dense compact little structure as a typical *Micromonospora* colony has already been indicated. It seemed advisable therefore to have recourse to sectioning methods.

Preparation of stained paraffin sections. A review of the work of earlier investigators of colonial organization of bacteria and other micro-organisms has been given by Greene (1938). The technique recommended by him and earlier by Hutchinson (1907), of covering the colonies with lukewarm agar to preserve the surface intact, was employed. It was later found that with

the firm compact *Micromonospora* colonies this complete embedding was not necessary, provided care was taken in handling the specimen. Colonies of varying ages from 3 days to 5 months were removed with a portion of the agar in which they had grown, and transferred to the fixing agent. This and subsequent liquids were pipetted in and out of the phial in order to avoid disturbance of the colony. The standard medium for growth was Czapek's sucrose agar. Bottom and surface colonies from liquid Czapek's sucrose media were also prepared for sectioning.

Acetic acid-formalin-alcohol, Bouin's, and Karpechenko's fluids proved equally good as fixatives. The most satisfactory dehydration was obtained by means of the well known alcohol, cedar-oil technique. Paraffin sections were cut with a rotary microtome at a thickness of 5 micra. Differential staining was effected by means of Mallory's and Flemming's triple stains, by iron-hematoxylin counterstained with safranin or orange G, and by erythrosin counterstained with Grübler's "Nachtblau." In all cases the spores took an intense stain and were readily distinguished from the vegetative mycelium. About 150 sections were cut.

Examination of sections. The most interesting fact which emerged from a study of these sections was the appearance of a well-marked zonation of spore-bearing hyphae within the colony. In the earlier paragraphs devoted to direct microscopic observation of living material it was noted that sporulation generally takes place in the centre of the colony on about the second day, and that the growth then becomes heaped up—or, alternatively, develops into the medium—thus impeding vision, while it is not until about the second week or later that the glistening superficial spore layer, which is usually associated with dark pigmentation, is produced. The series of vertical sections of colonies of different ages would, it was hoped, reveal whether there was any organized structure within the colony resulting in this production of a superficial spore layer.

Figure 9, which represents a 5-day-old colony of Me9, shows that, while the young colony is more or less homogeneous near the surface—occasional spores being produced anywhere in that

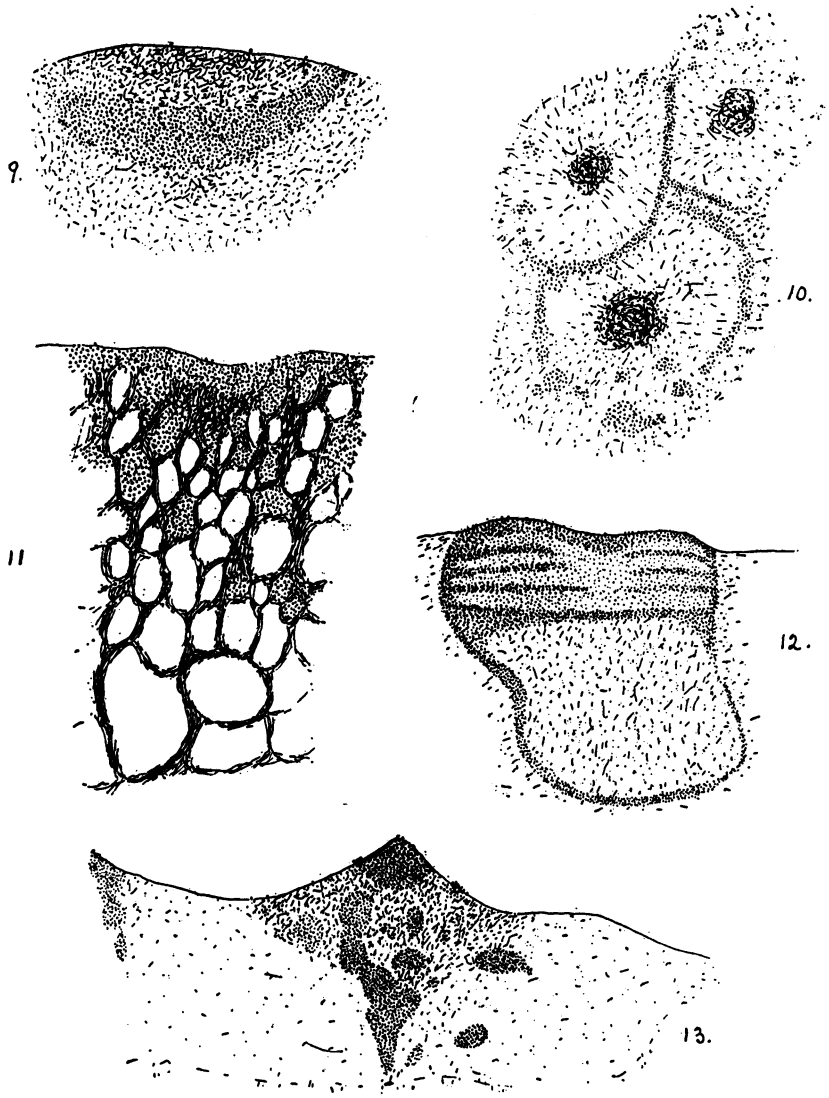


FIG. 9. Me9: Vertical section of colony showing sub-surface sporulating zone, 5 days 37°C., ×500; stained by modified Mallory's triple stain; medium Czapek sucrose agar.

FIG. 10. Me1: Horizontal section of cluster of colonies showing radial distribution of sporulating material, 3 months 25°C., ×500; stained by iron-haematoxylin, counterstained orange G; medium Czapek sucrose solution.

FIG. 11. Me10: Vertical section showing development of air spaces, 4 months 25°C., ×1250; stained by erythrosin, counterstained Grübler's Nachtblau; medium Czapek sucrose agar.

FIG. 12. Me6: Vertical section showing elliptical route traced by sub-surface sporulating zone, 7 weeks 25°C., ×1250; stained Flemming's triple stain; same medium.

FIG. 13. Me3: Section showing conical development sporulating hyphae, 6 weeks 25°C., ×1000; stained erythrosin and Nachtblau.

area—there is a definite sub-surface zone of more abundant sporulation which has reached the surface in one direction. This sub-surface initiation of sporulation, as indicated in the sections by a more or less well-marked zone, was found to occur consistently in all strains with colonies of from 4 to 12 days old. Referring again to the directly observed living growth, it would seem that the young, centrally sporulating mycelium of figure 2 must give rise to the picture shown in figure 9 as the result of active ramification of vegetative hyphae *above* the central sporulating area, which now as it grows in extent becomes the well-marked sub-surface zone. This sub-surface zone may rapidly lose definition, as in microphotograph 1, portraying a 12-day-old colony of Me3, where the dark masses of spore-bearing material are irregularly assembled near the crest of the colony. Figure 13 illustrates a later phase in the life of the same strain, in which a conical development of sporulating hyphae has taken place beneath each crest while the surrounding vegetative filaments have more or less disintegrated (6 weeks). On the other hand, figure 17 depicts another aspect of this same strain, in which the sub-surface sporulating zone has persisted after three crests have been produced (2 weeks).

This persistence of the sub-surface sporulating zone, from which fresh surface growths arise, is on the whole the most common feature of the sections. Thus, figure 12 (microphotograph 6 represents same strain Me6—7 weeks) shows a peculiar elliptical route traced by the sub-surface zone. The more usual course, roughly parallel to the superficial zone, is seen in microphotograph 2 (strain Me8—4 weeks) and in figure 14 (Me2—6 weeks). A lateral extension of the colony by means of a more or less scalloped sub-surface zone can be found in figure 15 (Me5—5 weeks). Even after two months (microphotograph 5—strain Me8) traces of the sub-surface zone, which connects a lateral outgrowth with the original colony, may still be seen. Figure 19 (magnified in figures 20 and 21—strain Me7—4 months) represents an extreme case of periodicity; here there are three sub-surface sporulating zones with occasional small clusters of spores

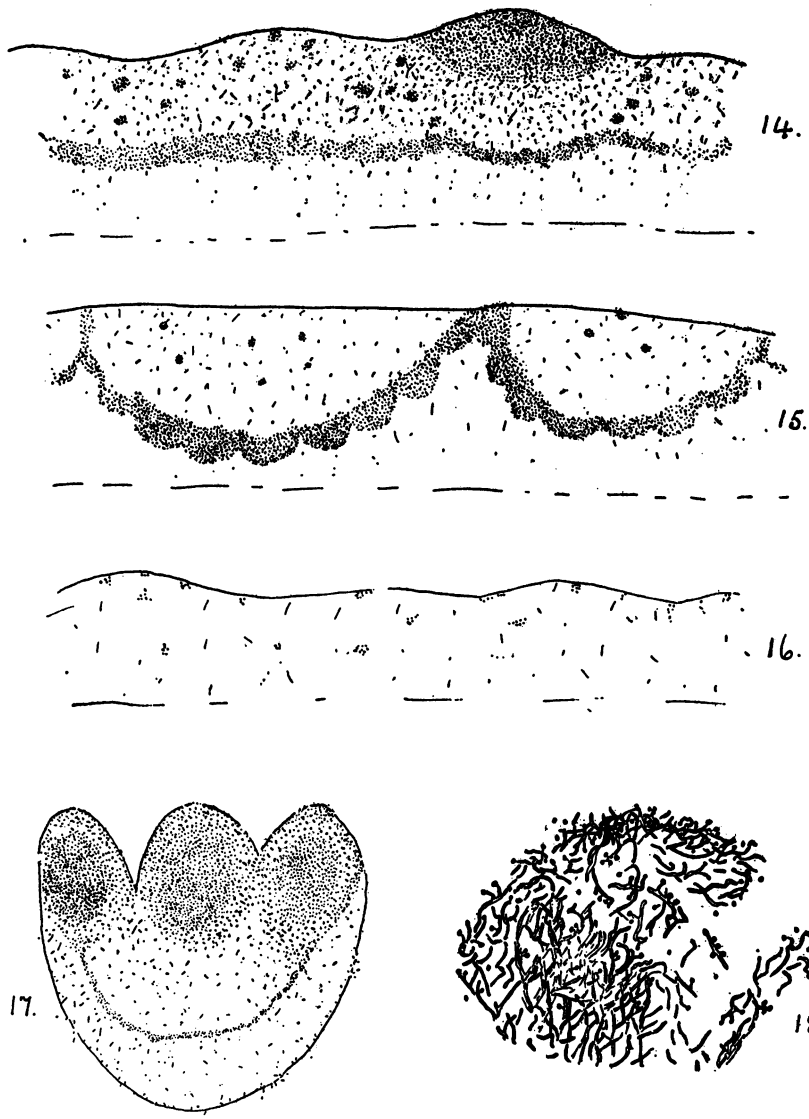


FIG. 14. Me2: Section showing sub-surface sporulating zone roughly parallel to surface, 6 weeks 25°C., ×500; stained iron-haematoxylin and safranin.

FIG. 15. Me5: Section showing lateral extension of colony by means of scalloped sub-surface sporulating zone; 5 weeks 25°C., ×500; stained modified Mallory's triple stain.

FIG. 16. Me8: Section showing irregular clusters of spores in amorphous vegetative mass, 6 months 25°C., ×500; stained modified Mallory's triple stain.

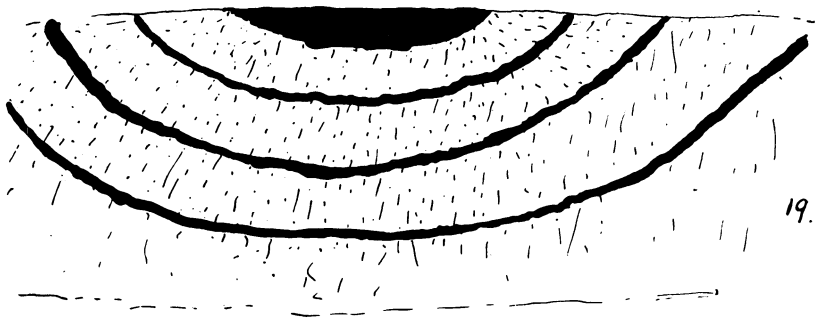
FIG. 17. Me3: Section showing three crests of colony filled with dense sporulating material and connected by sub-surface sporulating zone, 2 weeks 25°C., ×500; stained modified Mallory's triple stain.

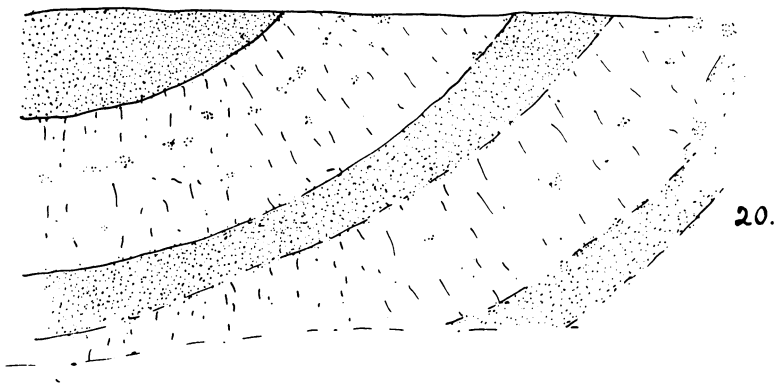
FIG. 18. Me3: Portion of same serial section, illustrating part of one of the crests ×1250; stained carbol fuchsin and nigrosin.

between the arc-like bands (fig. 20), while the intervening vegetative filaments are almost completely disintegrated. Occasionally, with growths of this age, a tissue-like appearance is given (fig. 11 —strain Me10—4 months) resulting from the formation of large air-spaces, which increase in size with depth. With still older growths (fig. 16—strain Me8—6 months) there may often be no organization left, merely irregular clusters of spores in an otherwise amorphous vegetative mass.

The density of the spores in zones such as have been described above is so great that it is usually impossible to discern individually their attachment to lateral branches. That they are so attached can, however, be seen in microphotograph 7 (magnified in microphotograph 8—strain Me1—5 weeks), in which zonation is not evident; also in fig. 18 (a magnification of figure 17—Me3—2 weeks), which was slightly ruptured when the slide was smeared with nigrosin for a spore stain. Microphotograph 8 also demonstrates the way in which the vegetative hyphae at the base of the colony grow into the medium and so anchor the growth.

Fewer sections were cut of material derived from liquid cultures; this was due to the slowness with which such growths increased in size and the difficulty of manipulating the minute colonies. They grew horizontally rather than vertically, and figure 10 represents a horizontal section of three colonies in a cluster (strain Me1—3 months). It will be evident that there is a marked radiate arrangement of the spore-bearing hyphae. Smear preparations made from bottom growths in liquid cultures have revealed an irregular production of spores at all ages. Growths which were attached to some mechanical support above or near the liquid level, in general showed much more abundant sporulation. The stimulating effect of oxygen was particularly noted in the case of strains Me4 and Me9 which, as mentioned earlier, were subjected to aeration for 6 days; very profuse sporulation was observed in this instance. On the other hand, the minute colonies produced after relatively long anaerobic cultivation were poor in spores.





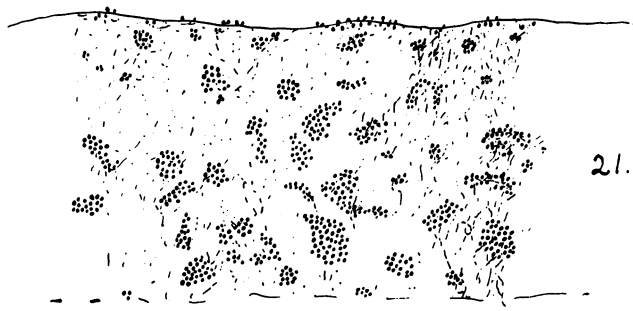
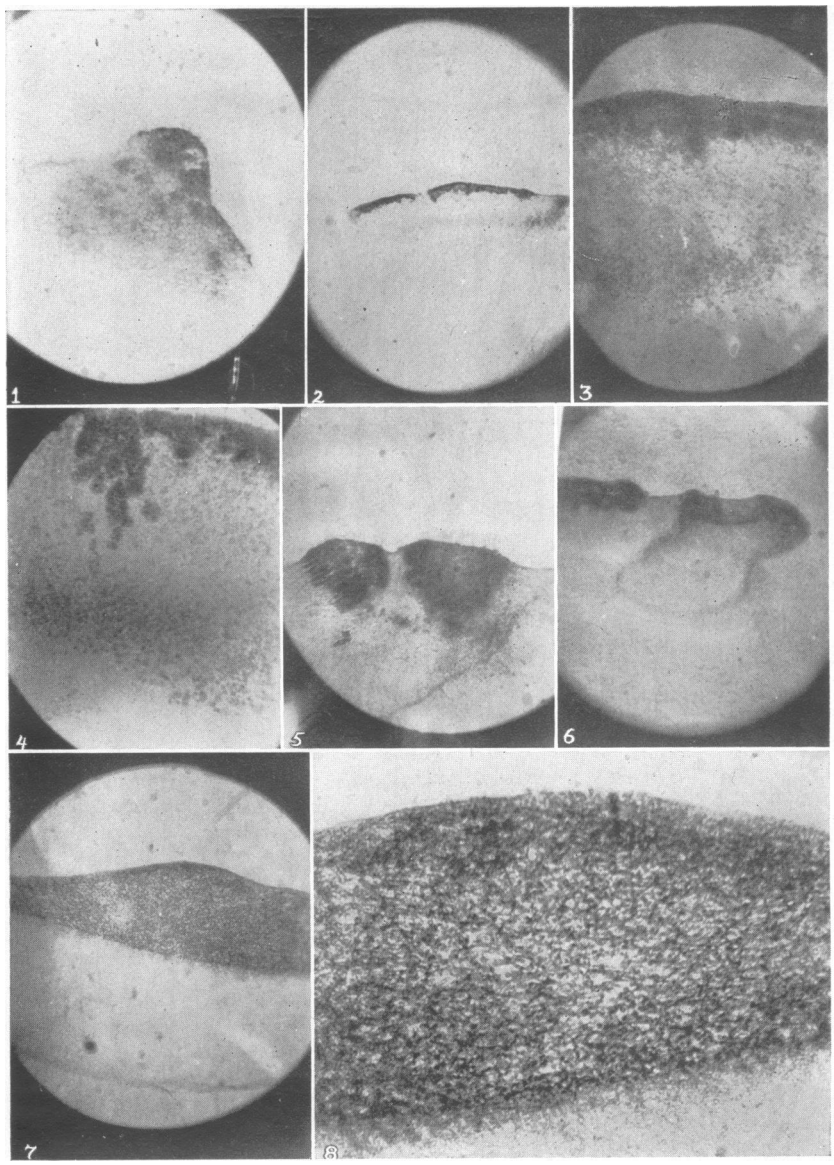


FIG. 19. Me7: Diagrammatic representation of section of colony demonstrating extreme periodicity, with 3 sub-surface sporulating zones, 4 months 25°C., ×100; stained Mallory's triple stain.

FIG. 20. Me7: Same, ×500, showing occasional clusters of spores intermediary between the sporulating zones.

FIG. 21. Me7: Same, ×1250, showing distribution of spores within more or less disintegrated vegetative mycelium of central surface zone.



MICROPHOTOGRAPH 1. Me3: Section showing dark masses of spore-bearing material irregularly distributed near crest, 12 days 25°C., ×500; stained iron-haematoxylin and safranin.

MICROPHOTOGRAPH 2. Me8: Section showing superficial and sub-surface sporulating zones, 4 weeks 25°C., ×100; stained erythrosin and Nachtblau.

(*d*) *Resistance to drying and temperature requirements*

As has been pointed out by many authors, the Actinomycetes as a whole are very resistant to drying. Resistance of Micromonosporae has been tested by Ørskov (1923) who stated that the spores are viable for at least eight months, and by Jensen (1930) who found that the spores generally did not resist three months' drying, while the vegetative mycelium survived for five weeks. In the case of the present lake-mud strains, spores were picked from colonies six months old which had dried on to the walls of test-tubes, and which had been grown on Czapek sucrose agar media; they were then sown on individual slide cultures of the same medium. Some, though not all, of the spores spread on each strip of medium were observed germinating within the next 24 hours; this applied to all strains. The impossibility of being certain that vegetative mycelium only, devoid of any spores, is picked from even young colonies which show no superficial spore layer, will be apparent from the preceding observations on colonial morphology, in which the early production of a sub-surface sporulating zone was noted. No attempt, therefore, was made to isolate fragments of vegetative hyphae in order to ascertain their resistance, but the examination of sections of older colonies has revealed that quite often the vegetative filaments are disintegrated while clusters of spores remain intact.

As regards temperature requirements, these strains are ordinary mesophilic organisms. In view of their source, from the bottom of a lake in which almost freezing temperatures are maintained for a considerable portion of the year, attempts were made to

MICROPHOTOGRAPH 3. Me8: Same, ×500, showing intense staining of superficial zone compared with the scattered distribution of spores in the center.

MICROPHOTOGRAPH 4. Me8: Same, ×500; another portion of serial section showing the irregular depth of the intensely staining superficial sporulating zone.

MICROPHOTOGRAPH 5. Me8: Section illustrating traces of sub-surface zone still connecting lateral outgrowth with dense central sporulating mass, 2 months 25°C., ×500; stained modified Mallory's triple stain.

MICROPHOTOGRAPH 6. Me6: Section showing peculiar route followed by sub-surface sporulating zone, 7 weeks 25°C., ×500; stained erythrosin and Nachtblau.

MICROPHOTOGRAPH 7. Me1: Section showing even distribution of spores on lateral branches within the mycelium, 5 weeks 25°C., ×500; stained modified Mallory's triple stain.

MICROPHOTOGRAPH 8. Me1: Same, ×1500; demonstrating attachment of individual spores to branches, and at the base of section faintly staining vegetative hyphae which anchor colony to medium.

grow them in the cold room (2°C.) on chitin, cellulose, and sucrose. Very little growth was observed after one month; but when the tubes were then placed in the incubator at 37°C., abundant growth appeared within two days on the chitin strips and also on the liquid surrounding them, and within nine days on the filter paper and to a lesser extent on the sucrose.

3. DISCUSSION

Although it must be left for future work to determine the exact role, if any, of these Micromonosporae in lacustrine ecology, it would seem from the results of this study that they are admirably adapted for life under the conditions obtaining at the bottom of Lake Mendota. The extensive researches of Birge and Juday (1911) on the dissolved gases of this hard-water lake have established that, as regards temperature and dissolved oxygen, the most favorable period of the year for aerobic bacterial life is after the autumn overturn when the temperature is as high as possible, when the water is saturated or nearly saturated with oxygen, and when the pH is slightly on the alkaline side of neutrality. Growth commenced at this time could then, according to the experimental studies, survive prolonged low temperatures and absence of oxygen during the winter, being again stimulated possibly for a very brief period during the vernal circulation of the waters. During the summer, when maximum temperatures obtain but when there is no dissolved oxygen in the bottom layers and the pH is on the acid side, there could be very little active growth.

It should be remembered that the favorable conditions obtaining during the autumnal circulation would also be those which favor the growth of ordinary aerobic bacteria, which are probably much faster growers than the Micromonosporae, other things being equal. Here it may be that considerable importance attaches to the ability of these Micromonosporae to utilize resistant substrates like chitin and lignin, especially lignin which according to Steiner and Meloche (1935) constitutes 30 to 48 per cent of the total organic deposit in the lake mud. Such substrates are not readily attacked by most aerobic bacteria and

might very well, therefore, act as selective enrichment media for these organisms. It is probable also that during and immediately after the autumn overturn there is an abundance of cellulosic, chitinous, and lignin-containing material at the bottom of the lake, furnished by plankton which after the summer's activity has sunk to the bottom, and also by leaves which, blown by the wind from the trees bordering the lake, have become water-logged and in like manner have been carried to the bottom.

Another possibly advantageous feature of the Micromonosporae is the resistance of their spores, and the development of those spores surrounded by vegetative mycelium which, even when partly disintegrated, thus affords a means of protection to the clusters of spores that persist—as demonstrated by sections—for many months. Compared with the aerially borne spores of the other Actinomycetes, these embedded spores of the Micromonosporae may well be better adapted for life under aquatic conditions.

4. SUMMARY

Ten strains of the species-group *Micromonospora chalceae* (Foulerton) Ørskov, derived from lake mud or in one instance from lake water, have been examined in detail. Physiologically, they have been found capable of growing on a very large variety of more or less resistant organic compounds, being especially active in the decomposition of chitin, cellulose, and, to a lesser extent, lignin. Morphologically, they have been shown, by means of thin sections, to possess a definite colonial organization characterized by the zonation of sporulating material. Their possible rôle in lacustrine ecology has been discussed.

ACKNOWLEDGMENTS

This work has been made possible through the generous hospitality of the Department of Agricultural Bacteriology, University of Wisconsin, to whose staff I wish to express my sincere appreciation. I am especially grateful for the many kindnesses received from Dean E. B. Fred, Professor E. G. Hastings, Drs. E. McCoy, and W. W. Umbreit. I should also like to acknowl-

edge my indebtedness to Dr. Louise Wipf of the Genetics Department for her kind advice on sectioning methods.

REFERENCES

BIRGE, E. A., AND JUDAY, C. 1911 The Inland Lakes of Wisconsin. The dissolved gases of the water and their biological significance. Madison, Wisconsin.

ERIKSON, D. 1935 The Pathogenic Aerobic Organisms of the Actinomyces Group. Med. Research Council (Brit.) Special Rept. Series No. 203.

ERIKSON, D. 1940 Pathogenic Anaerobic Organisms of the Actinomyces Group. Med. Research Council (Brit.) Special Rept. Series No. 240.

FOULERTON, A. G. 1905 New species of streptothrix isolated from the air. Lancet, **83**, i, 1200.

GREENE, H. C. 1938 Colony organization of certain bacteria with reference to sporulation. J. Bact., **35**, 261–273.

HENRICI, A. T., AND MCCOY, E. 1938 The distribution of heterotrophic bacteria in the bottom deposits of some lakes. Trans. Wisconsin Acad. Sci., **31**, 323–361.

HUTCHINSON, H. B. 1907 Ueber Form und Bau der Kolonien niederer Pilze. Zentr. Bakt. Parasitenk., II, **17**, 417–427, 593–604.

JENSEN, H. L. 1930 The genus Micromonospora Ørskov—a little known group of soil microorganisms. Proc. Linnean Soc. N. S. Wales, **55**, 231–248.

JENSEN, H. L. 1932 Further observations on the genus Micromonospora. Proc. Linnean Soc. N. S. Wales, **57**, 173–180.

MCCLUNG, L. S., MCCOY, E., AND FRED, E. B. 1934 Studies on anaerobic bacteria. II. Further extensive uses of vegetable tissue in anaerobic systems. Zentr. Bakt. Parasitenk., II, **91**, 225–227.

MIEHE, H. 1907 Die Selbsterhitzung des Heues. G. Fischer, Jena.

ØRSKOV, J. 1923 Investigations into the Morphology of the Ray Fungi. Copenhagen.

SCHÜTZE, H. 1908 Beiträge zur Kenntnis der thermophilen Aktinomyzeten und ihrer Sporenbildung. Arch. Hyg., **67**, 35–56.

SNOW, L. M., AND FRED, E. B. 1926 Some characteristics of the bacteria of Lake Mendota. Trans. Wisconsin Acad. Sci., **22**, 143–154.

STEINER, J. F., AND MELOCHE, V. W. 1935 A study of ligneous substances in lacustrine materials. Trans. Wisconsin Acad. Sci., **29**, 389–402.

TSIKLINSKY, P. 1899 Sur les mucedinées thermophiles. Ann. inst. Pasteur, **13**, 500–505.

WAKSMAN, S. A. 1940 On the classification of actinomycetes. J. Bact., **39**, 549–558.

WAKSMAN, S. A., UMBREIT, W. W., AND CORDON, T. C. 1939 Thermophilic actinomycetes and fungi in soils and in composts. Soil Sci., **47**, 37–61.