Myxobacters of a freshwater lake and its environs

Edmund Everett Jeffers

Iowa State University

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Iowa State University of Science and Technology, Ph.D., 1964
Bacteriology

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MYXOBACTERS OF A FRESHWATER LAKE AND ITS ENVIRONS

by

Edmund Everett Jeffers

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Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean/Chair Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1964
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INTRODUCTION

The myxobacters of freshwater habitats have been little studied. The species reported are few in number and almost all have been placed in the genera *Cytophaga* Winogradsky 1929, 577, *Chondrococcus* Jahn 1924, 85, *Dactylocoena* Enderlein 1924, 6, and *Sphaeromyxa* Bauer 1962, 399.

To gain information about the myxobacters of an aqueous environment a project was started in the summer of 1960 and continued through the next four years. The area investigated was a lake in Minnesota. Collections were made in the summer months.

The work accomplished over the four years became extensive and the data voluminous. The aqueous habitat posed special problems calling for special techniques. The number of strains of the many species found far exceeded the number expected and data on these accumulated rapidly. Moreover, new species were found and special treatment of each of these had to be improvised.

Some of the data are organized and presented herein. Background information derived from the total study is used as a guide to the present account. Some of the additional information is introduced at times to support or clarify a determination or conclusion but organization of the additional data on aquatic myxobacters must be delayed for presentation under separate titles.

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1 Supported in part by grant number E-3124 of the Institute for Allergy and Infectious Diseases of the U.S. Public Health Service of the Department of Health Education and Welfare.
Myxobacters as Aquatic Organisms

Most investigators to the time of Stanier (1940) confined their research to myxobacters from soil and non-aqueous sources and their accounts were largely restricted to discussions of species that produce myxangia.

Some early references to aquatic organisms, possibly non-myxangia-producing myxobacters, were made however. The work of Frankland and Frankland (1894) includes descriptions which suggest myxobacters but which were regarded by the authors as species of the genus Bacillus. Eisenberg (1891), Lustig (1893), Mez (1898), Tataroff (1891) and Zimmermann (1890) include descriptions some of which indicate that myxobacters may have been present among organisms they were studying.

Jordan (1890) reported on organisms from sewage and described a number of new species. The possibility that non-myxangia-producing myxobacters were present is difficult to dismiss as his descriptions are studied.

Merker (1911) almost unquestionably reported a species of myxobacter in Elodea leaves when he described Micrococcus cytophagus. Stanier (1942) regarded it as identical with Sporocytophaga myxococccoides.

The next aquatic myxobacter to be described was Bacillus columnaris Davis 1922. Davis discovered the organism in lesions of diseased fish in Iowa and studied its peculiar motility without being able to isolate the organism.

Reports of aquatic organisms, some of which were probably myxobacters, appear in the literature sporadically through the decades 1930 and 1940. One account, that of Geitler (1924) who described the species Polyangium parasiticum found on an alga (cladophora) is concerned with myxangia-
producing forms. Lundestad (1929) provisionally identified strains of *Cytophaga* and *Sporocytophaga* in his collection of marine bacteria. Johnson (1932) observed some non-myxangia-producing forms on crabs in her study of chitin-digesting strains. Anne Benton (1935) may have had strains of *Cytophaga* or *Sporocytophaga* in her collection of organisms from Lake Alexander and Lake Minnetonka in Minnesota.

After 1940 a number of reports of identified and unidentified myxobacters appeared. With rare exceptions all were descriptions of marine non-myxangia-producing forms.

Stanier (1940) described *Cytophaga krzemieniewskae* and *C.* *diffluens*, agar-digesting bacteria from sea water. In 1946 Humm named *C.* *sensitiva*, an agar-digesting species from Atlantic coastal waters. A cold water myxobacter, *Cytophaga psychrophila*, was named and described by Borg (1948). Kadota (1953) isolated and described *Cytophaga haloflava* which he secured from deteriorating fishing nets. A variety, *C.* *haloflava* var *non-reductions*, was described by Kadota (1954). In the same year he reported the discovery of *C.* *rosea* in sea water.

The fermentative myxobacter, *Cytophaga fermentans*, was isolated from marine mud by Bachman (1955). Kadota (1956) in a long discussion of aerobic, marine, cellulose-digesting bacteria reviewed his own previous work and included a detailed account of the morphology and physiology of a number of eubacterial cellulose-digesters. Velankar (1957) published a lengthy list of organisms from marine mud and sea water of Mandapan. Among these were some organisms he regarded as probable strains of the genus *Cytophaga*.

Anderson and Ordal (1961) described *Cytophaga, succinicans* and paid
particular attention to its ability to ferment certain carbohydrates. This was a fresh water form from the Snake River in Idaho. Knorr and Graf (in Graf 1962) described *Sporocytophaga cauliformis* from fresh water. The distribution and biology of the species was discussed at length. It was reported as a stalked form showing creeping motility.

Bauer (1962) described the new genus, *Sphaeromyxa*, with one species, *S. xanthochlorus*. He found "sphaeroids" and a developmental cycle not characteristic of other genera among the myxobacters. The organism was discovered in study of myxobacters from trickling filters.

**Myxobacters in Diseases of Fish**

The literature on fish diseases also contains pertinent information about aquatic myxobacters. After the report of Davis (1922) attention was turned to these organisms. Migrelli found *Bacillus columnaris* Davis in lesions of catfish and bullheads in a New York aquarium. Ordal and Rucker (1944) reported *Chondrococcus columnaris* (Davis) in lesions of trout and salmon on the Columbia River. They presented evidence that *C. columnaris* caused these lesions characteristic of "columnaris disease".

Through the next fifteen years *C. columnaris* and its presence in gills, skin, and various lesions of fish was reported by many investigators including Davis (1946), Slater (1948), Davis (1949), Foster and Olson (1951), Bryant (1951), Johnson (1951), Johnson and Brice (1952), Rucker, Earp and Ordal (1954) and Griffin (1954). There is little question that the organism is wide-spread in American waters. A review of foreign investigations of fish diseases yielded no statement about the presence of *C. columnaris* in other parts of the world.
C. columnaris has been the object of more studies and discussion than other aquatic myxobacters. Only one other myxobacter has been implicated as a possible cause of disease in fish. Borg (1948) believed Cytophaga psychrophila to be the cause of a cold-water disease of trout and salmon of the Northwest.

Rucker, Earp and Ordal (1954) reviewed the literature on bacterial diseases of fish and remarked that myxobacters were common in an aqueous environment. Despite this, very little concerning myxobacters of water has been reported and almost nothing has been said about the individual species except C. columnaris, an occasional species of Cytophaga, and the species, Sphaeromyxa xanthochlora (Bauer 1962).

The Aquatic Habitat Surveyed for Myxobacters

Birch Lake, located in Cass County, Minnesota, was chosen for the study. It is the second in a chain of lakes linked by the Little Boy River draining spring-fed Ten Mile Lake, the source. The stream flows in a tortuous channel through a muskeg-sphagnum bog for about three miles.

Birch Lake is a highly productive body of water, a typical eutrophic lake for that region. It is quite irregular with a maximum length and width of three miles. The maximum depth is about 30 feet but it is rarely more than 15 to 20 feet deep. There are numerous bays, some quite shallow, with abundant submerged and emergent vegetation which becomes particularly dense in places. The zone of emergent vegetation includes species of Sagittaria, Scirpus, Typha and Pontederia. At the inlet a heavy growth of species of Nymphaea and Nuphar occurs. The rooted plants which are wholly submerged or nearly so include species of Potamogeton, Ruppia,
Ceratophyllum, Najas, Utricularia and Myriophyllum, together with such green algae as several species of Chara. Along the southern shore is a wide band of wild rice (Zizania aquatica).

Phytoplankton of the littoral zone include species of Spirogyra, Cladophora, Rhizoclonium, Draparnaldia, Hydrodictyon, Nostoc, Anabaena, Oscillatoria, Aphanizomenon and Scenedesmus. Diatoms are abundant on submerged rocks, twigs, branches and debris. The pools along the Little Boy River are particularly rich in desmids.

Development and Evaluation of Special Media

First attempts to isolate myxobacters from the fresh water habitat in question were unrewarding. Media usually employed for isolating these organisms were not satisfactory. Motile eubacteria from the lake water overwhelmed the myxobacter colonies. Often the presence of myxobacters could be determined only by microscopic examination of wet mounts for observation of the creeping motility of cells.

Throughout the first year of the study and most of the second extensive work had to be done to discover adequate media both for isolating and characterizing the organisms. The lake had a large population of flagellated bacteria and most conventional media favored these. The polar flagellated forms in particular grew luxuriantly and spread rapidly on the media usually used for isolation of the creeping bacteria. The search for suitable media produced much information not only about media but about the characteristics of the selected test organisms.
Additional Criteria for Characterization of Myxobacters

Criteria for distinguishing species of myxobacters have never been great in number. For some forms myxangia have been stressed for characterization and other features given secondary consideration or none. In the classification of the species not producing myxangia only a few physiological characteristics have been noted as important.

An attempt was made to increase the number of criteria available for characterization of myxobacters and to try to reduce emphasis on characterization by use of a relatively few diagnostic features. More than 200 synthetic and non-synthetic media were formulated and tested. The possibility that colony differences among species could be used as an aid to distinguish the myxobacters had never been examined. It became apparent that colony morphology could be important in characterization of these bacteria. Detailed examination of colony structure was made and a list of names and definitions colony morphotypes was developed.

Species Isolated

More than 2000 plates were made with many different agar-containing media. These were inoculated with material from the lake and environs and in 4 cases from other localities. Over 500 plates were found by microscopic examination of colonies or cells to include myxobacters. Two hundred and fifty-one strains were isolated.

As these strains were cultured an attempt was made by use of presumptive tests to identify the species. Seventy-six of these strains were selected for more intensive study with the following data resulting.
<table>
<thead>
<tr>
<th>Name</th>
<th>Number of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytophaga (species not determined)</td>
<td>133</td>
</tr>
<tr>
<td>Sporocytophaga <em>myxococcoides</em></td>
<td>5</td>
</tr>
<tr>
<td>Sporocytophaga (species not determined)</td>
<td>1</td>
</tr>
<tr>
<td>Archangium <em>flavum</em></td>
<td>2</td>
</tr>
<tr>
<td>Archangium (species not determined)</td>
<td>2</td>
</tr>
<tr>
<td>Podangium <em>lichenicolum</em></td>
<td>1</td>
</tr>
<tr>
<td>Polyangium (species not certain)</td>
<td>1</td>
</tr>
<tr>
<td>Chondrococcus <em>coralloides</em></td>
<td>12</td>
</tr>
<tr>
<td>Chondrococcus <em>blasticus</em></td>
<td>9</td>
</tr>
<tr>
<td>Chondrococcus <em>cerebriformis</em></td>
<td>2</td>
</tr>
<tr>
<td>Chondrococcus (probably <em>C. columnaris</em>)</td>
<td>15</td>
</tr>
<tr>
<td>Dactylocoena (new species)</td>
<td>7</td>
</tr>
<tr>
<td>Dactylocoena (new species)</td>
<td>4</td>
</tr>
<tr>
<td>Myxococcus <em>fulvus</em></td>
<td>11</td>
</tr>
<tr>
<td>Myxococcus <em>virescens</em></td>
<td>5</td>
</tr>
<tr>
<td>Myxococcus <em>ovalisporus</em></td>
<td>5</td>
</tr>
<tr>
<td>Myxococcus <em>stipitatus</em></td>
<td>1</td>
</tr>
<tr>
<td>Myxococcus <em>cruentus</em></td>
<td>1</td>
</tr>
<tr>
<td>Myxococcus <em>xanthus</em></td>
<td>3</td>
</tr>
<tr>
<td>Myxococcus (new species)</td>
<td>8</td>
</tr>
<tr>
<td>Genera uncertain (probably Cytophaga and Chondrococcus)</td>
<td>23</td>
</tr>
</tbody>
</table>

Of these 251 strains, 219 were isolated from water. All strains of *Cytophaga* were cultured from Birch Lake as were the five strains of *Sporocytophaga myxococcoides*. The other species of *Sporocytophaga* was
isolated from Lake Luverne on the Iowa State University campus in Ames, Iowa.

The greater number of myxangia-producers (strains producing fruiting bodies) also came from Birch Lake though some were from non-aquatic habitats.

Seven of the 11 strains of *M. fulvus* but none of *M. xanthus* were isolated from lake water. Of the three strains of *M. xanthus* two were isolated from muskrat dung on an island in the lake and one was found on elm bark by Dr. Lois Tiffany of the Botany Department, Iowa State University, Ames, Iowa.

Three strains of *M. ovalisporus* and the lone strain first identified as *M. stipitatus* were isolated from algae washed up on shore. The other two strains of *M. ovalisporus* were found in a fen near Silver Lake in northwestern Iowa by students of Dr. John Dodd of the Botany Department, Iowa State University, Ames, Iowa.

All five strains of *M. virescens* were isolated from non-aquatic sources near Birch Lake. Five of six strains of the new species of *Myxococcus* were isolated from sites in the lake. The other strain was terrestrial. The species presumptively identified as *M. cruentus* was isolated from slime under a rock in the lake.

The genus *Chondrococcus* Jahn was well represented by several species and many strains in water. *C. coralloides* was found seven times in various habitats in the lake. *C. blasticus* was almost as common. The two strains of *Chondrococcus cerebriformis* were found in algae along the lake shore. *C. columnaris* was found only in water.

Five of the seven strains of a new species of *Chondrococcus* were
isolated from sources in the lake. The sixth strain appeared on a plate inoculated with scrapings from an agaric growing on a clay bank above the lake. The seventh came from soil. Four strains of another new species of Chondrococcus were isolated. All of these were taken from sites in the lake.

The single strain of Polyangium was isolated from an owl pellet on an island in Birch Lake. This may be *P. vitellinum*. The strain of Podangium lichenicololum was isolated from decaying wood on a bank above the lake. All strains of Archangium were cultured from algae in the lake.

**Disposition of Isolates**

From the 251 isolates 14 were selected early in the project for the investigation of media and to assess the value of new criteria for characterizing myxobacters. A representative sample was desired. To meet this requirement, the following organisms were chosen: 3 strains of Cytophaga, 3 strains of *M. fulvus*, 2 strains of *M. virescens*, 2 strains of *M. ovalisporus*, 1 strain of *M. stipitatus*, 1 strains of *C. coralloides*, 1 strain of *C. blasticus* and 1 strain of a probable *C. columnaris*.

Following the studies involving evaluation of media and criteria for characterizing the 14 strains above, 62 more were taken from the collection to be characterized. These 62 organisms were: 37 strains of Cytophaga, 4 strains tentatively identified as *C. columnaris*, 3 strains of a new species of Chondrococcus, 2 strains of a second undescribed species of Chondrococcus, 3 strains of a new species of Myxococcus, 1 strain of *M. virescens*, 2 strains of *M. xanthus*, 3 strains of *M. ovalisporus*, 2 strains
of *C. blasticus*, 1 strain of *Archangium flavum*, 1 strain of a species probably of the genus *Archangium*, and 3 strains of *Chondrococcus coralloides*.

In all, 76 strains were subjected to an intensive study for purposes of characterization and for evaluation of media. The latter assessment was carried on primarily with the 14 selected strains noted above.

**Descriptions of New Species**

In the study of the 76 strains some new species were discovered. Among these were two species of *Dactylocoena* Enderlein 1924 (*Chondrococcus Jahn 1924*). These were selected for special study together with a new species of *Myxococcus*. The first two are to be described in subsequent pages as are two other species of *Dactylocoena* included for comparative purposes.
METHODS AND MATERIALS

Stains

Gram staining

The crystal violet solution of Hucker (1922) was used.

Routine staining

A modification of the phenol-erythrosin stain of Winogradsky (1929) was employed for routine use. It was prepared as follows:

- Erythrosin bluish C. I. No. 45430
- Double distilled water, 430 ml.
- Add an excess of erythrosin
- Shake at intervals for 3 days
- Add 0.5% phenol, 70 ml.
- Filter and add 500 ml. 95% ethyl alcohol.

Special staining

Giemsa stain, Wright's stain, and three staining methods using iron hematoxylin were used occasionally. Iron hematoxylin was tried in attempts to clarify spore-formation and spore-germination in several species.

Fixatives

At the outset the problem of fixing smears was troublesome. Many solutions were tested. Among these were Bouin's, Zenker's, AFA, methyl alcohol, and ethyl alcohol (95% and absolute). Eventually absolute ethyl alcohol was chosen as satisfactory for routine use.
Preparation of Smears

After a number of trials with different methods wet mounts were made by placing a sterile cover-slip on a colony or on one to a number of myxangia and pressing gently. The cover-slip was carefully lifted off with a forcep and turned over on droplet of 0.1% peptonized milk solution on a sterile slide. This impression mount was studied with a phase microscope. Cells and spores were measured from such mounts.

To make permanent preparations, impression smears were dipped in absolute alcohol, held for 3 minutes, touched to absorbent paper and flamed. The alcohol was burned off and the flame extinguished within 3 seconds. This procedure yielded excellent results.

Staining Procedures

A fixed preparation was made as shown above and flooded with carbol-erythrosin for 10 minutes. The stain was washed off with distilled water and Hucker's crystal violet applied for 3 minutes. This was washed with tap water. The smear was allowed to dry in the air, flamed gently, and mounted in a synthetic mounting fluid or in Canada balsam.

Preparation of Plates

Plates were poured to contain not less than 18 ml and not more than 22 ml of medium. They were used after standing inverted at 25°C overnight. No plates over 24 hours old were used in experimental work. The effort to standardize this aspect of culturing the myxobacters arose when it was found desirable to gain information as to the timing of changes in colony
morphology.

It was important also to define the time of appearance of myxangial initials (immature fruiting bodies) or perceptible papillae. Another concern was that of variants. Occasionally a rapid series of successive transfers from day to day on very moist media led to colony variation attended by a change in cell morphology.

Inoculation and Transfers

Transfers were made from the leading edge of colonies not over 8 days old or from the distal margin or a myxangium. For studies of spore-germination, plates or liquid media received inocula from myxangia over 4 months old or from spore suspensions, unheated, or heated in a water bath at 62°C for 5, 10, 25, 30, 60, and 120 minutes.

Spore suspensions for use in physiological tests and spore-germination studies were made in the following manner. Spores from myxangia at least 3 months old, on plates in which the medium was nearly dehydrated, were placed in distilled water at pH 6.8. In many cases entire myxangia were included. The suspension was shaken and allowed to stand at 5°C for 4 to 7 days with intermittent shaking. The tubes were then centrifuged, the water removed, and enough 0.0025% peptonized milk added to make a cloudy suspension of spores. This was held in the cold room at 10°C. Inoculations from these to PMA were made periodically to check loss of viability. New lots were made from time to time.

To determine numbers of spores inoculated into any liquid media, a direct microscope count was made within 8 hours. When the spore suspensions were inoculated into liquid media in physiological tests calling
for a determination of population increase, 0.01 ml of the stored sus-
pension was used.

Because the distribution of spores in any inoculum was uneven, the
initial counts were not more than rough estimates. The problems of dis-
tribution of spores was particularly difficult in the species of Dactylo-
coena because of the toughness of the slime. Many attempts to release
spores from these myxangia were made but none was wholly satisfactory.

Cell transfers were made with a loop while being viewed at a magnifi-
cation of 7X or 15X, and at times, 80X. A point on the receiving plate
was inoculated. Streaking or smearing was avoided unless the purpose was
evaluation of culture purity. In assessing the purity of a culture,
inocula were placed in 3 ml of 0.1% peptonized milk (PMS) and shaken in-
termittently about 10 minutes during a period of an hour. In some trials
3 ml of a 0.25% solution of aesculin were used in place of PMS in an
effort to break up slime. In a later series of attempts to get an even
distribution of cells 0.25% aesculin was used in 0.1% peptonized milk
solution. It was found that the two together led to a more even distribu-
tion of colonies.

Inoculated solutions were viewed at 24 hours for growth by use of wet
mounts and a phase microscope. The tubes were held for another 2 days and
checked again. Spore-germination, if any, was noted also and cell measure-
ments were made.

Microscopy

A Bausch and Lomb stereoscopic microscope giving magnifications of 7X
and 15X was employed to observe colonies and myxangia. A 100 watt bulb in
a goose-neck lamp provided illumination from a distance 10 to 12 inches back of the stage and 4 to 5 inches above it. Colony colors were determined using the color standards of Ridgeway (1912). Transmitted light was avoided in these color determinations.

A Cycloptic microscope with a Spencer fluorescent illuminator No. 640 attached to the objective system served in the photography of myxangia and colonies. Additional illumination was provided by a Bausch and Lomb lamp with a 100 watt projection bulb. The lamp was placed 8 inches to the right of the stage and 2 inches above it. Colors of colonies and myxangia were reassessed at magnifications of 20X, 40X and 80X.

Cell and spore photography required the use of a phase microscope and also an oil immersion system to view stained preparations.

Incubation of Cultures

Unless otherwise stated, the results of experiments are those from cultures incubated between 26° and 30°C at a relative humidity of about 50%. In a number of experiments, cultures were incubated at 25° or 32°C. The humidity was brought to near saturation at 27°, 32° and 37°C for some determinations.

Growth in Liquid Media

In early efforts to measure population increase in liquid cultures a Coleman Universal Spectrophotometer No. 14 was employed. Because most cultures of myxangia-producers grew only on the wall of culture tubes use of the spectrophotometer was not in consequence completely satisfactory.
Also in early experiments with liquid cultures growth was not heavy enough for effective use of the spectrophotometer.

Noren (1952) in his contribution to an understanding of myxobacters, used the spectrophotometric method to read population density and slime production on the walls of tubes. His technique was tried as described by him and several early determinations were made with it. However it proved to be impractical in an investigation involving numerous strains and a search for a more convenient method of assessing population density was sought.

A modification of the direct microscopic method of Breed (1928) was then tried and found reasonably satisfactory in giving an estimate of the numbers of cells and spores. The technique was particularly useful in spore-germination experiments for in many cases a suspension of spores was used as the inoculum.

Media

All media unless otherwise specified were autoclaved at 15 psi for 15 minutes. The pH of all media was held to about pH 6.8. To make this adjustment 1/N NaOH and 1/N HCl were used. To cover a pH range from 3.0 to 12.0, the acid-base indicators used were thymol blue (acid range and alkaline range) methyl orange, brom cresol green, phenol red and thymol phthaleine. A Coleman Metrion pH-meter was also employed to check results. For the most part the Coleman Metrion pH-meter and phenol red were adequate.

Numerous media were prepared from autoclaved or Millipore filtered solutions of mineral salts and carbon compounds. These were prepared in 10 per cent, 1 per cent and 0.1 per cent concentration whenever possible.
A measured amount of each solution to be tested was added to sterilized distilled water or to a 1.5% agar solution in distilled water. Additions were made to the agar solutions after they were cooled to 50°C following autoclaving. All filtered or autoclaved solutions of carbon compounds and mineral salts were held at 250°C, rather than in the cold, to avoid the formation of precipitates. The carbon compound used in the various media were: arabinose, xylose, glucose, fructose, galactose, sorbose, sucrose, lactose, cellobiose, melibiose, melizitose, raffinose, rhamnose, mannitol, dulcito, dextrin, starch, inulin, cellulose, chitin, alginic acid, agar, asparagine, creatine, aesculin, salicin and glycerol. Also, filtered and autoclaved solutions of the following complex nitrogen-containing substances were made: BBL milk protein hydrolysate, Bacto tryptone, Bacto tryptose, Bacto neopeptone, Bacto peptone and Bacto peptonized milk. Early in the project 10%, 1.0% and 0.1% solutions of skim milk were prepared and used also. More specific methods and materials are reported in connection with specific descriptions of taxa.

**Peptonized milk agar (PMA)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto peptonized milk</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Bacto agar or Oxoid Ionagar No. 2</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0 m</td>
</tr>
</tbody>
</table>

The formation of PMA was the result of many trials with various combinations of agar and nutrients in an endeavor to develop a satisfactory general purpose medium. This medium seemed to afford optimum conditions in that it permitted the rapid spread of colonies of myxobacters without stimulating the spread of colonies of motile eubacteria common in the lake.
Myxobacter colonies on this medium showed the most clearly defined differences between species even when crowded by contaminating eubacteria. This was the only medium, among the many studied, which quite uniformly favored the production of myxangia.

This medium is quite transparent and particularly suited to the observation of colonial morphology. Colonies of myxobacters growing on PMA in most cases can be readily distinguished from colonies of other bacteria even when contaminating eubacterial colonies are present.

A literature search showed no previous use of peptonized milk for cultivation of myxobacters. Probably the first investigator to use milk in any form in the study of myxobacters was Kofler (1913) who employed the skim milk medium of Hastings (1903). Hastings used nutrient agar, 1000.0 ml and skim milk, 100 to 120 ml.

On PMA the type of colony appearing on the original isolation plates made directly from the source of the organism in nature was retained in most cases without significant change through successive transfers.

**Mineral salt peptonized milk agar (SPMA)**

- Bacto peptonized milk: 1.0 g
- Agar (Oxoid Ionagar No. 2 or Bacto Agar): 15.0 g
- Distilled water: 1000.0 ml

Autoclave at 15 psi for 15 minutes

Cool to 50°C

Add from separately prepared autoclaved solutions:

- \((\text{NH}_4)_2\text{SO}_4\): 0.10 g
- \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\): 0.50 g
FeCl$_3$·6H$_2$O 0.01 g
CaCl$_2$ 0.25 g
MnCl$_2$ 0.0001 g
K$_2$HPO$_4$ 0.25 g

SPMA proved particularly suitable for the preservation of stock cultures. Some are viable after three years. This medium stimulated the more rapid spread of colonies and emphasized the distinguishing characters. Pigmentation of the myxangia of some strains was enhanced on SPMA.

A disadvantage of SPMA was suggested when some strains of the genus Cytophaga, two strains of M. fulvus, and one strain of M. stipitatus lost their ability to produce obviously veined or ridged colonies, and in two species of Myxococcus, to form myxangia. Apparently mineral salts may be adjusted to induce a loss of ability to produce myxangia and a critical balance of salts may exist with respect to maintenance of stable veined colonies and the continued production of myxangia. The formula used in SPMA was determined after numerous experiments with mineral salt-containing media reported by other investigators and by modification of these media in the course of the present study.

**Peptonized milk solution (PMS)**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto peptonized milk</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0 ml</td>
</tr>
</tbody>
</table>

PMS was prepared to determine the ability of various strains of myxobacters to grow in a liquid medium. It was helpful also in determining the presence of contaminants among myxangia-producers. The solution was employed routinely as a suspending medium in which cells or spores were shaken before being placed on an agar-containing medium. An inoculated
solution of PMS was shaken and plates were streaked within the first half-hour and another set of plates streaked from the same solution at 18 to 24 hours.

All available strains of myxangia-producers grew in PMS and most of them formed well-developed myxangia.

**Peptonized milk solution with mineral salts (SPMS)**

SPMS was an improvement over PMS for growth of myxangia-producers in liquid culture. It contained 0.1% PMS and one of the salt mixtures listed on page 30.

**Crystal violet PMA (CVPMa)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto peptonized milk</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>60.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>4000.0 ml</td>
</tr>
</tbody>
</table>

Separate into equal amounts in 4 flasks

Add saturated alcoholic solution of crystal violet, 0.1 ml to flask 1, 0.05 ml to flask 2, 0.001 ml to flask 3, and 0.0001 ml to flask 4. Autoclave at 15 psi for 15 minutes.

CVPMa was prepared in a search for criteria to differentiate the species of Cytophaga and to develop a selective medium for isolation of these myxobacteria from fresh water. Later the medium was found to have value in the differentiation of some of the species of myxangia-producers.

**PMA with varying agar concentrations (VAPMA)**

The standard PMA medium was modified by employing the following agar concentrations: 0.5 per cent, 1.0 per cent, 1.5 per cent, 2.0 per cent and 2.5 per cent.

Soft agar, 0.5 per cent, permitted the rapid spread of the colonies
of most strains of myxobacters but sharply limited some. It was almost useless for isolation when flagellated contaminants were present. For freeing cultures from such contaminants, 1.5 per cent agar was found to be optimum. Soft agar was generally unfavorable to the development of myxangia. For production of spores and myxangia, 0.5 per cent agar was superior. In some cases it was found that certain myxobacters which tended to spread very slowly or to develop sharply circumscribed colonies, when placed on soft agar were able to spread much more rapidly. This was particularly true of two strains of Dactylocoena and 5 strains of Cytophaga.

PMA with varied pH (PHPMA)

The standard PMA medium was modified by adjusting the pH to 4.5, 5.0, 6.0, 6.5, 7.0, 8.0, 9.0 and 9.5.

PHPMA was found to be useful in differentiating some of the strains of the genus Cytophaga and from most of the myxangia-producers. After a study of the effect of pH on the available strains a pH of 6.8 was chosen as satisfactory for all organisms.

Peptonized milk agar with varying concentrations of peptonized milk (PPMA)

Early in the project peptonized milk was found to constitute an excellent substrate for growth of myxobacters. Determination of the optimum concentrations for general and special use was needed for characterization of strains and species. Concentrations of 0.01 per cent to 10 per cent were prepared. The 0.1 per cent concentration was determined to be the best for general use. High concentrations such as 1.0 per cent materially increased production of slime and decreased the longevity of the culture.

Skim milk agar (SMA)

Preliminary tests demonstrated the usefulness of low-fat milk powder
as a nutrient. Kofler (1913) had shown this to be a possibility for growing myxobacters when he employed the milk agar of Hastings (1903). Concentrations of 1.0 per cent, 2.5 per cent, 5.0 per cent and 10.0 per cent skim-milk agar were prepared with agar at 1.5 per cent. These media were not found to be adequate for isolation of myxobacters or for study of colony morphology. A concentration of 2.5 per cent was the most useful, but valuable primarily for study of casein proteolysis. Some strains failed to produce myxangia on SMA. When myxangia were produced they had brighter colors than on PMA.

**Dung decoction agar of Beebe (DDA)**

To make possible a comparison of the available strains with those described by earlier investigators, media as nearly like those described in the literature were prepared. Among those media was the dung decoction agar of Beebe (1941a).

Rabbit pellets (dry) 100.0 g
Distilled water 1000.0 ml
Boil
Allow to stand 24 hours at 27°C
Filter through four layers of gauze
Bring volume to 1000.0 ml with distilled water
Agar 15.0 g
Autoclave at 15 psi for 30 min.
Adjust pH to 6.8

DDA was employed initially in a comparison with other media in isolation of myxobacters from the mixtures of organisms in lake water. Later it was used to compare the morphology of myxangia of different strains.
In general the medium proved to be too opaque for determination of colony morphology.

Dilute dung decoction agar (DBA)

DDA was modified by diluting dung decoction 1 to 10 in 1.5 per cent agar. DBA supported the growth of all strains that grew on DDA. The tendency of some myxobacters to produce an abundance of slime was eliminated. The colors of myxangia were as bright as those on DDA and myxangial morphology was the same. It had greater value than DDA because colony morphology could be determined more easily on a medium allowing the passage of more light.

Bacto nutrient agar (BNA)

Thaxter (1892) 400, 401, used a "nutrient agar" when he described Chondromyces crocatus and C. aurantius. Later (1897) he referred to "nutrient agar" as a medium on which Myxococcus stipitatus grew luxuriantly. The composition of Thaxter's nutrient agar is not given. Presumably it included Witte's peptone. Apparently the medium was not adequate for general purposes, for some species failed to grow on it.

To compare results with those of Thaxter and others, Difco nutrient agar was used. The concentration of nutrients in this medium proved to be too high to permit satisfactory differentiation. As a result the medium was diluted as shown below.

BNA served throughout the project in tests of culture purity.

Diluted Bacto nutrient agar (DBNA)

BNA was diluted 1 to 10 with distilled water and the agar concentration adjusted to 1.5 per cent.

DBNA was designed to reduce the concentration of nutrients to a point
which would limit the growth of contaminating eubacteria and to enable the
myxobacters to produce a characteristic spreading colony. DBNA was pre-
ferable to BNA although myxangia were formed by only a few strains. All
strains of myxobacters grew on DBNA but the similarity of the colonies
from species to species was such that DBNA was useless for characterization
on the basis of colony morphology.

Grated potato agar (GPA)

Thaxter (1892), Quehl (1906) and Vahle (1909) used potato agar. These
authors did not give the details of the preparation of this medium.

Various potato agar preparations were formulated. The one which gave
the best results had the following composition.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly peeled and finely grated potato</td>
<td>50.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0 ml</td>
</tr>
</tbody>
</table>

Most myxobacters produced no myxangia on this medium. Colonies were
more intensely colored than on any other nutrient substance but they were
thick, slimy and heavily ridged in almost all cases and could not be used
for characterization. The veins or ridges of the colonies of some strains
tended to become knotted in places as if initiating myxangia-production.
A microscopic study of these knots revealed no spores characteristic of
the strain. The cells in the knots were swollen and twisted.

Oatmeal infusion agar (OIA)

It was early evident in the project that relatively few media had been
described in the literature which were satisfactory for studying the growth
and differentiation of fresh water myxobacters and particularly for
securing pure cultures from mixed cultures in lake water. Agar-containing infusions of many plant materials such as tissues of emergent lake vegetation, algae (diatoms and species of *Anabaena*, *Cladophora*, *Spirogyra*, *Rhizoclonium* and *Chara*), grains and many foodstuffs were prepared. Among these some showed promise and contributed to an understanding of the great range of variation that can occur in color and shape of myxangia even of a single strain. But the medium which proved the most useful was an oatmeal infusion agar having the following composition.

```
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oatmeal (rolled oats as Quick Quaker Oats)</td>
<td>100.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0 ml</td>
</tr>
<tr>
<td>Boil 5 minutes</td>
<td></td>
</tr>
<tr>
<td>Filter hot through two layers of gauze</td>
<td></td>
</tr>
<tr>
<td>Add distilled water to make a total volume of 1000.0 ml</td>
<td></td>
</tr>
<tr>
<td>Autoclave at 14 psi for 15 minutes</td>
<td></td>
</tr>
<tr>
<td>Hold at 10°C for 5 days or more</td>
<td></td>
</tr>
<tr>
<td>Three layers form in the flask</td>
<td></td>
</tr>
<tr>
<td>Draw off the top clear layer</td>
<td></td>
</tr>
<tr>
<td>Decant the middle white mildly gelatinous layer</td>
<td></td>
</tr>
<tr>
<td>Discard the bottom layer</td>
<td></td>
</tr>
<tr>
<td>Measure 50 ml of the middle layer and dissolve in the following</td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0 ml</td>
</tr>
<tr>
<td>Autoclave at 15 psi for 15 minutes</td>
<td></td>
</tr>
</tbody>
</table>
```

Myxangia that developed on this medium were usually brightly colored.

The medium proved valuable for the maintenance of stock cultures of some
strains of myxangia-producers which were viable after two years on slants of OIA.

Some myxobacters failed to grow on the medium. Some grew but did not produce myxangia.

OIA was too opaque to be useful in a study of colony morphology. In general, attempts to isolate some myxobacters were facilitated because the colonies of these strains spread more rapidly than motile eubacteria.

Nellis and Garner (1964) found a cooked oatmeal agar useful in the study of species of the genus Chondromyces. This medium is not the OIA described above.

**Bacterial cell agar (BCA)**

It has long been known that certain myxobacters are capable of lysing bacterial cells. Among the authors who have recorded bacterial cytolysis myxobacters in a medium with bacterial cells or cell products, are Vahle (1909), Pinoy (1913), Solntzeva (1939), Beebe (1941b), Snieszko, McAllister and Hitchner (1941), Singh (1947) and Oetker (1953). Loebeck and Klein (1956) used constituents of *E. coli* and found that some of these were metabolized. Beebe and Snieszko, McAllister and Hitchner placed some emphasis on the possible use of cell suspensions of different bacterial species for the characterization and taxonomy of myxobacters. The possibility that lysis of bacterial cells of different species of eubacteria might be employed in the differentiation of myxobacters made it desirable to develop suitable culture media for that purpose. This part of the attempt to characterize the available strains of myxobacters was not emphasized, however, as other facets were taken up.

A number of eubacterial species were prepared as cell suspensions in
early attempts to test the differential value of cell suspensions for characterizing myxobacters. The eubacteria used were *Escherichia coli*, *Bacillus subtilis*, *Aerobacter aerogenes*, *Sarcina lutea* and *Aeromonas hydrophila*. The medium finally made in sufficient quantities for a determination of the lytic ability and other activities of many strains of myxobacters was an agar-containing medium with a suspension of *Aeromonas hydrophila*. The medium was prepared as follows.

Bacto tryptose blood agar base w/o agar 33.0 g

Distilled water 1000.0 ml

Autoclave at 15 psi for 15 minutes

Inoculate with a 24-hour culture of *Aeromonas hydrophila*

Incubate, with occasional shaking, at 30°C for 72 hours

Distribute this liquid culture in 40 ml amounts in 50 ml centrifuge tubes

Centrifuge for 30 minutes at 3000 rpm

Resuspend the sediment and repeat centrifuging and washing four times

Resuspend the final sediment in 25 ml of distilled water, shake, and pour contents into tubes

Autoclave at 15 psi for 15 minutes

Autoclave 250 ml of distilled water containing 1.5 per cent agar at 15 psi for 15 minutes

Add 25 ml of autoclaved bacterial cell suspension

The medium should be nearly opaque. Not all strains of myxobacters capable of producing myxangia were able to grow on the medium. Myxangia, when produced, were usually brightly colored and well developed. This was an important aid in distinguishing strain differences. Not all strains
lysed the bacterial cells in the medium. Colony morphology could be studied effectively in those organisms which cleared the medium.

**BBL tryptcase soy broth (TSB)**

This medium was prepared in accordance with the instructions in the BBL manual. TSB was employed primarily to determine the purity of cultures. In general myxobacters capable of producing myxangia do not grow in this medium. Species of the genus *Cytophaga* grew well in TSB. This was another aid in determining the presence of members of this genus in cultures of myxangia-producers none of which would grow on EMB.

**Bacto NIH Thioglycollate broth (TB)**

The instructions in the Difco manual were followed in the preparation of this medium. Contaminants that might have been overlooked by other techniques were sought by use of TB.

**Tryptone agar of Ordal and Rucker (TA)**

Ordal and Rucker (1944) reported success in the isolation and cultivation of a species of fresh water myxobacters by using 0.9 per cent agar and 0.25 to 0.50 per cent tryptone in distilled water. Thaxter (1892) earlier used a peptone and Vahle (1909) also observed that peptone seemed necessary.

TA was used in this project first as a medium containing 0.25 per cent tryptone and 0.9 per cent agar and, shortly thereafter, as a medium containing 0.50 per cent tryptone and 0.9 per cent agar. Later the agar concentration was raised to 1.5 per cent. All of these combinations were tested in early attempts to isolate myxobacters from diseased fish. Motile eubacteria often obscured the colonies of myxobacters on TA.
Eventually other media being investigated were chosen for isolation of myxobacters. TA was retained for comparative studies of the colony morphology of myxangia–producers.

**Variable salt agar (VSA)**

Part of the attempt to characterize the available strains of myxobacters involved the use of media of known chemical composition. Certain combinations of mineral salts were employed with known carbon compounds. The final formula for such a medium was derived from preliminary studies of media described by other investigators including Hutchinson and Clayton (1919), Stapp and Bortels (1934), Solntzeva (1939)(1940), Stanier (1942), Oxford (1947), Noren (1952), Oetker (1953) and to some extent, Dworkin (1962).

The following media were tested with 14 strains of myxobacters.

<table>
<thead>
<tr>
<th>Medium</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>1000.0 ml</td>
<td>1000.0 ml</td>
<td>1000.0 ml</td>
<td>1000.0 ml</td>
</tr>
<tr>
<td>Ionager No. 2</td>
<td>12.5 g</td>
<td>12.5 g</td>
<td>12.5 g</td>
<td>12.5 g</td>
</tr>
<tr>
<td>Autoclave</td>
<td>0.50 g</td>
<td>0.50 g</td>
<td>0.50 g</td>
<td>0.50 g</td>
</tr>
<tr>
<td>Add:</td>
<td>0.01 g</td>
<td>0.15 g</td>
<td>0.25 g</td>
<td>0.50 g</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>0.01 g</td>
<td>0.15 g</td>
<td>0.25 g</td>
<td>0.50 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.0001 g</td>
<td>0.0001 g</td>
<td>0.0001 g</td>
<td>0.0001 g</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.01 g</td>
<td>0.15 g</td>
<td>0.25 g</td>
<td>0.50 g</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0.01 g</td>
<td>0.15 g</td>
<td>0.25 g</td>
<td>0.50 g</td>
</tr>
<tr>
<td>FeCl₃·6H₂O</td>
<td>0.01 g</td>
<td>0.01 g</td>
<td>0.01 g</td>
<td>0.01 g</td>
</tr>
<tr>
<td>M SO₄·7H₂O</td>
<td>0.01 g</td>
<td>0.15 g</td>
<td>0.50 g</td>
<td>1.00 g</td>
</tr>
</tbody>
</table>

The test strains of *Cytophaga* grew well on all four media in the presence of a suitable carbon source of known composition. The same
strains also grew in the plates with 0.0025 per cent peptonized milk.

In general Media C and D were superior to Media A and B. Where concentrations of 0.01 per cent and 0.005 per cent peptonized milk were present, this superiority was most evident.

**Variable salt agar with different protein hydrolysates (VSAH)**

As reported on page 10, eleven strains of myxangia-producing species and three strains of Cytophaga were used in early and continuing attempts to find the most satisfactory general purpose media. This included mineral salt media to be used with the most effective protein hydrolysate. These protein hydrolysates were tested: Bacto tryptone, Bacto tryptose, Bacto neopeptone, Bacto peptone, Bacto peptonized milk, and BBL milk protein hydrolysate (MPH).

Determinations as to the most satisfactory medium for growth were made by judging rapidity of colony spread, time of appearance of the colony, extent of colony spread, distinctiveness of the colony, time of appearance of myxangia, number size, variety and morphology of myxangia, stability of colonies, and longevity of cells on the medium. At first measurements of colony spread were made in millimeters. Later this was abandoned as unnecessary and at times misleading.

Bacto tryptone, tryptose, neopeptone and peptone were rejected early as not as effective as the other hydrolysates, either in any of the 4 salt combinations or alone in any of the 3 concentrations of the hydrolysates. Myxangia-producers grew in tryptone and neopeptone about equally well at the same concentrations. Peptone and tryptose were more inhibitory than tryptone and neopeptone. These two were optimum at 0.25%. Above that
concentration colony distinctiveness was obscured partly because slime production was enhanced.

After initial tests at concentrations of 1.0%, 0.5% and 0.1%, peptonized milk and MPH were examined in concentrations of from 1.0% to 0.001% in the 4 salt mixtures. Peptonized milk was found to be superior at 0.1% to 0.25% in VSA-C and VSA-D. At concentrations below 0.0025% peptonized milk, most myxangia producers failed to show a response to salts. M. ovalisporous and M. stipitatus were exceptions, but their growth apparently depended upon their ability to use agar. At concentration of 0.005% peptonized milk, all species showed some response to salts.

Tests to ascertain the limiting dilution of MPH were not conclusive. Differences in the response of different species were encountered and more detailed studies were not made at a later date as was the case with peptonized milk.

**Variable salt media with different protein hydrolysates (VSMH)**

Protein hydrolysates giving most effective results by measurement of population increase in liquid media were sought. Results of studies with VSAH were relied upon to give direction to experiments using liquid media.

Tubes with 5 ml of 1.0%, 0.5% and 0.1% each of peptone, neopeptone, tryptone and tryptose were inoculated and agitated in a shaking machine 14 to 21 days. Growth occurred in tryptone and neopeptone but was very scant in all of the dilutions used. The salt solutions were those of the VSM constellation. These were varied by changes in composition in later trial and error attempts to secure the best growth of the test organisms but without success. In general the results with these hydrolysates were not satisfactory in agitated tubes with the VSM complex or without. M. fulvus
and *M. virescens* grew more abundantly than *D. coralloides* and *D. blastica* which always showed poor results.

Bacto peptonized milk and BBL milk protein hydrolysate were then investigated and at greater length. Peptonized milk was found to be superior to MPH at the optimum concentration of 0.1% for peptonized milk and 0.333% for MPH. Peptonized milk appeared to be most effective in VSA-C and MPH in VSA-A with 5 times the concentration of MgSO$_4$·7H$_2$O usually in VSA-A. Both supported growth without added salts. Best results occurred in stationary tubes or flasks. MPH showed a tendency to give somewhat different result with different strains depending upon the salt mixture used. The effect of magnesium sulfate on MPH in different salt mixtures needs further study.

At the time of these experiments growth in relation to oxygen tension had not been explored. The need for such an investigation became apparent when stationary tubes were used and differences in depth of the medium found to make a difference in growth of the submerged culture. A number of adjustments of the level of the liquid were made before an optimum of 3 to 4 mm was found. It is likely that early experiments with agitation of tubes with the protein hydrolysates did not give a true picture of the potential of these hydrolysates. This agitation probably was too rapid. More intensive study of the problem is needed.

VSA-C with 0.0025 per cent peptonized milk is designated VSAP-C. VSM-C with 0.0025% peptonized milk is designated VSMP-C.

*Rabbit pellets*

Rabbit pellets from animal house.

Remove hair and debris by flaming.
Autoclave at 15 psi for 6 hours.
Dry in vacuum for 3 hours.
Place a pellet to the side on any agar containing medium as this medium begins to gel in the plate.

Rabbit pellets were used primarily in the study of myxangia. Also the extractives entering the medium from the pellet provided special conditions at the base for the growth of both myxobacter colonies and myxangia.

Rabbit pellets inhibit the growth of some strains of myxobacters. This peculiarity was used as an aid in the differentiation of some of the species.

Commercial preparations of cellulose such as Avicel and Solka Floc (BW-100)

Make up cloudy suspension and place in petri dishes.

Autoclave at 15 psi for 15 minutes.
Dry in vacuum for 2 hours.
Add to selected liquid media to make a slightly cloudy suspension or scatter on the surface of a solid medium.

These products were used with mineral-salts base media to be tested as possible carbon sources or to serve as dispersed particles for growth of microcolonies.

Avicel and Solka-Floc were among many other finely divided particles used in liquid media in an attempt to give the myxangia-producers a surface for formation of microcolonies. There is evidence that myxangia-producing species resemble certain aquatic eubacterial species in their need for a surface upon which to grow (See Zobell (1946) 124).
**Antibiotics** Rimocidin sulphate was used often in PMA to act as a fungus inhibitor. It was learned early that Rimocidin did not act as a substrate and did not inhibit myxobacters.

Penicillin and streptomycin either singly or in combination were used occasionally in various concentrations in PMA to inhibit the growth of certain contaminants. They were ineffective in inhibiting growth of contaminating organisms the more difficult to control.

Sensitivity discs with various antibiotics were tried in an attempt to differentiate species of myxobacters. It was found that myxobacters in general are quite uniform in their response to a great variety of antibiotics.
DISCUSSION

Characterization of the Genus Dactylocoena Enderlein (Myxobacterales) by use of Newly Described Media and Techniques

Among the strains isolated from Birch Lake and its immediate vicinity were many later identified as species of Dactylocoena particularly Dactylocoena coralloides and Dactylocoena blastica (Beebe) comb nov. Some strains of Dactylocoena could not be identified with named species. Characterization studies showed that 2 were new species.

Two strains of D. coralloides, two of D. blastica, two of new species number one and three of new species number 2 were selected for intensive study.

In discussing the new species details of colonial and myxangial morphology are stressed. An attempt is made in the characterization of these species to use all available information about nutrition and to apply it to the taxonomy of the organisms.

Dactylocoena tarda sp. nov.

Etymology Tardus. L. adj., late, slow.

Sources Of the 2 strains of D. tarda selected for study, strain 14 was isolated from slime on a log in Birch Lake and strain 129 from the surface of the carapace of a living crayfish found near shore. Both

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*The author recognizes that names of new species of bacteria and of new combinations under the rules of the International Code of Nomenclature of the Bacteria and Viruses are not validly published when included in an unpublished dissertation. The names and descriptions of the new species herein presented have been submitted for publication in the International Bulletin of Bacteriological Nomenclature and Taxonomy.*
strains of *D. coralloides* were cultured from the lake. One of the strains of *D. blastica* was isolated from a mass of *Rhizoclonium* sp. near shore, the second from a dock piling.

**Cell morphology** Cells of *D. tarda* from colonies on PMA were broad with rounded or slightly tapered ends and ranged from 5.5 to 7.8 μ in length and 0.6 to 0.8 μ in width (Plate 1, figures 1 and 2). In PMS the cells were somewhat longer in a 24 hour culture, measuring 6.2 to 12.0 μ in length and 0.45 to 0.70 μ in width. In one liquid culture many of the cells became very short (2.3 to 4.0 μ) within 48 hours after inoculation though they were of usual length at 24 hours. This sudden change was attended by a slight turbidity in the medium.

The cells of *D. tarda* were either straight or U-shaped, rarely more flexed, contorted or twisted. This was equally true of *D. blastica* and *D. coralloides*. Repeated examination showed the cells of the 3 species to be gram-negative and not acid-fast. They showed no flagella by Leifson's technique.

The cells of *D. tarda* stained rather poorly with safranine used in the gram staining procedure. This was characteristic also of *D. coralloides* and *D. blastica*. Cells of the species of *Myxococcus* stained more intensely with safranine.

The cells of *D. tarda* shortened perceptibly on the 12th to 14th day, those of *D. coralloides* on the 5th to 7th day and those of *D. blastica* as early as the 3rd day.

Cells of *D. tarda* from the margin of colonies on PMA showed very slow creeping and no flexing. They were the most inactive of all the available species of myxangia-producers. The cells of *D. blastica* and *D. coralloides*
were inconsistent in this respect.

A summary of the cell morphology and motility is presented in Table 1.

Table 1. Comparison of the cells of Dactylocoena tarda, D. coralloides and D. blastica from colonies on PMA

<table>
<thead>
<tr>
<th>Cell Characteristics</th>
<th>D. tarda</th>
<th>D. coralloides</th>
<th>D. blastica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>5.5 to 7.8 μ</td>
<td>6.0 to 8.7 μ</td>
<td>4.5 to 7.5 μ</td>
</tr>
<tr>
<td>Width</td>
<td>0.6 to 0.8 μ</td>
<td>0.5 to 0.65 μ</td>
<td>0.5 to 0.7 μ</td>
</tr>
<tr>
<td>Intensity with safranine</td>
<td>Poor</td>
<td>Fair</td>
<td>Fair</td>
</tr>
<tr>
<td>Appearance of short cells</td>
<td>12 to 14 days</td>
<td>5 to 7 days</td>
<td>3 to 5 days</td>
</tr>
<tr>
<td>Motility</td>
<td>Weak</td>
<td>Inconsistent</td>
<td>Inconsistent</td>
</tr>
</tbody>
</table>

Colony morphology on PMA The morphologies of the colonies of D. tarda and D. coralloides were nearly alike. Colony morphology differed sharply in D. blastica.

The colonies of D. tarda were of a design common to all myxangia-producing myxobacters 48 to 72 hours of age on PMA. They retained this structure while almost all other species showed major modification of the colony morphology on aging of the culture.

Over the period of two years of transfers from on PMA both strains of D. tarda showed great stability of colony structure. The colony was low, flat and spreading, with raised, narrow, cord-like veins in a thin film of slime. The veins branched and anastomosed and took a serpentine course toward the border of the colony to produce a medusa-like effect (Plate 1, figures 3 and 4). In general the veins of the colonies of D. tarda were heavier and much more obvious than those of the species of Myxococcus.
The colonies of *D. coralloides* were similar to those of *D. tarda* for the first 3 or 4 days. Later the central area of the colony lost its sharply raised narrow veins and more obscure venation occurred. The result was a somewhat glassy appearance of the central area where myxangia were forming. This central area of the colonies of *D. coralloides* rarely measured more than one-half the diameter of the colony.

In the course of a number of transfers on PMA two new colony types of *D. coralloides* developed. One stabilized as colony type like that formed by *D. tarda* and gave rise to very few myxangia thereafter. These few myxangia developed late, on about the 10th day, and remained small and biscuit-shaped. At the same time this culture upon repeated transfer failed to grow on rabbit pellets though the original culture of *D. coralloides* did so. At all times the cells were longer and more tapering.

The second new colony type of *D. coralloides* was formed following a loss of myxangia-production and a rapid extension of the outer band of raised veins. This colony type also remained stable upon transfer. A variety of efforts to make it revert to a myxangia-producer failed. In PMS and SPMS it formed small rounded hummocks without spores. It continued to produce a rapidly spreading colony on transfer back to solid media, particularly PMA. The cells remained normal in size and in most other respects. Also the strain retained the characteristics of *D. coralloides*.

The colonies of *D. blastica* were marked by the early formation of a well-defined central area and fine, delicate venation more characteristic of the genus *Myxococcus*. This was the only species of *Dactylocoena* to simulate the delicate-veined species of *Myxococcus* particularly *M. fulvus* and *M. xanthus*. A central area with a few low, obscure, glassy veins
appeared within 3 to 5 days and in this area innumerable myxangia emerged almost overnight.

The outer band of fine veins, often tightly interwoven into a closely-packed reticulum, was as great in width as the central zone in most cultures. Occasionally it was extensive, though it never became dominant at the expense of the central area. No myxangia were seen in this outer band.

The usual color of the colonies of *D. tarda*, *D. coralloides* and *D. blastica* on PMA was pale pink.

**Colony morphology on selected media** Differences of colony structure were observed in different media. On Bacto nutrient agar (DBA) *D. tarda* formed a raised, thick colony with coarse veins, usual for all species of *Dactylocoena* and *Myxococcus*. Nutrient agar was useless for purposes of characterizing these myxobacters. No myxangia were formed on these media by the three species.

On dilute Bacto nutrient agar (DBMA) a spreading colony, with coarser and heavier veins and more slime than the colonies on PMA, was produced by the three species. On this medium almost all myxangia-producers formed colonies very much alike. A pale pink color could be discerned when the colonies were heaped into a mound. Spores free from cells germinated on DBMA.

On oatmeal infusion agar (OIA) growth of *D. tarda* was inhibited. *D. blastica* grew well on OIA as did *D. coralloides*. None of the species clarified the opaque medium. The sharp difference in growth response in OIA, between *D. tarda* and the other two species, was a constant factor remaining 50 for three years of study. All colonies were pink against the white background of the medium.
On dilute dung decoction agar (DBA) colony growth of all three species was enhanced over undiluted dung decoction agar (DDA) as noted by colony spread and increase in the size of the veins. \textit{D. tarda} retained the colony structure seen on PMA but growth was inferior to that on PMA. \textit{D. coralloides} was similar to \textit{D. tarda} on both DDA and DBA except for more rapid spread of colonies at 20° and 25°. \textit{D. blastica} grew rapidly and abundantly on DDA in contrast to \textit{D. tarda} and \textit{D. coralloides}. On DBA, \textit{D. blastica} produced thin colonies but otherwise showed little change. \textit{D. blastica} was not inhibited on DDA or DBA at any incubation temperature from 20° through 32°.

On BCA \textit{D. tarda} produced colonies similar to those on PMA but the pink color was more intense. Lysis of the eubacterial cells did not occur at any incubation temperature. \textit{D. coralloides} and \textit{D. blastica} both grew more abundantly than \textit{D. tarda} forming colonies resembling those on PMA; some lysis of the eubacteria occurred. The veins of the colonies of both \textit{D. coralloides} and \textit{D. blastica} were enlarged on BCA.

Skim milk agar (SMA) was cleared slightly within 14 days by \textit{D. coralloides}, but not by \textit{D. tarda} or \textit{D. blastica}. This medium was somewhat inhibiting to colony spread and the size of the veins of all three species. An increase in the concentration of skim milk to 5.0%, then to 10%, did not improve the medium.

\textbf{Myxangia on PMA} Because myxangia have been used by taxonomists as the major diagnostic feature, often the only one, to characterize myxangia-producing species, it was necessary to investigate these structures in detail.

In general the myxangia of \textit{D. tarda}, \textit{D. coralloides} and \textit{D. blastica}
possessed tough or cartilaginous slime enclosing one or more spore masses. The slime was clear and the spore masses consistently pink to orange pink. No envelope or membrane enclosed these spore masses.

The myxangia of *D. tarda* were from one-half to fully developed by the 12th to 20th day. Before that time they appeared as small, slowly enlarging papillae which could be observed by the 7th to 12th day. *D. coralloides* produced mature myxangia by the 5th to 10th day and *D. blastica* by the 4th to 6th day.

The myxangia of *D. tarda* appeared gradually over a period of days rather than in a day or two as in the case of *D. blastica*. *D. coralloides* resembled *D. tarda*. *D. tarda* formed scattered myxangia as did *D. coralloides* though the latter species showed some tendency to produce concentric rings. The myxangia of *D. blastica* occurred in enormous numbers closely-packed in the central area. A profusion of myxangia produced in close proximity to each other appearing quite abruptly was a constant and distinctive feature of all strains of *D. blastica*.

Six myxangial morphologies of *D. tarda* were distinguishable on PMA.

1. Small, pale pink, and abruptly raised or biscuit-shaped. Length 50 to 125 µ; width 50 to 95 µ; height 50 to 75 µ (Plate 2, figure 5).

2. Dome-shaped myxangia with gently sloping sides; occasionally flat on top but usually rounded. Diameter midway between base and tip 50 to 100 µ; height 50 to 100 µ. (Plate 2, figures 6 and 7).

3. Columnar myxangia similar to fingers extending from the agar. These looked like slender, elongate versions of No. 1. Diameter 35 to 75 µ; height 50 to 125 µ.
4. Columnar myxangia showing a tendency to bend or form a U-shaped arc. Size as in No. 3. (Plate 2, figure 8).

5. Columnar and globular or otherwise swollen heads on a low "foot". Length, width and height as above. "Foot" longer than head (Plate 3, figure 9). This "foot" may be swollen to form a definitely expanded base for 2 or more columnar myxangia (Plate 3, figure 10).

6. Forked or multilobed myxangia were most common in cultures of D. tarda (Plate 3, figure 11).

Myxangia on rabbit pellets A significant feature of D. tarda was its tendency to form only a few myxangia and often none on a rabbit pellet. Those that did occur belonged to morphotypes No. 1 and No. 2. Usually they were not shiny or glistening as on PMA but pitted and dry in appearance. When rabbits pellets were soaked overnight in cold water, sterilized, and dried in a vacuum, the myxangia of D. tarda appearing on pellets became glistening. Few if any myxangia were formed at the base of rabbit pellets.

The tendency to produce few myxangia was seen also in some strains of D. coralloides, though myxangia-production on a rabbit pellet was unquestionably less inhibited in the latter species and one strain always formed at least a moderate number of myxangia, usually many.

D. blastica grew abundantly and produced many myxangia on pellets but they were smaller than on PMA.

All three species showed some reluctance to produce large numbers on rabbit pellets. This appeared to be a general feature of all species of Dactylocoena including D. columnaris. The probability is that a pH near 8.5 or above may have been partly responsible for this. However there is
evidence that the species of _Dactylocoena_ are among the most susceptible of all myxangia-producers to high concentrations of nutrients. This was observed repeatedly in the experiments designed to secure the most satisfactory concentration of nutrients for a general purpose medium such as PMA.

**Myxangia on selected media**  
No myxangia were formed by _D. tarda_ on BNA, DBNA, TA, OIA, or GPA. On DDA myxangia were usually morphotypes 1 and 2 and not abundant. The diluted dung decoction encouraged the appearance of all five kinds of myxangia but results were inconsistent. Myxangia of morphotypes 3, 4, 5 and 6 were occasionally abundant on SMA. Myxangia-production was sometimes delayed at all incubation temperatures for at least 3 weeks and the resulting myxangia were very small. Bacterial cell agar stimulated the production of myxangia of morphotype No. 5.

_D. coralloides_ consistently produced myxangia on OIA, DDA, and DBA but never on BNA and rarely on DBNA. The myxangia in OIA and DDA were smaller than on PMA. On OIA the spore masses took on an orange cast.

Bacterial cell agar was noteworthy in causing myxangia of _D. coralloides_ to show a wide range of forms and intensification of color. Angular forms were common. This medium was similar to PMA in its support of diverse myxangial shapes. No myxangia appeared on GPA.

_D. blastica_ was similar to _D. coralloides_ on the above media.

**Spores from old myxangia (over 4 months) on PMA**  
The spores of _D. tarda_ were round to oval measuring 1.47 to 1.8 μ by 1.4 to 1.6 μ (Plate 3, figure 12). _D. coralloides_ and _D. blastica_ produced spores of the same shape and size as those of _D. tarda_.
Growth on PMA with different hydrogen-ion concentrations (PHPMA)

To measure the effect of hydrogen-ion concentration PMA was adjusted to different pH levels (PHPMA). Table 2 shows the pH effects on this medium.

Table 2. Determinations of the influence of different hydrogen-ion concentrations on growth of *D. tarda*, *D. coralloides*, and *D. blastica* cultured on PHPMA at 27°C

<table>
<thead>
<tr>
<th>pH</th>
<th>4.5</th>
<th>5.0</th>
<th>6.0</th>
<th>7.0</th>
<th>8.0</th>
<th>9.0</th>
<th>9.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. tarda</em></td>
<td>0</td>
<td>0</td>
<td>G</td>
<td>E</td>
<td>G</td>
<td>S</td>
<td>0</td>
</tr>
<tr>
<td><em>D. coralloides</em></td>
<td>0</td>
<td>0</td>
<td>E</td>
<td>E</td>
<td>G</td>
<td>S</td>
<td>F</td>
</tr>
<tr>
<td><em>D. blastica</em></td>
<td>0</td>
<td>S</td>
<td>E</td>
<td>E</td>
<td>G</td>
<td>S</td>
<td>F</td>
</tr>
</tbody>
</table>


Evidence derived from experiments on PHPMA was not precise. If allowed to stand two or three days, the more alkaline media dropped in pH regardless of the presence of colonies. If growth started after 5 days the results were questionable. Many trials had to be made to discover the pH range tolerated by these three species and the approximate optimum pH for growth. The evidence indicates that these three species were much alike in the optimum pH for growth between pH 6.0 and 7.0.

Growth on PMA with different agar concentrations (VAPMA) All three species spread slowly on PMA with agar concentrations of 0.5% and 1.0%. A thin film occurred on the surface. Myxangia were formed on 1.0% but not 0.5% VAPMA. The results on agar at 2.0% and 2.5% in PMA were identical with those on 1.5% agar. Previous results showed that neither 0.5% agar nor 1.0% agar could be used in isolation of these forms from
lake water because motile eubacteria obscured the myxobacter colonies.

**Growth on PMA with different concentrations of crystal violet (CV:PMA)**

A search for media to inhibit contaminating eubacteria and to serve in characterization studies led to the use of many different dyes in different concentrations in PMA. Crystal violet had the most promise. Table 3 shows the results of 4 experiments with the three species.

Table 3. Influence of different concentrations of crystal violet in PMA on growth of *D. tarda*, *D. coralloides* and *D. blastica*

<table>
<thead>
<tr>
<th>Species</th>
<th>Percent concentrations of agent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0001</td>
</tr>
<tr>
<td><em>D. tarda</em></td>
<td>+</td>
</tr>
<tr>
<td><em>D. coralloides</em></td>
<td>+</td>
</tr>
<tr>
<td><em>D. blastica</em></td>
<td>+</td>
</tr>
</tbody>
</table>

*0 - no growth, + slight growth, + - evident growth*

The greater hardiness of *D. blastica* was first recognized in studies of its response to different mineral salts in several concentrations. This was revealed again in experiments on the effect of heat on spores and in the response of *D. tarda* to crystal violet. Growth occurred at a concentration of 0.005%.

An effort to evaluate the effect of crystal violet was made later in the liquid media PMS and SPMS. The results are seen in Table 4.

Crystal violet had a more toxic effect in PMS and SPMS than in PMA.
Table 4. Influence of different concentrations of crystal violet in SPMS on cultures of D. tarda, D. coralloides and D. blastica at 27°C

<table>
<thead>
<tr>
<th>Species</th>
<th>Per cent concentration of dye</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00005</td>
</tr>
<tr>
<td>D. tarda</td>
<td>+</td>
</tr>
<tr>
<td>D. coralloides</td>
<td>+</td>
</tr>
<tr>
<td>D. blastica</td>
<td>+</td>
</tr>
</tbody>
</table>

a0 - no growth, + - growth evident

Growth in 0.1% peptonized milk solution (PMS) and (SPMS) The search for a clear liquid medium giving abundant and relatively rapid growth of myxangia-producing strains led to the routine use of 0.1% peptonized milk solution (PMS) and PMS with mineral salts (SPMS) after it was learned that stationary and slanted tubes or flasks of this medium were more satisfactory than agitated tubes or flasks for cultivation of myxobacters.

None of the available test strains of the three species grew as well or as promptly as any of the strains of Archangium or Myxococcus. The test strains of these two latter genera grew well from the time of the first inoculation into PMS and SPMS and growth improved upon continued passage.

The three species of Dactylocoena showed the poorest growth of all of the myxobacter species tested in liquid media. D. tarda did not produce myxangia. Small papillae formed in PMS and SPMS but these papillae did not contain spores. An attempt to stimulate spore-formation by the addition of 6 month old myxangia from PMA was a failure. Spores (500,000 per ml)
from 4 to 6 month old myxangia did not germinate. Addition of sterile aesculin to make a final concentration of 0.25% did not influence the results. Spores from young myxangia (3 weeks old) quick-frozen at -10°C and held for 24 hours, then inoculated in lots of about 1 million per ml, remained in the liquid medium and failed to germinate but grew on PMA when transferred from the PMS or SPMS after 21 days.

Growth in peptonized milk agar with mineral salts (SPMA) The addition of mineral salts to PMA improved growth of all three species. In this medium viability of the cultures over long periods was preserved and the color of myxangia intensified.

To make test of viability cultures of D. tarda, D. coralloides and D. blastica three were held on slants. Tubes were of the screw-cap variety. One set of tubes was inoculated and the caps screwed down tightly. In the second set of inoculated slants the caps were loose. Each of the 2 sets of tubes was divided into 3 lots for storage at 10°, 20° and 27°C. This was repeated with 4 media, PMA, SPMA, DDA and DBNA. The cultures had been held at 27° until growth was abundant and myxangia had formed (if this was a characteristic of the strain) before they were set aside for the viability tests at 10°, 20° and 27°C.

Table 5 shows the results of viability tests for stock cultures on the four different media.
Table 5. Longevity in months of *D. tarda*, *D. coralloides* and *D. blastica* on four media at 10°C in sealed (S) and unsealed (U) tubes

<table>
<thead>
<tr>
<th>Species</th>
<th>PMA</th>
<th>DDA</th>
<th>DBNA</th>
<th>SPMA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>U</td>
<td>S</td>
<td>U</td>
</tr>
<tr>
<td><em>D. tarda</em></td>
<td>30.0</td>
<td>36.0</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td><em>D. coralloides</em></td>
<td>28.0</td>
<td>36.0</td>
<td>8.0</td>
<td>12.0</td>
</tr>
<tr>
<td><em>D. blastica</em></td>
<td>36.0</td>
<td>36.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Table 6. Longevity in months of *D. tarda*, *D. coralloides* and *D. blastica* on four media at 20°C in sealed (S) and unsealed (U) tubes

<table>
<thead>
<tr>
<th>Species</th>
<th>PMA</th>
<th>DDA</th>
<th>DBNA</th>
<th>SPMA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>U</td>
<td>S</td>
<td>U</td>
</tr>
<tr>
<td><em>D. tarda</em></td>
<td>15.0</td>
<td>18.0</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td><em>D. coralloides</em></td>
<td>20.0</td>
<td>20.0</td>
<td>6.0</td>
<td>3.0</td>
</tr>
<tr>
<td><em>D. blastica</em></td>
<td>20.0</td>
<td>36.0</td>
<td>10.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Table 7. Longevity in months of *D. tarda*, *D. coralloides* and *D. blastica* in four media at 27°C in sealed (S) and unsealed (U) tubes

<table>
<thead>
<tr>
<th>Species</th>
<th>PMA</th>
<th>DDA</th>
<th>DBNA</th>
<th>SPMA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>U</td>
<td>S</td>
<td>U</td>
</tr>
<tr>
<td><em>D. tarda</em></td>
<td>3.5</td>
<td>12.0</td>
<td>3.0</td>
<td>12.0</td>
</tr>
<tr>
<td><em>D. coralloides</em></td>
<td>6.0</td>
<td>36.0</td>
<td>3.0</td>
<td>15.0</td>
</tr>
<tr>
<td><em>D. blastica</em></td>
<td>6.0</td>
<td>36.0</td>
<td>3.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

The addition of mineral salt solution to PMA favored viability of cells of all three species. Sealed tubes were not as satisfactory as
those with loose caps. This was particularly evident at higher holding temperatures. PMA and SPMA were not greatly different in supporting stock cultures for long periods of time at lower holding temperatures. DDA and DBNA were not as effective as PMA and SPMA for holding cultures at any temperature.

**Growth on variable salt agar (VSA)** On these 4 solid media with no added carbon compound all strains showed a suggestion of growth at the point of inoculation. This occurred regardless of the purity of the agar, the combination of salts, or the strain. There is no evidence that the organisms used agar as a nutrient. The colony did not spread significantly but remained confined and thin. The medium beneath it became mildly opalescent. Growth appeared early, developed for 4 to 7 days, and then ceased. *D. coralloides* and *D. blastica* showed more growth than *D. tarda* but less than strains of *Myxococcus*.

Myxobacteria will grow on very dilute media. It is not surprising that the impurities in the purest commercial agars should be sufficient to support some growth of these forms.

**Growth on VSA containing different protein hydrolysates** Bacto peptone, tryptone, tryptose and neopeptone were used in 0.1%, 0.5% and 1.0% concentrations alone and on the four variable salt combinations (VSA). *D. tarda* showed poor growth on the media regardless of the kind of added hydrolysate. Tryptone and neopeptone, at concentrations from 0.25% to 0.50%, in a salt mixture or not, were more effective than the other 2 hydrolysates. Myxangia were formed but remained few and scattered. *D. coralloides* and *D. blastica* produced heavier and more extensive colonies.
than D. tarda.

When BBL milk protein hydrolysate and Bacto peptonized milk were used alone and in the above salt mixtures, the growth response and distinctiveness of the colony were improved over those conditions on tryptone and neopeptone. MPH gave the best response in VSA-A and VSA-B (both with 5 times the MgSO$_4$.7H$_2$O concentrations of VSA-A) at concentrations near 0.5%. Addition of salt mixtures made a noticeable improvement over none at all. Peptonized milk was most effective at 0.1% in the salt mixtures VSA-C and VSA-D.

MPH and peptonized milk were assessed in concentrations of 1.0% to 0.001% in VSA-A and VSA-C. Little growth of the three species occurred in VSA mixtures with 0.0025% peptonized milk or less but the colony showed evidence of spread and an increase in density with 0.005% peptonized milk particularly in VSA-A and VSA-D. About the same response occurred in 0.005% peptonized milk without mineral salts.

MPH was ineffective at concentrations below 0.01% regardless of the salt mixture used. At this concentration, VSA-A with 0.50% MgSO$_4$.7H$_2$O per 1000 ml of solution was superior to mineral salt mixture VSA-C and to absence of a salt mixture. The most effective concentrations of MPH were 0.333% and 0.5% in VSA-A with the MgSO$_4$.7H$_2$O concentration noted above.

Growth on VSA-C with added carbon compounds The carbon compounds given in Table 8 were added to make a concentration of 0.333% of each in VSA-C in the investigation of growth. Growth was not stimulated sufficiently to make accurate determinations possible.

Growth on variable salt agar, medium C, with 0.0025% peptonized milk (VSAP-C) The 3 species were grown on VSAP-C without the addition of
Table 8. Influence of filtered (F) and autoclaved (A) carbon compounds in VSAP-C on the growth of *D. tarda*, *D. coralloides* and *D. blastica*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>D. tarda F</th>
<th>D. coralloides F</th>
<th>D. blastica F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Xylose</td>
<td>0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
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^0 - no growth, - - growth inhibited, + - slight growth, + - evident growth, NT - no test

special carbon sources. Growth was negligible. A thin film limited to the vicinity of the colony developed on plates inoculated with *D. coralloides* and *D. blastica*.

Growth on VSAP-C containing added carbon compounds When the various carbon compounds were added to this medium in a concentration of
0.333% of each *D. tarda* was not stimulated to grow regardless of the carbon source. Salicin was inhibiting.

*D. coralloides* showed evidence of stimulation on VSAP-C with melezitose, dextrin, starch, arabinose and xylose.

*D. blastica* was stimulated by raffinose, dextrin and creatine. Filtered xylose gave inconsistent results. Autoclaved xylose appeared to be mildly inhibitory. Creatine stimulated most cultures of *D. blastica* sufficiently to be considered the most effective carbon compound in the list.

Table 8 shows the results of experiments with the various carbon compounds in VSAP-C. The differences between filtered and autoclaved compounds are tabulated also.

**Growth in variable salt media (VSM)** Growth did not occur in any of the 4 different mineral salt solutions.

**Growth in VSM with different hydrolyzed proteins (VSMH)** Bacto tryptone, Bacto tryptose, Bacto peptone, and Bacto neopeptone were used in 3 concentrations in tubes with 7 ml of 2 VSM mixtures VSM-A and VSM-C. Two series of such tubes were prepared. The tubes of one series were inoculated with cells from young colonies of *D. coralloides* on PMA. The second series was inoculated with *D. blastica*. *D. tarda* was not used. All tubes were shaken for 14 days. Insignificant growth occurred in all salt combinations regardless of the concentration of the hydrolysates. A second and a third experiment gave equally poor results. In these latter experiments the (NH₄)₂SO₄ concentration was altered from none to 1.0% in VSM-A and VSM-C without change in the response.

The above hydrolysates were abandoned in favor of Bacto peptonized milk and BBL milk protein hydrolysate (MPH). These were more effective
than the above hydrolysates in supporting growth. The 2 hydrolysates, MPH and peptonized milk, were then examined in dilutions of 1.0%, 0.5%, 0.25%, 0.333%, 0.1%, 0.25%, 0.005%, 0.0025% and 0.001% in VSM-A, VSM-C and alone. The MgSO₄·7H₂O concentration of VSM-A was not altered as in tests using VSA-A and MPH. *D. coralloides* and *D. blastica* failed to grow in MPH at concentrations from 0.001% to 0.25%. Sparse growth occurred in VSA-A and VSA-C with 0.1% MPH and improved slightly in concentrations above that. Peptonized milk was superior to MPH at the most effective concentration of both, 0.5% for MPH and 0.1% for peptonized milk. Neither *D. blastica* nor *D. coralloides* grew well in any liquid media.

**Growth in VSM-C with added carbon compounds** The carbon compounds noted in Table 9 were added to make a final concentration of 0.333% in VSM-C. The tubes were inoculated and agitated. No growth was observed.

**Growth in VSM-C with 0.0025% peptonized milk (VSM-P-C)** Slanted stationary tubes with medium 3 to 4 mm deep were used. No growth of *D. tarda* or *D. coralloides* occurred. *D. blastica* gave inconsistent results. In one of five trials the count increased about two-fold by the fifth day and then fell off.

When the peptonized milk was increased to 0.005% *D. blastica* showed a thin film on the third day. *D. coralloides* failed to show significant growth though cell counts increased slightly from the third to the sixth day. Apparently some stimulation occurred.

Near the end of the experiments designed to evaluate the limiting or extinction dilution of peptonized milk, it was learned that the sterile 30 ml plastic bottles used in tissue-culture investigations were superior to glass tubes. At that time it was discovered that glass tubes differed
Table 9. Influence of carbon compounds filtered (F) and autoclaved (A) in VSMP-C on the growth of D. tarda, D. coralloides and D. blastica.

<table>
<thead>
<tr>
<th>Compound</th>
<th>D. tarda</th>
<th>D. coralloides</th>
<th>D. blastica</th>
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<td></td>
<td>F</td>
<td>A</td>
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*0 - no growth, + - slight growth, + - evident growth, NT - no test

considerably in their effect on growth. Chromic acid cleaning solution did not alter this effect. The plastic containers gave more consistent results. Peptonized milk at concentrations of 0.005% and above stimulated D. coralloides and D. blastica to produce thin film on the walls of plastic flasks but not on the walls of glass tubes. D. tarda failed to grow in plastic containers.
Growth in VSMP-C with added carbon compounds

The carbon compounds were employed in stationary tubes of VSMP-C. The results are seen in Table 9.

D. tarda failed to show significant growth in any tube or flask.

D. coralloides failed to use melizotose in liquid culture though it did so in VSAP-C but fructose, filtered and autoclaved, stimulated formation of a fine but perceptible film. Dextrin gave inconsistent results. Arabinose was stimulatory in all tests. Xylose was ineffective. One test suggested the use of asparagine by D. coralloides. Efforts to repeat this result with asparagine failed.

D. blastica remained closer to the determinations made on VSAP-C. Raffinose, creatine, and dextrin caused mild response that started on the fifth day and continued through the 14th day with a slow increase in numbers of cells. A final 7-fold increase in cell numbers occurred in the presence of 0.333% creatine. D. blastica used asparagine as well as creatine in liquid culture if the strains had been adapted to PMS or SPMS. The non-adapted cultures did not show this ability.

Spore-germination D. coralloides and D. blastica were observed in the process of spore-germination in cultures from a number of solid media and two liquid media, PMS and SPMS. Spore-germination in D. tarda was observed only in cultures on PMA. The observations to be discussed are those from cultures on PMA. Though there is limited evidence on the germination of D. tarda, it appeared to take the course of D. coralloides and D. blastica.

Early in the project investigation of spore-germination in D. coralloides and D. blastica showed results that cast doubt upon the
conclusion that germination occurred by simple elongation of the spore. Later and more intensive investigations showed that a ruptured spore wall or shed coat was visible and that the cell emerged to increase in size thereafter.

This evidence was not wholly clear however. Though the cultures were prepared on agar blocks, in deep-well slides, or in specially made chambers on plain glass slides, and observed at intervals from 4 hours to 21 days, the course of spore-germination by rupture of spore case or the shedding of a spore coat could not be followed adequately enough to give wholly conclusive results. In an effort to clarify this problem other species of myxangia-producers were studied in the same was as D. coralloides and D. blastica. It was discovered that a strain of a myxobacter, probably a new species of Arthangium, gave clear evidence of a ruptured wall with the emergence of a cell. When later the process of germination of D. coralloides and D. blastica and D. tarda were compared with these results close similarity to the process seen in the species of Arthangium seemed to be evident.

Microscopic study always showed that many bodies, probably protoplasts and spheroplasts, were present. Also there were pieces of "fabric" or what seemed to be torn coats or wrappings which were more or less flat. These might have been shed spore-wall elements and their fragments. Together with these were the open "hulls" of the spores.

In the cultures of all strains studied were small cells, some about the size of Hemophilus influenzae, some larger, and showing one or more deeply stained bodies. Often there were 2 of these bodies giving the cell
a bipolar effect. Some cells appeared to be emerging from a spore.

**Spore-formation**  Spore-formation in *D. tarda*, *D. coralloides* and *D. blastica* appeared to be similar to that of other species of myxangia-producers. The cells shortened perceptibly about a day before the ultimate change to spores. The entire process was not clarified.

When the process was watched by making impression smears every hour for 48 hours and staining with carbol-erythrosin and crystal violet, a granular mass in the cell staining pink was seen to encircle darker violet-stained material. This was observed frequently in observations of strains of *Myxococcus* and *Archangium*.

**Optimum incubation temperature**  Growth of all three species occurred from 34°C to 15°C. Optimum growth occurred near 25°C.

**Diagnostic Summary**

*Dactylocoena tarda* sp. nov.

**Etymology**  *Tardus*., L. adj. Late, slow.

**Cells on PMA**  Rods 5.5 to 7.8 μ long and 6.0 to 8.7 μ wide.

Straight to U-shaped. Gram-negative. Not acid-fast. Stain weakly with safranin in gram stain. Short cells appear in abundance on 12th to 14th day on PMA.

**Motility from colonies on PMA**  Slow creeping. No flagella by Leifson's technique.

**Colonies on PMA**  Flat and spreading with corded, narrow, raised venation persisting. No significant central area developing.

**Myxangia on PMA**  Six morphological groups.
1. Small, 50 to 125 μ long, 50 to 95 μ wide and 50 to 75 μ high. Biscuit-shaped. Single or multiple. Color pale pink to pink.

2. Dome-shaped with sloping sides. 55 to 150 μ long, 35 to 125 μ wide and 50 to 100 μ high. Pale pink.

3. Columnar, similar to fingers rising from substratum. Resembling those in many species of Myxococcus and Dactylocoena; also in Podangium lichenicolum. Usual also in cultures of M. fulvus. Diameter 35 to 75 μ. Weight 40 to 175 μ. Milky to pink.

4. Similar to No. 3 but recurved.

5. Columnar, globular, club-shaped, anvil-shaped and otherwise swollen heads on a low "foot". Size as in any of the above groups. The "foot" may be swollen to constitute a primary myxangium supporting secondary myxangia as columnar, globular, club-shaped and anvil-shaped heads. Pale pink to pink.

6. Forked on a low to high foot or primary myxangium. Size of columns as individual prongs of a fork, 30 to 65 μ in diameter and 40 to 100 μ in height. Columns often with very few spores. A very characteristic feature of the species. Milky to pink.

Myxangia on a rabbit pellet
Numbers 1 and 2 usual. Number 3 rare. Never many of any group; often absent. A dry and pitted appearance usual.

Myxangia on selected media
No myxangia on BNA, DBNA, OIA and TA. Scattered myxangia numbers 1 and 2 on DDA and DBA with numbers 3 and 4 occurring at times on the latter medium.

Myxangia in general
Slime hard and clear. Inner spore mass or masses pale pink to pink. Probably no membrane around spore masses. A PMA plate may look like it was covered with tiny fragments of splintered
glass when myxangia of morphotype No. 3 are abundant on the medium.

Spores from myxangia on PMA Round to oval, 1.4 to 2.0 μ by 1.4 to 1.6 μ.

Effect of pH Grows best between pH 6.0 and 7.0.

Effect of crystal violet in PMA and PMS No growth in 0.0005% crystal violet in PMA but growth in 0.0001%. No growth in 0.0001% crystal violet in PMA.

Growth in 0.1% peptonized milk solution (PMS) An adapted strain grew weakly in stationary tubes or flasks of PMS. No myxangia.

Growth on PMA with mineral salts (SPMA) Survival of cultures for at least 3 years in loosely capped tubes at 10°C. Death rate increased in loosely capped tubes at 20° and 25°C and much increase in tightly capped tubes.

Growth on variable salt agar, medium C (VSA-C) Minute thin colony at site of inoculation.

Growth on VSA-C without added carbon compounds No growth.

Growth on VSA-C with 0.0025% peptonized milk (VSAP-C) Growth not stimulated by 0.0025% peptonized milk alone.

Growth on VSAP-C with added carbon compounds No growth of D. tarda occurred in VSAP-C containing the following agents: arabinose, xylose, glucose, fructose, galactose, sorbose, lactose, sucrose, cellobiose, melibose, melezitose, raffinose, rhamnose, dulcitol, mannitol, glycerol, salicin, aesculin, dextrin, starch, inulin, asparagine, creatine, alginic acid, agar or cellulose.

Growth in variable salt media (VSM) No growth.
Growth in VSM with carbon compounds used as above with VSAP-C No growth.

Growth in VSM with 0.0025% peptonized milk (VSMP-C) No growth.

Growth in VSMP-C containing the carbon compounds used with VSAP-C No growth.

Optimum temperature 25° to 27°C.

Source Two strains used in study, both from sites in Birch Lake. Strain 14 from an underwater log and strain 129 from the carapace of a living crayfish.

Type Strain 14 is designated as the type.

Dactylocoena cyclangioides sp. nov.

Etymology Gr. n. cyclus - circle. Gr. n. angium - vessel.

Cyclangioides M.L. adj., resembling a circular vessel.

Sources Three strains were selected for study. One, No. 60, was used in all experiments. The other two were included at intervals to assess their status and to evaluate the conditions of the experiments. The first strain, No. 60, came from the alga Chara in Birch Lake. Of the two strains used intermittently, No. 101 was isolated from a decaying log on a bank deep in the trees above the lake and No. 307 taken a year later from the same Chara bed as No. 60. Strain 60 is designated the type strain of the species.

Cell morphology from colonies on PMA The cells were broad with rounded ends becoming tapered a day or two before spore-formation. Measurements showed the cells to be 4.0 to 6.5 μ long and 0.8 to 1.3 μ wide, and generally shorter and plumper than the cells of D. tarda, D.
coralloides and D. blastica. Also they were more variable, some cultures possessing thin cells having the appearance of the cells of the stock strains of Cytophaga.

Shortening of the rods could be seen on the 4th day and a mixture of normal cells, with oval to round spores was evident at that time.

Most cells were straight to U-shaped though some were S-shaped (Plate 4, figures 13, 14, 15, and Plate 5, figure 16).

D. cyclangiodes was gram-negative and not acid-fast. No flagella could be demonstrated by Leifson's technique.

Motility of cells from colonies on PMA. The cells of D. cyclangiodes were relatively inactive when isolated but on successive transfers they showed increased motility becoming as active as some strains of Cytophaga.

Colony morphology on PMA. The colonies of D. cyclangiodes were distinctive though they possessed many of the characteristics of the colonies of D. coralloides.

The colonies of D. cyclangiodes had a narrow band of raised, corded veins making a rim around an extensive glassy almost veinless central area which developed on the 3rd or 4th day (Plate 5, figures 17, 18, 19, and Plate 6, figure 20). Often the change from a corded, veined central zone to a glassy one was attended by myxangia-formation so that within 2 days after myxangia started forming the zone was crowded with them. The largest myxangia were at the midpoint of the colony (Plate 6, figure 21). As this took place clouding occurred in the medium beneath the central area.

Microscopic study of the clouded agar showed many shortened cells,
some nearly oval staining intensely with crystal violet. Cell fragments were found also together with cells similar to those in surface colonies. The pink colony color so usual among myxangia-producers was more intense in *D. cyclangiodes* than in the other species studied.

**Colony morphology on selected media**  
*D. cyclangiodes* grew well on all the media provided in the investigation of colony structure. In this respect it showed a close relationship to the available species of *Archangium*.

On OIA the colony was structurally similar to that of *M. fulvus*, lacy in fine or delicate venation. Vague clearing of the opaque medium was seen by the 5th day but clarification did not continue to transparency.

Colonies on BNA were like those of other species of myxangia-producers, coarse and not distinctive. On DBNA a coarse colony also occurred. Colonies like those of *D. coralloides* occurred on both dung decoction agars DDA and DBA. On these 2 media the central area of the colony became intensely cloudy. This colony was dominant also on SMA. Clouding of the medium in the central area of the colony occurred and casein was hydrolyzed.

On BCA the cells of *Aeromonas hydrophila* were lysed. The colony resembled that of *D. coralloides*.

**Myxangial characteristics on PMA**  
The myxangia of *D. cyclangiodes* were as characteristic as the colonies. They appeared as a whorl over the entire central zone on the fourth to sixth day (Plate 6, figure 21). After the first few developed in the very center of the colony others appeared rapidly and in profusion over the entire central zone. Within 48 hours this area was filled with myxangia. The most centrally located
myxangia were usually elongate and huge (Plate 6, figure 22). Inter­
spersed among them were myxangia of lesser size, some of them ellipsoidal to round (Plate 7, figure 23). Measurements of the larger and elongate myxangia showed them to be 510 to 1400 μ long, 180 to 230 μ wide and 100 to 140 μ high. Their appearance was that of an inflated vein with clear slime enclosing one or more pink to blood-red spore masses. The spaces between the spore masses were marked by a shallow indentation, rarely by a deep cleft. Angularity was not characteristic. Finger-like extensions of the main body along the agar were common.

When prodded with a loop the entire myxangium moved. The slime was soft. Deliquescence did not occur. Myxangia with soft non-deliquescent slime were characteristic also of the available species of Archangium.

Myxangia rarely appeared on rabbit pellets and were small somewhat like the sloping forms of D. tarda. Usually myxangia in the vicinity of a rabbit pellet, if formed at all, were small and pale. In this respect D. cyclangioides resembled the species of Dactylocoena and Archangium flavum.

Myxangial characteristics on selected media

Myxangia were small and pale on OIA.

On BCA most myxangia were round rather than elongate and of a bright pink to red color.

This species was one of two that produced myxangia on BNA. These myxangia were pink to orange and long and followed the course of certain veins. Round to elongate, pink to red myxangia were formed on DBNA but the whorls seen on PMA were lacking. In general the myxangia were smaller than on PMA.
On DDA a limited number of moist, small, round, pale pink myxangia were produced. Confluence of adjacent myxangia occurred.

Elongate to round myxangia with a pink cast were formed when DDA was diluted to make DBA. At 20° (the characteristic whorl seen on PMA) was apparent but at 25° and 27°C this was not observed; the myxangia, usually only a few, were scattered.

On SMA blood red to pink myxangia were produced as on PMA.

Spores from 4 month old myxangia on PMA Spores were oval to ellipsoidal and sometimes elongate 1.4 to 2.6 μ long by 1.0 to 1.6 μ wide (Plate 7, figure 24). The elongate spores were similar to those of D. cerebriformis and the available species of Archangium.

Growth on PMA with varied pH (PHPMA) PMA was altered to make different series of plates, each series adjusted from pH 4.5 to 9.5. A departure from the results found in the study of D. tarda, D. coralloides and D. blastica was found. D. cyclangiodes grew best above pH 7.0 and probably had an optimum near pH 7.5 or 8.0. Good colony growth was seen on PHPMA with a pH of 9.0. No growth occurred at pH 4.5 or 5.0 but was evident at pH 6.0.

These results were sufficiently different from the usual for the species of Dactylocoena to make tests in liquid media desirable. These were made first in PMS then in SPMS. First trials in agitated tubes were failures probably because aeration was not controlled.

Stationary tubes were then used. Under these conditions D. cyclangiodes showed about the same growth potential as on PHPMA at pH 6.0 to pH 8.0 with an optimum at about pH 7.5. Similar results were recorded
also for the species of Archangium and for all but two of the available species of Myxococcus, M. stipitatus and M. ovalisporus.

Growth in PMA with different agar concentrations (VAPMA) D. cyclangiodes spread rapidly on PMA with 0.5% agar. Dense clouding of the medium with 1.0% agar occurred beneath the colony. On both media a thin surface film appeared. On the medium with 1.0% agar cells tended to be thinner than those from PMA with 1.5% agar if they were observed from the 3rd to the 5th day. Myxangia occurred on 1.0% but not on 0.5% agar.

On PMA with 1.5% and 2.0% agar growth did not differ. Colony spread on 2.5 agar was limited and myxangia were scattered and small.

Growth on PMA with different concentrations of crystal violet (CVPMA) Growth occurred on PMA with 0.0001% crystal violet, but at concentrations of 0.0005% and 0.001% there was no growth. The colonies became pale purple. This was seen in certain other species also, particularly species of Cytophaga and Archangium.

Growth in 0.1% peptonized milk (PMS) and peptonized milk with mineral salts (SPMS) Early attempts to grow D. cyclangiodes in liquid media did not yield satisfactory results. Successive transfers led to increasing growth in PMS and SPMS particularly in 30 ml tissue-culture flasks. In these D. cyclangiodes produced myxangia which tended to be globular or nearly so and similar to the low mounds on PMA. The abundance of these red to pink myxangia was particularly great in SPMS and PMS.

Growth on variable salt agar (VSA-C) A suggestion of growth occurred at the site of inoculation on all salt agars. This growth was more evident than that of D. tarda, of D. coralloides and of D. blastica.
on the same media. Agar apparently was not a source of carbon for *D. cyclangiodes*. In general *D. cyclangiodes* showed less growth at the site of inoculation than the species of *Myxococcus* and *Archangium*. In this respect it resembled other species of *Dactylocoena*.

**Growth on VSA-C with added carbon compounds**  
When the carbon compounds listed in Table 11 were added to VSA-C to make concentrations of 0.333% no significant increase in growth at the site of inoculation was seen.

**Growth on VSA-C with 0.0025% peptonized milk (VSAP-C)**  
Addition of peptonized milk to make a concentration of 0.0025% did not significantly change the amount or rate or growth seen on VSA-C. On VSA-C growth at the site of inoculum did not progress after the 4th or 5th day. On VSAP-C this was not the case, growth, though thin or sparse, continued to about the 14th day. This was true also of the strains of *Archangium*.

**Growth in variable salt medium, medium C (VSM-C)**  
No growth was observed at any of the usual 5 incubation temperatures.

**Growth in VSM-C with 0.005%, 0.0025% and 0.001% peptonized milk**  
The three concentrations indicated were tested in stationary 30 ml tissue-culture flasks. Growth of the strains adapted to PMS or SFMS occurred at a concentration of 0.005% but not at 0.0025% peptonized milk.

**Growth in different concentrations of BBL milk protein hydrolysate (MPH) and Bacto peptonized milk with or without VSA-A or VSA-C**  
In tests of nitrogen sources suitable for growth *D. cyclangiodes* showed no evident stimulation by MPH at concentrations of 0.01% in VSA-A and VSA-C.

Optimum growth occurred at concentrations of MPH from 0.333% to 0.5%
in both salt mixtures and without salts.

The same experiments were repeated using different concentrations of peptonized milk. Incubation was carried out at 20°, 27° and 32°C. Growth was evident at these three temperatures when the concentrations of peptonized milk were 0.005% and above in salts or without a salt mixture. The results are given in Table 10.

Table 10. Influence of different concentrations of BBL milk protein hydrolysate in VSA-A and Bacto peptonized milk in VSA-C on the growth of D. cyclangiodes at 27°

<table>
<thead>
<tr>
<th>Per cent concentrations</th>
<th>MPH</th>
<th>Peptonized milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0010</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.0025</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>0.0050</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.0100</td>
<td>+</td>
<td>+++M</td>
</tr>
<tr>
<td>0.0500</td>
<td>+</td>
<td>+++M</td>
</tr>
<tr>
<td>0.1000</td>
<td>+++M</td>
<td>+++M</td>
</tr>
<tr>
<td>0.2500</td>
<td>+++M</td>
<td>+++M</td>
</tr>
<tr>
<td>0.5000</td>
<td>+++M</td>
<td>+++M</td>
</tr>
<tr>
<td>1.0000</td>
<td>+M</td>
<td>+++M</td>
</tr>
</tbody>
</table>

*0 - no growth, + - slight growth, + - evident growth, ++ - good growth, +++ - excellent growth, M - myxangia*

Stock cultures were maintained on SPMA. After 3 years *D. cyclangiodes* was readily grown from slant-cultures on this medium. The techniques of cultivating and holding were the same as those used for other species including *D. tarda*, *D. coralloides* and *D. blastica*. Inoculations were made on slants and the cultures grown at 27° for about 3 weeks. The slants were then separated into lots and held at 10°, 20° and 25°C.
Two series of tubes were used. One series was capped tightly to eliminate air and the second was capped gently to allow some exchange of gases but reduce loss of moisture. Cultures in tightly capped tubes held at 25° and 20°C died within a year. Those at 10°C were viable after 3 years when the last determination was made. In loosely capped tubes survival was the same from 10° through 25°C.

Growth on VSAP-C with 0.5% gelatin Gelatin was liquefied in 3 to 4 days.

Growth on VSAP-C with added carbon compounds D. cyclangiodes was stimulated by dextrin, asparagine, rhamnose, aesculin, arabinose and starch. Dextrin and rhamnose appeared to give optimum results. Dulcitol, glycerol, salicin, and xylose were inhibitory. In one culture creatine stimulated growth. When this experiment was repeated stimulation did not occur. Repeated trials with creatine suggest that it might have been consistently stimulator if the MgSO₄·7H₂O concentration were doubled.

Table 11 shows the results of determinations using different carbon compounds.

Growth on VSAP-C with insoluble carbon compounds Chitin and cellulose were not used by D. cyclangiodes.

Growth in VSMP-C containing added carbon compounds In the liquid medium above, VSMP-C, D. cyclangiodes showed an increase in cell numbers along the walls of glass tubes or plastic bottles when dextrin and asparagine were added together to make a final concentration of 0.4% (0.2% of each). Asparagine and raffinose together served the same purpose.

Table 12 shows the results of determinations with carbon sources used separately or in combinations.
Table 11. Influence of carbon compounds at concentrations of 0.333% in VSAP-C on the growth of *D. cyclangiodes*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Filtered</th>
<th>Autoclaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fructose</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>Galactose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sorbose</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td>Lactose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Melibiose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Melezitose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Raffinose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td>Solcin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aesculin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dextrin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>NT</td>
<td>+</td>
</tr>
<tr>
<td>Inulin</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Asparagine</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Creatine</td>
<td>?</td>
<td>NT</td>
</tr>
</tbody>
</table>

*0 - no growth, + - evident growth, - - inhibition, NT - no test*
Table 12. Influence of carbon compounds at concentrations of 0.333% in VSMP-C on growth of *D. cyclangiodes*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Filtered</th>
<th>Autoclaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Xylose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fructose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Galactose</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Sorbose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lactose</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mellibiose</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Melezitose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+?</td>
<td>0</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dulcitor</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Salicin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aesculin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dextrin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td>Inulin</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Asparagine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Creatine</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dextrin + asparagine</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Raffinose + asparagine</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Dextrin and raffinose with MgSO₄ at 0.5%</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Dextrin and raffinose with MgSO₄ at 1.0%</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*0 - no growth, + - evident growth, NT - no test*

**Spore germination** Spore germination was not studied.

**Spore formation** Cells shortened on the 5th day and progressed to spores (Plate 7, figure 25). Cells often stamped intensely with crystal violet on the 4th or 5th day (Plate 7, figure 26).

**Effect of incubation temperature** On PMA *D. cyclangiodes* grew from
20° through 32°C. Better growth occurred at 20° than 32°C. The optimum 
incubation temperature may be 25° to 27°C.

**Dactylocoena cyclangiodes sp. nov.**

**Etymology**  Gr. **Cyclus**-circle **Angium**-vessel.

**Cyclangiodes**  M.L. adj., resembling a circular vessel.

**Cells**  Rods with rounded ends and occasionally a slight taper.

Shorten perceptibly in cultures on PMA on 5th to 7th day. Rather plump 
cells from most PMA cultures 4.0 to 6.5 μ long and 0.8 to 1.3 μ wide.

Some cultures with longer thinner cells occurred. Cells straight to 

**Motility from colonies on PMA**  Inactive when isolated; later, one 
of the most active of myxangia-producing forms. No flagella by Leifson's 
technique.

**Colonies on PMA**  Spreading, flat, with narrow, corded, branching 
venation for first 3 or 4 days after which a central glassy area occurs. 
Glassy area becomes rapidly extensive leaving a narrow, corded band as a 
border around the central area. Myxangia appear as the central area is 
formed and quickly fill it and appear in a whorl.

**Myxangia on PMA**  Large, bright, pink to blood red. Occasionally 
huge. Largest near mid-point of colony. Low and elongate to round with 
fingers from main spore mass along agar. Some dome-shaped with single to 
many spore masses in a myxangium. Slime soft but not deliquescent sug-
gesting the species of Archangium. Length of larger myxangia, 50 to 1400 
μ; width 180 to 230 μ; height 10 to 180 μ. Spore masses usually round in 
the clear slime.
Myxangia on a rabbit pellet  Usually no myxangia.

Myxangia on selected media  On oatmeal infusion agar and bacterial cell agar myxangia tend to be round, small and pale. Myxangia form on Bacto nutrient agar as long, narrow, orange to red bodies like thickened veins. Small, round, pale scattered myxangia occur on dung decoction agar and diluted dung decoction agar. Blood red, pink and elongate myxangia occur on skim milk agar. Casein hydrolysis occurred.

Spores from myxangia on PMA  Round to oval to ellipsoidal, some like shortened cells, 1.4 to 2.6 μ long by 1.0 to 1.7 μ wide.

Effect of pH  Optimum growth between pH 7.0 and 8.0, probably pH 7.5.

Growth in PMA containing different concentrations of crystal violet (CVPMA)  Growth in crystal violet at a concentration of 0.001% but not 0.0005%.

Growth in 0.1% peptonized milk solution (PMS) and (SPMS)  Good growth with myxangia production in stationary tubes or flasks at 25 and 27°C. Cells become somewhat narrower. Myxangium globular. Myxangia abundant in submerged culture in SPMS.

Growth on variable salt agar, medium C (VSA-C)  No growth beyond a minute colony at site of inoculation.

Growth on VSA-C with added carbon compounds  No response to arabinose, xylose, glucose, fructose, galactose, sorbose, lactose, sucrose, cellobiose, melibiose, melizitose, raffinose, rhamnose, dulcitol, mannitol, glycerol, salicin, aesculin, dextrin, starch, inulin, asparagine, and creatine.

Growth on VSA-C with 0.0025% peptonized milk (VSAP-C)  Growth not
enhanced.

Growth on VSAP-C with carbon sources as above at a concentration of 0.333% Arabinose, rhamnose, aesculin, dextrin, starch and asparagine stimulate growth. Dulcitol, xylose, salicin, glycerol probably toxic.

Growth on VSA-C with BBL milk protein hydrolysate (MPH) Optimum growth at 0.333%. No growth at 0.01% MPH.

Growth on VSAP-C with 0.5% gelatin Gelatin digested.

Growth in variable salt medium, medium C (VSM-C) No growth.

Growth in VSM-C with 0.0025% peptonized milk (VSMP-C) No growth.

Growth in VSMP-C with carbon compounds as in VSA-C In stationary tubes or flasks, dextrin and asparagine cause a response. Dextrin and asparagine combined, and raffinose and asparagine combined, cause a greater response than any carbon compound used alone. An increase in MgSO₄·6H₂O may improve the response of raffinose and asparagine used together.

Spore germination Unknown.

Optimum incubation temperature 25 to 27°C.

Source Three strains, No. 60 from a species of Chara; No. 101 from a decaying log on bank above lake; No. 307 from above Chara bed a year later.

Type Strain 60 is designated as the type.
CONCLUSIONS

In a survey of a Minnesota lake and its environs, 251 strains of myxobacters were isolated. Most of these were members of the genera Cytophaga and Dactylocoena. Other genera in the aqueous habitat were: Myxococcus, Sporocytophaga and Archangium.

The most abundant species, other than those in the genus Cytophaga, were Dactylocoena coralloides, D. columnaris, D. blastica, Myxococcus fulvus and M. ovalisporus.

Some new species were encountered. These included at least two new species of Dactylocoena, one each in the genera Myxococcus and Archangium, and several in the genus Cytophaga.

In a review of the record on the immediate sites from which all 251 forms were isolated it became apparent that the organisms were present in the lake on logs, rocks, posts, debris, flora, and fauna, all of which had a coat of slime. It was possible to return to these sites within the immediate collection period or from one year to the next to find myxobacters similar to those previously isolated at the same point. Apparently they persisted in an aqueous habitat provided they had a surface for maintaining themselves.

For purposes of isolation and characterization of the myxobacters of the lake, new and better media were desirable and others needed re-evaluation and at times modification. In all, about 200 different media and combinations of nutrients were developed or modified. About 40 of these proved useful in a study of growth characteristics of 76 strains of myxobacters chosen from the 251 isolates for preliminary testing. Of these 20
strains were studied intensively.

Final emphasis was placed upon a study of 4 species of the genus Dactylocoena. Two of these were new species. The differential characteristics of the two species were studied in detail with constant comparisons with the species Dactylocoena blastica and D. coralloides.

Attention is called to the fact that the generic name Chondrococcus Jahn 1924, usually applied to this taxon of myxobacteria, is illegitimate and must be replaced by Dactylocoena Enderlein 1924.

Among the characteristics of the two newly-described species, the following may be pointed out as particularly significant. Dactylocoena tarda is considered first.

Dactylocoena tarda was so named because of its relative inactivity on many media stimulating other myxangia-producers, and because its myxangia developed late even on the most satisfactory media. Also it was the least resistant of the species of Dactylocoena to known toxic agents and compounds not toxic to the other species.

D. tarda formed a colony structurally like those of D. coralloides and D. blastica on peptonized milk agar (PMA) for the first three to four days of incubation. However the colonies of the last two species changed after about 4 days and those of D. tarda remained unaltered as if persisting in a primitive state. This colony was formed of narrow, corded, anastamosing veins throughout.

The myxangia of D. tarda were cartilaginous and of at least six morphological types. Usually they were small; often they were numerous. Most distinctive were the myxangia formed as forked columns arising from a foot or a primary myxangium. These forked myxangia often had few spores
and when abundant on a plate produced the effect of tiny particles of frosted glass scattered over the agar. *D. blastica* was similar in this respect but the myxangia were distinctly different in detail and in time and manner of appearance on a plate. Moreover, they formed abundantly on rabbit pellets and those of *D. tarda* often failed to form.

Physiologically *D. tarda* was almost inert compared with *D. blastica* and *D. coralloides*. This was a persisting diagnostic feature. It probably accounted for the fact that the strains were so long overlooked in early efforts to isolate myxobacters from water.

*D. cyclangiodes*, the second new species, was noted as different from all other myxangia-producers from the time the strains were isolated. However, it was difficult to place in a genus because it possessed features of species in the genus *Archangium* as well as the genus *Dactylocoena*. Some characteristics were similar to those of *A. gephyra*; others were similar to those of *A. flavum* and the other species which Jahn 1924 classified with hesitation in the genus *Archangium*. After intensive study of *D. cyclangiodes* and comparisons with *A. flavum*, *D. cerebriformis*, species of *Myxococcus*, and a possibly new species of *Archangium*, the decision was made to place *D. cyclangiodes* where it is with a view to future reconsideration of it together with *D. cerebriformis*, *A. flavum*, *D. cirrhosa* and *D. cruentus*. A new genus to contain these species seems to be desirable if they are not retained finally in the genus *Dactylocoena*.

*D. cyclangiodes* formed a colony like that of *D. coralloides* and the species of *Archangium*. Cells from the colony were plump, the spores often rather elongate. Myxangia were soft and usually large and long with little
angularity. Convolutions were rare. The spore masses in the slime of a myxangium were never separated by deep clefts. From one to seven such masses occurred in the slime. These long myxangia had the appearance of swollen veins. Fingers usually led away from these myxangia. A very distinctive characteristic was the tendency for the myxangia to appear in a large whorl starting near the center of the plate. Bright pink to blood red colors predominated.

Physiologically *D. cyclangiodes* was active compared with *D. tarda*, *D. coralloides* and *D. blastica* which form a compact group. It was stimulated by several carbon compounds in mineral salt media and grew well in certain liquid media where it also formed myxangia.
ACKNOWLEDGMENTS

Work does not go forward without the insight and assistance of other people. To acknowledge the support of the many who contributed to the present work would require many pages. If, then, these many are not recognized by name here they are not forgotten for the aid and advice they gave.

To Dr. R. E. Buchanan go the greatest thanks for guidance, perseverance and encouragement throughout this project.

Particularly good fortune prevailed in the form of the assistance and understanding of the members of the writer's doctoral committee, Dr. W. R. Lockhart and Dr. R. J. Beers of the Department of Bacteriology, and Dr. Lois Tiffany and Dr. John Dodd of the Department of Botany.

Directive help and answers to many questions were provided over the four years of the project by Dr. John Holt, Dr. L. Y. Quinn and Dr. P. A. Hartman of the Department of Bacteriology. In particular, Dr. Holt's knowledge of myxobacters was invaluable.

It is desired that recognition be accorded the members of Dr. Buchanan's staff particularly Mrs. Lyle McConnell as well as Mr. Don Draper, a capable, resourceful and indefatigable technical assistant. The same recognition goes to the members of the technical staff of the Department of Bacteriology and to Mary R. Barron and LaDena F. Bishop of the staff of the Iowa State University Library.

By no means forgotten are Martha, Nancy, Cynthia and Mary.

The very capable typist was Mrs. Natalie T. Skola of Ames, Iowa.
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PLATES AND FIGURES
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Figure 1. 3 day cells of D. tarda grown on PMA Modified Winogradsky stain (2400X)

Figure 2. 10 day cells of D. tarda grown on PMA Modified Winogradsky stain (2400X)

Figure 3. 7 day colony of D. tarda on PMA (10X)

Figure 4. 7 day colony of D. tarda on PMA (10X)
Figure 5. 4 week old biscuit-shaped myxangium of *D. tarda* on a rabbit pellet (80X)

Figure 6. 4 week old dome-shaped myxangium of *D. tarda* on PMA (80X)

Figure 7. 4 week old columnar myxangium of *D. tarda* on PMA (80X)

Figure 8. 4 week old curved myxangium of *D. tarda* on PMA (80X)
Plate 3

Figure 9. 5 week old anvil-shaped myxangium of *D. tarda* on a foot of slime on PMA (80X)

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Figure 12. Mature spores from myxangia of D. tarda grown on PMA Modified Winogradsky stain (2400X)

Figure 13. 2 day cells of D. cyclangiodes grown on PMA Modified Winogradsky stain (2400X)

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Figure 17. 5 day colony of *D. cyclangiodes* on PMA showing outer venous band and a limited part of the central area with myxangia (20X)

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