MYXOBACTERS OF A FRESHWATER LAKE
AND ITS ENVIRONS

I. New and Modified Culture Media Found Useful in Isolation
and Characterization of Freshwater Myxobacters.

Edmund E. Jeffers

Department of Bacteriology, Iowa State University
Ames, Iowa

SUMMARY. Twenty media have been developed that have proved particularly useful for isolation and characterization of myxobacters from a northern freshwater eutrophic lake. Formulae are given and special uses of each discussed. In most cases the media described have not previously been used. Some media (such as peptonized milk agar (PMA)) have proved useful because well adapted to growth of most species. Other media are important in determining the purity of cultures, particularly for detecting contaminating eubacteria. Certain media stimulate the development of myxangia (so-called "fruiting bodies" of myxobacters). Many formulae are primarily useful in differentiation and identification of species.

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The present communication is an introduction to a series of publications relating to the characterization of the myxobacters isolated from a freshwater lake. The primary objectives were to get pertinent information as to the abundance, importance and taxonomy of strains found in such waters.

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 which drains spring-fed Ten Mile Lake, which is its 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 is found. 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.

From various sites in or near the lake, 251 strains of myxobacters have been isolated. Seventy-six of these have been studied relatively intensively. Among the latter some undescribed species and subspecies have been found.

Search of the literature failed to reveal satisfactory media, materials and techniques for the isolation of freshwater myxobacters. Much time in the beginning was used in the development of new and satisfactory media. To meet the difficult conditions of isolating these organisms from a productive lake, and to make possible comparison with the data of earlier investigators, many media were evaluated.

MEDIA

All media, unless otherwise specified, were autoclaved at 15 psi for 15 minutes. The pH of the agar-containing media, except those used in determination of the effect of hydrogen-ion concentration, was adjusted to 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 were thymol blue (acid range and alkaline range), methyl orange, brom cresol green, phenol red and thymol phthalein. A Coleman Metrion pH-meter was also used to check

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results. For most of the determinations the Coleman 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 as 10%, 1% and 0.1% solutions wherever 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 25°C rather than in the cold room to avoid the formation of precipitates. The carbon compounds used in the various media were: arabinose, xylose, glucose, fructose, galactose, sorbose, sucrose, lactose, cellobiose, melibiose, melezitose, raffinose, rhamnose, mannitol, dulcitol, dextrin, starch, inulin, cellulose, chitin, alginic acid, agar, asparagine, creatine, aesculin, salicin and glycerol.

This comparative study of media described in the literature and the new media formulated herein was made with an intent to answer the following queries.

1. What is the effect of each medium on growth, colony morphology, sporulation, spore-germination and production of myxangia (fruiting bodies)?
2. Is the medium selective, favoring the growth of myxobacters over other microorganisms?
3. Does it contribute to distinguishing the taxa of myxobacters?
4. Does continual growth on the medium contribute to stability of morphology and physiology?
5. Is the medium of use for a wide variety of myxobacters or is it selective for certain species or groups of species?
6. Do the physical characteristics of the medium obscure growth characteristics of the bacterial colonies?
7. Can the medium be so described that it may be prepared by other workers?
8. What are the toxic levels and the minimal levels of mineral salts and carbon compounds that are used to make a definitive medium?

9. What are the concentrations of mineral salts and a carbon source to make a balanced definitive medium for the growth of myxobacters?
10. What are the conditions necessary to favor growth of myxangia-producing myxobacters in a liquid medium?

MEDIA FORMULATION AND USE

(1) PEPTONIZED MILK AGAR (PMA)

Bacto peptonized milk	1.0 g
Bacto agar or Oxoid Ionagar No.2	15.0 g
Distilled water	1000.0 ml

The formulation 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 so 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. In that respect it is similar to Bacto nutrient agar diluted one to ten. 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. Oxford (1947) used casein hydrolysate with mineral salts and asparagine supplied by the Glaxo Laboratories in an attempt to grow myxobacters in a liquid medium. Noren (1952) used Bacto Casamino Acids, mineral salts and asparagine in his study of the growth of myxangia-producers. Dworkin (1962), using Myxococcus xanthus, tested a variety of enzymatic and acid

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hydrolysates of protein including Bacto Casitone, BBL Milk Protein Hydrolysate, BBL Lactalysate, Bacto Casamino Acids and BBL Acidase. These were used with a standard mineral salt base. The last three investigators were less interested in the isolation and differentiation of myxobacters than in the physiology of Myxococcus virescens, M. xanthus, M. fulvus and Dactylocoena coralloides (Chondrococcus coralloides Jahn).

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 (with some, more than 100).

PMA has one disadvantage in that cultures grown on it tend to be shorter-lived than those grown on the medium SPMA next described.

(2) 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 sterile solutions:	
(NH ₄) ₂ SO ₄	0.10 g
MgSO ₄ ·7H ₂ 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

As noted above, SPMA proved 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 the 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.

(3) PEPTONIZED MILK SOLUTION (PMS)

Bacto peptonized milk	1.0 g
Distilled water	1000.0 ml

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.

(4) CRYSTAL VIOLET PMA (CVPMA)

Bacto peptonized milk	4.0 g
Agar	60.0 g
Distilled water	4000.0 ml

Separate into equal amounts in 4 flasks

Add saturated alcoholic solution of
crystal violet, 0.1 ml to flask 1,
0.005 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 myxobacters from fresh water. Later the medium was found to have value in the differentiation of some of the species of myxangia-producers.

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AND TAXONOMY(5) PMA WITH VARYING AGAR CONCENTRATIONS
(VAPMA)

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

Soft agar, 0.5%, permitted 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% agar was found to be optimum. Soft agar was generally unfavorable to the development of myxangia. For production of spores and myxangia, 1.5% 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 five strains of Cytophaga.

(6) PMA WITH VARYING PH (VHPMA)

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.

Norén (1952) reviewed the literature on the effect of differences in hydrogen-ion concentration on myxobacters. He found the results of several investigators (DeKruyff 1908; Vahle 1909; Solntzeva 1940; Beebe 1941; Singh 1948; and Oxford 1947) to be conflicting.

VHPMA was found to be useful in differentiating some of the strains of the genus Cytophaga from each other 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.

(7) PEPTONIZED MILK AGAR WITH VARYING CON-
CENTRATIONS 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% to 10% were prepared.

The 0.1% concentration was determined to be the best for general use. Higher concentrations such as 1.0% materially increased production of slime and decreased the longevity of the culture.

(8) 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%, 2.5%, 5.0% and 10.0% skim-milk agar were prepared with agar at 1.5%. These media were not found to be adequate for isolation of myxobacters or for study of colony morphology. A concentration of 2.5% 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.

(9) DUNG DECOCTION AGAR OF BEEBE (DDA)

Bauer (1904), Quehl (1906), Jahn (1924), Krzemieniewska and Krzemieniewski (1925, 1927, 1928), and Beebe (1941), among others, grew myxobacters either on rabbit pellets or a dung decoction medium. 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 Beebe's dung decoction agar.

Rabbit pellets (dry)	100.0 g
Distilled water	1000.0 ml
Boil	
Allow to stand 24 hours at 27°C	
Filter through 4 layers of gauze	
Bring volume to 1000.0 ml with distilled water	
Agar	15.0 g
Autoclave at 15 psi for 30 minutes	
Adjust pH to 6.8	

DDA was employed initially in a comparison with other media in isolation of myxobacters from the mixtures of

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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 dark and opaque for determination of colony morphology.

(10) DILUTE DUNG DECOCTION AGAR (DBA)

DDA was modified by diluting dung decoction 1 to 10 in 1.5% 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.

(11) BACTO NUTRIENT AGAR (BNA)

Thaxter (1892, 400, 401) used a "nutrient agar" when he described Chondromyces crocatus and C. auranticus. He later (1897, 409) referred to "nutrient agar" as a medium on which M. 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, Difco nutrient agar was used. The concentration of nutrients in this medium proved to be too high to permit satisfactory differentiation of the myxobacters. As a result the medium was diluted as shown below.

BNA served throughout the project in tests of culture purity.

(12) DILUTED BACTO NUTRIENT AGAR (DBNA)

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

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 preferable 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.

(13) 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 best results had the following composition:

Freshly peeled and finely grated potato	15.0 g
Agar	15.0 g
Distilled water	1000.0 ml

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.

(14) 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 freshwater 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

* 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.

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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:

Oatmeal (rolled oats as Quick Quaker Oats)	100.0 g
Distilled water	1000.0 ml
Boil 5 minutes	
Filter hot through two layers of gauze	
Add distilled water to bring to 1000.0 ml	
Autoclave at 15 psi for 15 minutes	
Hold at 10°C for 5 days or more	
Three layers form in the flask	
Draw off the top clear layer	
Decant the middle white mildly gelatinous layer	
Discard the bottom layer	
Measure 50 ml of the middle layer and dissolve	
in the following:	
Agar	15.0 g
Distilled water	1000.0 ml
Autoclave at 15 psi for 15 minutes	

Myxangia that developed in 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.

(15) 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, or myxobacters in a medium with bacterial cells or cell products, are Vahle (1909), Pinoy (1913), Solntzeva (1939), Beebe (1941), Snieszko, McAllister and Hitchner (1941), Singh (1947) and Oetker (1953). Loebeck and Klein (1956) used constituents of Escherichia coli and found that some of these were metabolized. Beebe

(1941) and Snieszko, McAllister and Hitchner (1941) 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 A. hydrophila
Incubate, with occasional shaking, at 30°C for 72 hrs
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 distilled water containing 1.5%
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

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strains lysed the bacterial cells in the medium. Colony morphology could be studied effectively in those organisms which cleared the medium.

(17) BBL TRYPTICASE 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.

(18) 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 means were sought by use of TB.

(19) TRYPTONE AGAR OF ORDAL AND RUCKER (TA)

Ordal and Rucker (1944) reported success in the isolation and cultivation of a species of fresh water myxobacter by using 0.9% agar and 0.26 to 0.50% tryptone in distilled water. This was not the first indication of the significance of a peptone in cultivating myxobacters. Thaxter (1892) used a peptone and Vahle (1909) observed that a peptone seemed necessary.

TA was used in this project first as a medium containing 0.25% tryptone and 0.9% agar and, shortly thereafter, as a medium containing 0.50% tryptone and 0.9% agar. Later the agar concentration was raised to 1.5%. 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.

(20) 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 a known carbon compound. 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), Norén (1952), Oetker (1953), and to some extent, Dworkin (1962).

The following media were tested with 14 strains of myxobacters.

	Medium			
	A	B	C	D
Distilled water (ml)	1000.0	1000.0	1000.0	1000.0
Ionagar No. 2 (g)	12.5	12.5	12.5	12.5
Autoclave				
Cool to 50°C				
Add:				
(NH ₄) ₂ SO ₄ (g)	0.50	0.50	0.50	0.50
KH ₂ PO ₄ (g)	0.01	0.15	0.25	0.50
CaCl ₂ (g)	0.01	0.15	0.25	0.50
MnCl ₂ (g)	0.0001	0.00001	0.0001	0.0001
FeCl ₃ ·6H ₂ O (g)	0.01	0.01	0.01	0.01
MgSO ₄ ·7H ₂ O (g)	0.01	0.15	0.50	1.00

A carbon compound was added to each. Those added to make a 0.333% concentration wherever possible are given on pp. 118-9. The 4 media were also prepared in separate lots to contain either 0.333% BBL milk protein hydrolysate or Bacto peptonized milk which was tested in four concentrations, 0.01%, 0.005%, 0.0025% and 0.001% to determine the lowest concentration no longer permitting growth. This was only roughly assessed because each strain varied in its capacity to grow at a low concentration. As a result the highest dilution of peptonized milk just failing to stimulate growth of any of the myxangia-producers was taken as the level sought. This was 0.0025% peptonized milk.

The 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%

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peptonized milk. BBL milk protein hydrolysate supported growth of all 14 strains tested. Colony spread and myxangia production of two strains were definitely limited in all four media.

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

Variable salt agar, Medium C, without peptonized milk or another complex carbon source is designated VSA-C; with 0.0025% peptonized milk the medium is designated VSAP-C.

(21) VARIABLE SALT MEDIA (VSM)

The two media, C and D, found superior in the determinations above were modified by the elimination of agar and distributed in 5 ml amounts in chemically clean test tubes. The strains of Cytophaga produced turbidity in both media in the presence of a suitable carbon source of known composition and at 3 of the 4 concentrations of peptonized milk tested: 0.01%, 0.005%, and 0.0025%. Four myxangia-producing strains produced perceptible growth on the sides of stationary slanted tubes containing 0.01% and 0.005% peptonized milk. None grew at a concentration of 0.0025%. BBL milk protein hydrolysate at 0.333% supported growth of the strains of Cytophaga. Two strains of myxangia-producers did not grow. The film produced by a third was nearly imperceptible.

A liquid medium using the salt concentrations of Medium C and 0.0025% peptonized milk was decided upon as optimum for a basal solution in determination of the effect of known carbon sources on the growth of myxangia-producers.

Variable salt, Medium C, without 0.0025% peptonized milk, is designated VSM-C; with 0.0025% peptonized milk the medium is designated VSMP-C.

SPECIAL ADDITIVES

(a) Rabbit pellets

Rabbit pellets from animal house
Remove hair and debris by flaming
Autoclave at 15 psi for 6 hours

INTERNATIONAL BULLETIN

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.

(b) Commerical 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-salt 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 these species are similar, in their need for a surface, to certain eubacterial species found in water, as reported by Zobell (1946, 124).

(c) 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. It was found that they were ineffective in inhibiting growth of contaminating organisms the more difficult to control.

Sensitivity discs with various antibiotics were tried in an

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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.

(d) BBL Milk Protein Hydrolysate

This was used in 5%, 1%, 0.05% and 0.1% concentrations to replace peptonized milk in PMA. At a concentration of 0.5% it might substitute for peptonized milk in an isolation medium. However, in pure culture work, colony differences from species to species were not as sharp and with some strains myxangia did not form. There was retardation of colony growth among some myxangia-producers. This occurred at all concentrations of the hydrolysate.

DISCUSSION AND CONCLUSIONS

The media herein described have been fruitfully employed in the isolation and characterization of myxobacters from a freshwater lake. These media for the most part have not been described in the literature of the Myxobacterales and have been newly formulated. Some are modifications of media described by earlier students.

For isolation of myxobacters from lake waters having high counts of motile eubacteria, and for characterization of these myxobacters the Medium (1), peptonized milk agar (PMA), was found to be most satisfactory. It was used as the general-purpose medium in the study of colony, myxangia and cell morphology and for observation of spore-formation and spore-germination. PMA was also modified by use of varying hydrogen ion concentrations found useful in characterizing and distinguishing the strains of myxobacters. Other modifications of PMA with differing concentrations of peptonized milk or agar were helpful in characterization of strains.

Several attempts to discover a selective medium that would inhibit some myxobacter strains and allow the growth of others depending upon the concentration of an inhibiting agent finally led to the finding that PMA modified by the addition of crystal violet (CVPMA) was quite satisfactory. Sharp inhibition of the growth of some strains as contrasted with relative resistance of others was marked. The concentration of crystal violet was tested in the range from

0.0001% to 0.001%. Most species of motile eubacteria were found to be inhibited at 0.001% crystal violet while some strains of Cytophaga grow luxuriantly at this concentration.

Other agar-containing media investigated for value in isolation and characterization included Bacto nutrient agar (BNA), full strength and diluted 1 to 10 (DBNA); Beebe's dung decoction agar (BBA), full-strength and diluted 1 to 10 (DBA); bacterial cell suspension agar (BCA); skim-milk agar (SMA); 0.25% and 0.5% tryptone agar (TA), with agar concentrations of 1, 0% and 1.5%; grated potato agar (GPA); and oatmeal infusion agar (OIA). Each supported the growth of some but not all strains of myxobacters. By use of these varied media many strains could be clearly distinguished.

Some strains grew but failed to produce myxangia on one or more of the media. Myxangia tended to show obvious color differences and differences in morphology from medium to medium. Occasionally these differences in color and shape of myxangia were striking.

The colors of myxangia were usually intensified on bacterial cell-suspension agar, oatmeal infusion agar and skim-milk agar. These media had other advantages. The presence or absence of casein hydrolysis was assessed with skim-milk agar. Lysis of bacterial cells was evaluated with bacterial cell agar. It is evident, as Beebe (1941), Snieszko *et al.* (1941), and Norén (1953) indicated, that a valuable aid to differentiation of myxobacter strains and species is the use of the cells of different bacterial species as substrates for myxobacter lysis.

Oatmeal infusion agar (OIA) is a relatively opaque medium. Some myxobacters clarified the medium around colonies. This clearing was more than a matter of starch hydrolysis apparently as shown by the iodine test. No strains of Cytophaga caused this clarification. OIA proved to be one of the most satisfactory isolation media because motile eubacteria did not spread rapidly while colonies of myxobacters tended to spread rapidly and "outrun" the contaminants. Further, colonies and myxangia, when produced, were usually bright and easily discerned. Nutrient agar media (BNA) at full strength and diluted in the range of 1 to 20 with uniform 1.5% agar concentration were used to make comparisons with the results primarily of Thaxter (1892, 1897, 1904). At a dilution of 1:10 the growth most closely approximated Thaxter's descriptions of new species, particu-

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larly that of M. rubescens Thaxter 1892, 403 and M. virescens Thaxter 1892, 404. On full strength nutrient agar, colonies of myxobacters are coarse and often mucoid with little difference from strain to strain. No myxangia were formed by any strain on the full-strength medium.

Grated potato agar (GPA) (an attempted approximation of the media reported by Thaxter (1892) and Vahle (1909)), Beebe's dung decoction agar (BBA) (a modification of the dung medium of Quehl (1906)), and the tryptone-containing agar of Ordal and Rucker (1944) (TA) were used routinely for observations of myxangia of all strains tested and compared with growth of strains of M. xanthus, M. fulvus and M. virescens.

DBA at full strength proved to be too dark and to give inconsistent results at times. Some strains of Dactylocoena failed to form myxangia on the full strength medium but did so when it was diluted 1 to 10. A rabbit pellet placed on a surface of peptonized milk agar permitted the diffusion of water-soluble extractives from the pellet. Myxangia formed in some cases on the pellet; with other strains the myxangia were on the agar at varying distances from the pellet.

Tryptone agar (TA) was not satisfactory for general purpose use. Myxangia were not produced by some strains. The medium was not favorable to the expression of strain differences in colony morphology. Motile eubacteria grow luxuriantly. GPA was useful only in that the colors of colonies were enhanced while myxangia production, with few exceptions, was inhibited.

Selected solid media were used in routine evaluations of culture purity. These were BNA, Bacto EMB, TSB and TA. Two liquid media were employed also for this purpose, TB and PMS. Occasionally BNA was employed to check the purity of cultures.

Among the liquid media evaluated for characterization of myxobacters, the most satisfactory was PMS. When in stationary slanted tubes or in flat-bottomed plastic bottles PMS solution in a depth of 3 mm supported good growth of the species that produced myxangia. Growth usually occurred as a film along the glass or plastic walls of the container. In rare instances a culture of an occasional strain would produce a slight turbidity. Submerged myxangia were formed by all but two species. Some strains which did not develop myxangia on PMA formed obvious mounds to small

papillae on the sides of the slanted tubes or plastic containers. Microscopic examination of these mounds or papillae showed them to contain small rounded cells to round or oval or ellipsoidal bodies identical in appearance to myxospores. From the slimy matrix between these papillae and mounds, short to long cells were present with few round bodies. Some of these strains had been characterized by previous study as strains of Cytophaga, Sporocytophaga and possibly Chondrococcus columnaris.

Different salts, singly and in different combinations and concentrations were used in agar-containing and liquid media.

As a result of many trials, it was found that a medium containing .0025% peptonized milk would not support growth of the myxangia-producing myxobacters, but growth was evident with many strains when a suitable carbon source was added. No purely synthetic liquid medium was found adequate for the study of all the available species as a substrate to determine growth response upon the addition of carbohydrates and other carbon compounds. A number of species responded but some did not.

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