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While the genus Cytophaga has been defined and classified on a morphological basis, knowledge of fresh water members of this group of myxobacteria is scanty. In the present study cultural and morphological characteristics were investigated in an attempt to classify a group of fresh water cytophaga.

Microscopic examination revealed all test isolates to exhibit morphological properties of myxobacteria. Vegetative cells were gram negative, long, slender, flexible rods showing gliding or creeping motility and low refractibility. On aging, cells showed various involution or degenerate stages, especially in rich media. As cells aged they lost their typical gliding motility but showed various other types of movement. Typical colonies at 24-48 hours were yellow-orange in color and showed morphological differences between strains. The nutritional content of media was found to be a

factor influencing colony morphology. More compact colonies were found on rich media and as the nutrients were diluted, colonies tended to spread and have less coloration. Variation in colony morphology were also noted on different types of media as well as different concentrations of the same medium. Only one strain was found to form fruiting bodies or columnar masses of cells; this organism however, did not form microcysts.

Test isolates could be divided into two groups on the basis of growth temperature. The psychrophilic group grew best at 18°C and failed to grow at room temperature; the mesophilic group grew best at room temperature. Members of the psychrophilic group were found to be more sensitive to increased salt concentrations or change in pH and were not able to grow anaerobically. Ten of the mesophiles were capable of growing anaerobically.

A number of macromolecules were degraded by the test isolates. Starch, gelatin, chitin, aesculin and tributyrin were hydrolyzed by various test organisms and most of the isolates lysed a variety of dead bacterial cells. Members of the psychrophilic group showed more limited ability to hydrolyze macromolecules than did those of the mesophilic group. Seven of the test organisms oxidized glucose, and seven fermented this sugar; however, none of these oxidizers or fermenters were psychrophiles.

Some of the organisms produced black pigment from tyrosine,

reduced nitrate, and possessed the enzymes catalase and cytochrome oxidase. All were negative for the production of acids or acetyl methyl carbinol from glucose, cadaverine from lysine, indol from tryptophan and ammonia from arginine. None of the test isolates could utilize glucose as a sole carbon source with ammonia as the sole nitrogen source. Nine amino acids tested would not support growth as the sole nitrogen and carbon source. Five of these were inhibitory for some organisms when added to peptone and mineral basal medium. The majority of the organisms were capable of growing on non-nutrient agar, even after several transfers.

Most of the organisms were found to be sensitive to dihydrostreptomycin, penicillin, tetracycline and erythromycin. Only five organisms were sensitive to neomycin. Another five organisms were sensitive to polymyxin B. Most of the isolates were resistant to novobiocin and bacitracin. The resistance to heat varied among the isolates. In general, the psychrophilic group were more sensitive to high temperatures than the mesophilic group. Since none of the organisms were able to survive a temperature of $70^{\circ}C$ for 15 minutes, it is likely that resistant forms are not produced by the isolates.

The results of this study indicate that cultural and biochemical data are useful in the classification of myxobacteria. Based on the results of this work and previously published data, a new taxonomic scheme for the cytophagas has been proposed.

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INTRODUCTION

Myxobacteria or "slime bacteria" is the name given to members of the order Myxobacterales. These organisms are procaryotic, nonphotosynthetic, gram negative rods of low refractility which lack the rigid cell wall of true bacteria. Characteristic of this order is its peculiar gliding motility over solid surfaces in the absence of flagella. While this type of motility is typical of the myxobacteria, it is not unique to this group. Gliding motility also occurs in other groups of organisms including the Cyanophyceae, Beggiatoaceae and Vitreoscillaceae. While the precise mechanism of this locomotion is still unsolved, a number of suggestions have been made. These include directed slime production, cellular contraction and the contraction of rhapidosomes (Dworkin, 1966).

A characteristic property of many myxobacteria is their ability to hydrolyze complex molecules such as cellulose, chitin, agar, starch, and a variety of proteins. Cellulose decomposing myxobacteria have been reported to be among the soil organisms most active in the decomposition of cellulose (Stanier, 1942). Many of the myxobacteria also appear to live in close association with other microorganisms. In fact, some have been shown to digest both

living and dead bacterial and fungal cells. Because of these properties, myxobacteria are also considered to play an important role in the biological equilibrium of the soil.

Among the aquatic myxobacteria, those which are pathogenic to fish have been studied most extensively. In 1944 Ordal and Rucker (1944) isolated an organism from sockeye salmon exhibiting the symptoms of columnaris disease. This organism was designated Chondrococcus columnaris and has been shown to be responsible for serious losses among natural and hatchery populations of fishes. Another myxobacterium, Cytophaga psychrophila, is the causative agent of bacterial cold water disease of fish. This organism is an important agent of disease in the hatchery and has caused serious mortalities in some populations of salmon.

The higher myxobacteria undergo two definite stages as a part of their life cycle. These include a vegetative stage in which the organism is a flexible rod exhibiting gliding motility. The second stage is the fruiting body. These structures are formed by the aggregation of thousands of vegetative cells into an organized body. In some cases the fruiting bodies are simple mounds of cells, in other instances they are elaborately sculptured structures. Within the fruiting body some of the vegetative rods enter a dormant stage by conversion to refractile, spherical to rod-shaped microcysts. With the exception of the family Cytophagaceae, all members of the

order Myxobacterales form microcysts.

The non-fruiting myxobacteria are placed in two groups, Sporocytophaga and Cytophaga (Stanier, 1940). The Sporocytophaga group produces microcysts among the vegetative cells without the production of fruiting bodies. This genus is placed in the family Myxococcaceae. Myxobacteria which form neither fruiting bodies nor microcysts are placed in the family Cytophagaceae.

Although the myxobacteria were described some time ago, there is still little information about the group. While considerable amount of work has been done with microorganisms of medical and industrial importance, little has been done with many of the morphologically unusual bacteria. Only recently the first cases of pathogenic anaerobic myxobacteria in humans were reported (Graf, 1961). One reason for the apparent lag of interest in the myxobacteria may be due to the fact that pure culture study was hampered by the lack of suitable culture media. The peculiar morphological traits of the myxobacteria may also have contributed to the lack of interest in the group since they aroused the curiosity primarily of the morphologist rather than the physiologist. This latter fact is evidenced by the predominantly morphological classification of the group and by the absence of biochemical and physiological differentiation of all but a few species. A recent attempt has been made to classify the organisms according to the structure of their genetic components

(Johnson and Ordal, 1966). Studies indicate that the DNA base composition of Cytophaga and Sporocytophaga is between 32-42% guanine plus cytosine while that of the fruiting myxobacteria, Polyangium and Myxococcus, is between 60-70% guanine plus cytosine. On this basis a reclassification of Cytophaga and Sporocytophaga has been suggested (Starr and Sherman, 1965).

The myxobacteria closely resemble the vegetative cells of rod shaped, true bacteria, but can be distinguished by their typical gliding movement and the lack of a rigid cell wall. The production of fruiting bodies and microcysts also sets them apart from eubacteria. Thus no clear relationship between true bacteria and myxobacteria is evident. The involved life cycle of many myxobacteria is similar to that of certain amoeba, but there is a great difference between the two groups with respect to vegetative morphology. Similarity in appearance and method of locomotion had also led certain writers to consider a relationship between myxobacteria and blue green algae. It is on this basis that some investigators feel that myxobacteria should be reclassified.

The family Cytophagaceae, with which this paper is concerned, differs from other families of the order by its lack of a fruiting body and microcysts. To date about 30 species have been described, 17 of which were isolated from the soil or wood and 10 of which were isolated from the marine environment. This family is divided into

species on the basis of morphological features, mode of locomotion, habitat, biochemical reactions and pigment production. The majority of the work on myxobacteria has been carried out on terrestrial isolates and little work on aquatic forms has been done. Although fresh water cytophaga occur, they are not included at present in Bergey's Manual of Determinative Bacteriology, 7th edition (1957).

While the definition of the genus Cytophaga proposed by Stanier (1942) has been accepted by most workers, the problem of the classification of this group has not been completely solved (Stanier, 1947; 1957). This is particularly true of the fresh water cytophagas where very little work has been done. The present study describes an attempt to classify a group of fresh water cytophagas using physiological and cultural characteristics as well as morphology. It is hoped that this work will contribute to the taxonomy and general knowledge of this group of organisms.

HISTORICAL REVIEW

Thaxter (1892) is credited with the first detailed description of myxobacterial species. He reported on a fungus-like growth found on decaying wood and fungi in New England and some southern states. Later this growth was found to be composed of bacterial-like organisms. Thaxter recognized that the myxobacteria constituted a distinct and large assemblage of bacteria which warranted their separation from other orders. He assigned the organisms to a new order, the Schizomycetes and proposed the name Myxobacteriaceae for this group. A number of characteristics of these bacterial-like organisms was noted by Thaxter which set them apart from other organisms of the class Schizomycetes. First, the life history was more complex than that of other bacteria and could be divided into two stages, a period of vegetation and a period of fructification. The peculiar morphology, motility and slime secretion of myxobacteria was also described. In fact, most of the characteristics of myxobacteria recognized today were noted and reported by Thaxter in 1892.

The first detailed taxonomic study of the myxobacteria was carried out by Jahn (Stanier, 1957). Four of the five families recognized in the current edition of Bergey's Manual of Determinative Bacteriology, 7th edition, were proposed. These include the

families Archangiaceae, Sorgangiaceae, Polyangiaceae and Myxococcaceae. All of the organisms in these groups produce fruiting bodies and microcysts.

The family Cytophagaceae and the genus Sporocytophaga have recently been added to the order Myxobacteriales (Stanier, 1940). Although these organisms do not form fruiting bodies, Stanier believed properties of the vegetative cell to be more important in identifying the myxobacteria. Thus the order Myxobacterales was redefined (Stanier and Van niel, 1941) on the basis of vegetative morphology emphasizing differences between myxobacteria and eubacteria. The most recent classification, as proposed by Stanier, is now listed in Bergey's Manual of Determinative Bacteriology (7th edition).

Two families of myxobacteria, Cytophagaceae and Myxococcaceae include species which are pathogenic to fish. The first myxobacterium found to be a fish pathogen was Chondrococcus columnaris, the etiological agent of columnaris disease. This disease was first reported by Davis in 1923; however, since his attempts to cultivate the organism were unsuccessful, he failed to recognize the organism as a myxobacterium. Characteristic of the organism was the development of columnar like masses of bacterial cells on bits of fish tissue taken from a lesion and placed in a wet mount. Because of this property, Davis proposed the name

Bacillus coumnaris for the organisms.

Ordal and Rucker (1944) were the first to isolate the etiological agent of columnaris disease in pure culture and positively identify it as a fresh water myxobacterium. On the basis of the shape of the microcysts, the species was assigned to the family Myxococcaceae of the order Myxobacterales. Because the fruiting bodies on agar were non-deliquescent and were surrounded by a firm membrane, the organism was placed in the genus Chondrococcus. The specific name proposed by Davis was retained, and the organism was named Chondrococcus columnaris. The morphology of the cells of this organism, the formation of columnar masses in wet mount and the type of lesions observed on infected fish were very similar to those reported earlier by Davis.

In 1945 Ganbjobst (1945) isolated a myxobacterium from infected bullheads which was similar to the organism described by Ordal and Rucker. Involution forms were noted in pure cultures of the organism; however, neither microcysts nor fruiting bodies were observed. For this reason Garnjobst named the organism Cytophaga columnaris. Apparently the only difference between Chondrococcus columnaris and Cytophaga columnaris is the absence of fruiting bodies and microcysts in the latter species. Dworkin (1966) pointed out that the two organisms may be the same, since repeated laboratory cultivation could result in a loss of the capacity to produce

fruiting bodies or microcysts.

Since the original description of the disease by Davis (1923), there have been a number of reports describing outbreaks of columnaris disease in both warm and cold water species of fish (Nigrilli, 1943; Ordal and Rucker, 1944; Garnjobst, 1945; Nigrilli and Hutner, 1945; Borg, 1960; and Rucker, Earp and Ordal, 1953). These reports all deal with similar organisms, which have been designated variously Bacillus columnaris, Chondrococcus columnaris and Cytophaga columnaris.

Another fish disease due to a myxobacterium was described by Borg (1960). This disease occurs in young salmon and trout during the spring, when water temperatures are low. The etiological agent of this disease was named Cytophaga psychrophila. This was the first time a fresh water cytophaga had been found, if one assumes that the species reported by Garnjobst was in reality a strain of Chondrococcus columnaris which had lost its ability to produce microcysts and fruiting bodies.

Three different groups of Cytophaga psychrophila were recognized by Borg. The first grew slowly at 20 to 22°C and formed non-spreading colonies. Organisms in this group failed to hydrolyze starch and gelatin and did not reduce nitrate. The second group grew rapidly at 20 to 23°C, hydrolyzed starch and gelatin, but did not reduce nitrate. The third group was distinguished from the

second by the fact that nitrate was reduced by these organisms.

In 1963 Ordal and Pacha (1963) described the occurrence of strains of Cytophaga psychrophila, identifiable as such by serological methods, which cause infection in young chinook and sockeye salmon. Mutants of Cytophaga psychorophila capable of growing at higher temperature were also reported by these workers.

Certain other myxobacteria of limited pathogenicity, such as those which cause gill disease, were mentioned by Rucker, Earp and Ordal (1953) in their review on infectious diseases of Pacific salmon. These workers also noted that large numbers of saprophytic and parasitic myxobacteria occur in fresh water and on the surface of fish.

In 1961 Anderson and Ordal (1961) described a nonpathogenic fresh water Cytophaga which could grow anaerobically and was able to ferment carbohydrates. During the course of their study, seventy cultures of Cytophaga were isolated from fish and twenty three were found to be fermentative. Three strains of fermentative Cytophaga isolated from widely separate sources and at different times were studied by these workers, with the result that a new species of saprophytic myxobacteria, Cytophaga succinicans, was proposed.

Except for the studies carried out by Anderson and Ordal (1961) very little work has been done on the fresh water

myxobacteria. The fact that large numbers of saprophytic myxobacteria exist in water and on the surface of fish often complicates studies on the pathogenic species. Adequate taxonomic studies on the saprophytic forms are needed to permit them to be easily differentiated from the pathogenic species. It is hoped that the present investigation will partially fill this need.

MATERIALS AND METHODS

At least 90 different cultural, morphological and biochemical reactions were used to examine each organism isolated and used in this study. The tests were repeated or carried out in duplicate.

History of Isolates

Forty-one myxobacterial isolates were studied. The organisms had been isolated over a period of years by Dr. R. E. Pacha from fish taken at various locations in the Pacific Northwest. The cultures had been preserved by lyophilization and were available for study. Table 1 provides a list of the organisms studied, together with the date of isolation, source and fish host.

Maintenance of Cultures

Cytophaga medium was used for liquid cultures, for routine plating, for growing cells for various studies, and for maintaining stock cultures. This medium consists of tryptone, 0.05%; yeast extract, 0.05%; beef extract, 0.02%; and sodium acetate, 0.02%; adjusted to pH 7 to 7.3.

Stock cultures were maintained by serial transfer in Cytophaga medium containing 0.4% Difco agar. The stock cultures are incubated at 18°C for two to five days and stored at 5°C until used.

Table 1. History of isolates.

Strain no.	Date of Isolates	Sources	Fish Host
1	March, 1965	Alsea Salmon Hatchery	Silver Salmon
2	March, 1965	Alsea Salmon Hatchery	Silver Salmon
3	March, 1965	Alsea Salmon Hatchery	Silver Salmon
4	March, 1965	Alsea Salmon Hatchery	Silver Salmon
5	Dec., 1964	Alsea Trout Hatchery	Rainbow Trout
6	May, 1963	Alsea Salmon Hatchery	Silver Salmon
7	May, 1964	Marion Forks Hatchery	Silver Salmon
8	March, 1965	Wizard Falls Hatchery	Atlantic Salmon
9	March, 1965	Sandy Hatchery	Silver Salmon
10	March, 1965	Siletz Fish Hatchery	Silver Salmon
11	Oct., 1963	Chilko Lake, British Columbia	Sockeye Salmon
12	April, 1963	Yakima R. Prosser, Wash.	Sucker
13	May, 1963	Hanford Slough, Richland, Wash.	Bass
14	May, 1964	Chilko Lake, British Columbia	Sockeye Salmon
15	May, 1964	Chilko Lake, British Columbia	Sockeye Salmon
16	March, 1965	Wizard Falls Fish Hatchery	Atlantic Salmon
17	Jan., 1965	OSU	Catfish
18	March, 1965	Siletz Fish Hatchery	Silver Salmon
19	March, 1965	Siletz Fish Hatchery	Silver Salmon
20	March, 1965	Siletz Fish Hatchery	Silver Salmon
21	March, 1965	Siletz Fish Hatchery	Silver Salmon
22	April, 1964	Snake River Ice Harbor Dam	Chinook Salmon
23	May, 1964	Snake River Ice Harbor Dam	Chinook Salmon
24	May, 1964	Chilko Lake, British Columbia	Sockeye Salmon
25	May, 1964	Chilko Lake, British Columbia	Sockeye Salmon
26	May, 1964	Chilko Lake, British Columbia	Sockeye Salmon
27	May, 1964	Chilko Lake, British Columbia	Sockeye Salmon
28	May, 1964	Chilko Lake, British Columbia	Sockeye Salmon
29	April, 1963	Chilko Lake, British Columbia	Carp
30	May, 1963	Hanford Slough, Richland, Wash.	Sucker
31	May, 1963	Hanford Slough, Richland, Wash.	Sucker
32	May, 1963	Hanford Slough, Richland, Wash.	Side Carp
33	May, 1964	Chilko Lake, British Columbia	Sockeye Salmon
34	April, 1964	Columbia R., Rock Island Dam	Whitefish
35	April, 1964	Columbia R., Rock Island Dam	Sunfish
36	April, 1964	Columbia R., Rock Island Dam	Sucker
37	April, 1964	Columbia R., Rock Island Dam	Sucker
38	May, 1964	Snake River Ice Harbor Dam	Sucker
39	May, 1964	Yakima River, Prosser, Wash.	Sucker
40	July, 1964	Columbia R. Rock Island Dam	Sockeye Salmon
41	May, 1964	Chilko Lake, British Columbia	Whitefish

Every thirty days cultures were transferred to new Cytophaga agar deeps.

Morphological Characteristics

The media used to examine morphological characteristics of the isolates were Cytophaga broth, Cytophaga agar, peptonized milk agar (PMA) (Jeffers, 1964) and 1/10 Cytophaga-peptonized milk agar (CPM), a medium developed by Dr. R. E. Pacha at Oregon State University. The composition of CPM is as follows: tryptone, 0.005%; yeast extract, 0.005%; beef extract, 0.002%; sodium acetate, 0.002%; bacto peptonized milk, 0.05%; and Bacto agar, 1.5%.

Cell Morphology

Air fixed smears were stained by Kopelloff's modification of the gram stain and examined under the light microscope for gram reaction. Cells from young Cytophaga broth at 18°C were used.

Cell size and morphology were determined by examination of wet mounts using the phase contrast microscope. An eye-piece micrometer was used for the size determinations.

The occurrence of gliding motility was determined using a procedure similar to that described by Stanier (1947). This procedure consists of placing a freshly prepared small agar square on a

sterile slide and inoculating the center of the agar block with a small number of cells. A cover glass is then placed on the top of the inoculum. Typical myxobacterial gliding motility can be seen after 8 to 32 hours using the oil immersion lens of a phase contrast microscope. Gliding motility can also be observed by examining the edge of a colony on Cytophaga agar, PMA or CPM with the high dry objective of a phase contrast microscope.

Colony Morphology

Colony morphology was observed on Cytophaga agar, PMA and CPM. The cultures were incubated 12 to 72 hours at 18°C before examination. Colony edges were examined either with phase contrast microscopy or by the agar fixation procedure described by Robinow (1944).

Fruiting Bodies

The ability of the organisms to produce fruiting bodies was tested using fish tissue submerged in water. The fish tissue was autoclaved by allowing the temperature to reach 110°C and then turning off the autoclave. Small pieces of the autoclaved tissue were inoculated and placed either in a petri dish with 15 ml of sterile tap water or in a 250 ml flask containing 25 ml of sterile tap water. The preparations were incubated at 18°C and examined

at regular intervals for the presence of fruiting bodies.

Environmental and Cultural Characteristics

The environmental and cultural characteristics of the organisms were tested, in most instances using Cytophaga broth as a basal medium. Unless otherwise stated, the cultures were incubated at 18°C.

Anaerobic Growth

The method of Anderson and Ordal (1961) was followed to test for anaerobic growth. The medium used consisted of peptone, 0.18%; yeast extract, 0.09%; beef extract, 0.09%; glucose, 0.9%; $NaHCO_3$, 0.18%. The glucose and sodium bicarbonate were filter sterilized and added to the basal medium aseptically. Tubes containing 10 ml of this medium were inoculated with two drops of Cytophaga broth culture and layered with sterile vaspar to exclude air. Turbidity was determined visually.

Resistance Tests

Sensitivity to antibiotics was tested using erythromycin, 2 mg; dihydrostreptomycin, 2 mg; bacitracin, 2 units; neomycin, 5 mg; novobiocin, 5 mg; tetracycline, 5 mg; polymyxin B, 50 units; and penicillin, 2 mg. Sensitivity discs (Difco) of the antibiotics were

used on Cytophaga agar after seeding with organisms. Inhibition of growth was indicated by a clear area around the discs.

Vegetative cells two to three days old were used for the inoculum in the heat survival tests at 37°C and 55°C. Two drops of young Cytophaga broth cultures were added aseptically to five ml of sterile Cytophaga broth and held in a water bath for 5 minutes and 15 minutes at the desired temperature. All tubes were then placed immediately into an ice bath to cool and subsequently incubated. Growth was determined by examining the tubes for turbidity.

Thirty day old Cytophaga broth cultures were used for heat survival tests at 50°C and at 70°C. After exposure to the desired temperature for either 5 or 15 minutes, aliquots were inoculated onto Cytophaga agar plates. Colony development was then checked after five days incubation.

Biochemical Characteristics

Carbohydrate Utilization

Acid production from D-glucose, D-galactose, sucrose, lactose, maltose and mannitol was examined using a modification of Hugh and Leifson procedure (1953). The medium consisted of peptone, 0.2%; K_2HPO_4, 0.03%; agar, 0.3%; bromothymol blue 0.0015%; filter sterile carbohydrate, 0.5%. All carbohydrates were

filter sterilized and added aseptically to the sterilized basal medium at a concentration of 0.5%. Final pH of all test medium used was 6.9 to 7.2. The results of the tests were read after 24 hours and at intervals up to two weeks.

The medium described by Anderson and Ordal (1961) was used in studies concerning the carbon dioxide dependent fermentation of glucose except that bromothymol blue was added. A color change of green to yellow indicates acid production.

Utilization of Macromolecules

The basal medium used in these tests consisted of Cytophaga agar unless otherwise stated.

Starch hydrolysis was detected using a medium containing 0.2% (w/v) soluble potato starch. After two to three days incubation the plates were flooded with an iodine solution. Colonies with the ability to hydrolyze starch produced clear areas in an otherwise blue medium.

Tributyrin hydrolysis was noted by clearing produced on a medium containing 0.2% (v/v) tributyrin. Plates were read after 48 hours to two weeks incubation.

Gelatinase production was tested using basal medium supplemented with 0.4% (w/v) gelatin. After two to three days of growth the plates were flooded with acid mercuric chloride (Frazier, 1926)

to show areas of gelatin liquefaction.

Casein hydrolysis was determined using a medium containing 2% (v/v) skimmed milk. After 24 to 48 hours of growth, the plates were examined for clear areas of hydrolysis in an otherwise opaque medium.

Hydrolysis of aesculin was checked using a medium containing peptone, 1.0%; aesculin, 0.1%; ferric ammonium citrate, 0.05%; and Bacto agar, 1.5%. The ability to split aesculin was demonstrated by the formation of a black precipitate around the colonies within 7 days (Sneath, 1956).

Chitin decomposition was tested using a suspension of chitin prepared according to the procedure described by Stanier (1947). The stock chitin suspension was prepared in the cold ($4^{o}C$) by dissolving 10 g of unbleached chitin in 200 ml of 50% sulfuric acid. This solution was filtered through six layers of gauze and diluted 15 times in distilled water. The white sediment of chitin which formed was collected by centrifugation and washed with distilled water until the pH was neutral. Five ml of agar containing peptone, 0.1%; chitin, 0.5%; and Difco agar, 1.0% were overlayed on non-nutrient agar plates and allowed to dry at $37^{o}C$ for one day. Cultures to be tested were spotted onto the surface of the overlay agar and hydrolysis was detected by the dissolution of chitin around the areas of growth.

The method of Emerson and Weiser (1963) was used to detect cellulose digestion. The mineral salts medium devised by Stanier (1942) was supplemented with 1.5% agar and used as a basal agar. This basal medium was inoculated with the test organisms and overlayed with 5 ml of 1.5% agar containing peptone, 0.1% and carboxymethylcellulose, 1.0%. The plates were examined once every two days for a month for depressions in the overlay surrounding the colonies.

Miscellaneous Physiological Studies

Urea breakdown within two days was checked using Christensen (1946) medium in liquid form. The medium was filter sterilized and distributed to sterile tubes aseptically.

Lysine decarboylase activity was tested by using a modification of the medium recommended by Calquist (1956). The medium employed contained pancreatic digest of casein, 1.5%; K_2HPO_4, 0.1%; filter sterilized glucose, 0.1%; and adjusted to pH 7.2. Five milliliter quantities of this broth containing 0.5% lysine were added to screw capped tubes and inoculated. Control tubes of the basal medium alone without the addition of lysine were also inoculated. After growth had occurred (2 to 5 days), one milliliter of 4N-NaOH was added and mixed followed by two milliliter of $CHCl_3$. The tubes were shaken vigorously and 0.5 ml of the chloroform

extract was removed and mixed with 0.5 ml of 1% ninhydrin in chloroform. This mixture was observed for 4 minutes at room temperature. A positive reaction is evidenced by the appearance of a deep purple color.

A modification of the procedure described by Niven (1942) was used to test for the production of ammonia from arginine. The medium consisted of casitone, 1.5%; dibasic potassium phosphate, 0.1%; glucose, 0.1%; L-arginine monohydrochloride, 0.5%; final pH adjusted to 7.2. The presence of ammonia was detected after two to five days growth with Nessler's reagent.

Tyrosine breakdown was measured by the disappearance of tyrosine (0.5% (w/v) suspended in Cytophaga agar medium) within two weeks (Park, 1961).

The ability to reduce nitrates was examined using a medium containing peptone, 0.5%; beef extract, 0.3%; potassium nitrate, 0.1%. The organisms were inoculated into tubes of the nitrate test medium containing a Durham vial and incubated for 1 to 3 days. The presence of nitrite was detected by the addition of equal volumes of sulfanilic acid and dimethyl-alpha-naphthylamine test reagents to the cultures. Positive tests are indicated by the development of a red color within a few minutes. The results of negative tests were confirmed by the addition of a small amount of zinc dust. The absence of a red color following the addition of zinc together

with the presence of gas in the Durham tube indicated the reduction of nitrate to free nitrogen or ammonia.

The following tests were carried out using procedures listed in the Manual of Microbiological Methods (1957); indol formation, Voges-Prokauer, and methyl red tests. Hydrogen sulfide production was measured by the procedure listed in the Manual of Microbiological Methods except that 0.4% tryptone broth was used as a growth media.

The presence of catalase was determined by mixing a loopfull of organisms grown on a Cytophaga agar plate in a drop of hydrogen peroxide solution (2%). Observations were made for gas evolution.

The cytochrome oxidase test of Gaby and Hadley (1957) was used to determine the presence of an oxidase. Two tenths ml of 1% alpha-napthol in 95% ethanol and 0.3 ml of 1% aqueous solution of para-amino-dimethylaniline oxalate were added to Cytophaga broth cultures. Appearance of a blue color within two minutes after shaking indicates the presence of cytochrome oxidase. Tests for cytochrome oxidase were also carried out on colonies grown on Cytophaga plates. For this test equal amounts of alpha-naphthol and para-amino dimethyl aniline oxalate reagents were mixed and several drops added to colonies on the plate. Colonies showing a positive reaction turned blue within one minute.

Nutritional Studies

The ability to use glucose as a sole source of carbon was tested using Stanier's mineral basal medium (Stanier, 1942) supplemented with 0.006M K_2HPO_4 and filter sterilized glucose at a concentration of 0.5%.

The utilization of citrate as a sole source of carbon was tested using Koser's citrate medium (Koser, 1923).

Utilization of glycine, alanine, arginine, asparagine, leucine, histidine, methionine, tyrosine and glutamic acid as sole carbon sources was tested. The amino acids were supplied in a concentration of 0.5% (w/v) in Stanier mineral base medium (Stanier, 1942).

Lytic Properties

Several gram positive and gram negative organisms were used to check the ability of the myxobacteria to lyse bacterial cells. Bacterial cell agar was prepared using washed cell suspensions. The growth obtained from 250 ml of nutrient broth was washed three times in distilled water, suspended in 160 ml of distilled water containing 1.5% agar and autoclaved at 15 pounds pressure for 45 minutes.

To test for lytic activity, non-nutrient agar plates were overlayed with 5 ml of bacterial cell agar and incubated. The plates

were checked for evidence of cell lysis for periods up to 5 days. Test eubacterial cultures included: Corynebacterium hoffmanii, Alcaligenes viscolactis, Proteus vulgaris, Serretia marcescens, Pseudomonas fluorescens, Pseudomonas aeruginosa, Aerobacter aerogenes, Escherichia coli, Streptococcus faecalis, Staphylococcus aureus, Sarcina urea, Mycobacterium smegmatis, Bacillus megatherium, and Bacillus subtilis.

RESULTS

Cell Morphology

The vegetative cells correspond to the classical definition of myxobacterial cells. The organisms were gram negative, slender, flexible rods with round ends. When measured with an eyepiece micrometer using phase-contrast microscopy, young vegetative cells of most of the organisms averaged 5 to 7 μ in length and 0.3 to 0.4 μ in width. In general, cells from young broth cultures were found to be longer than those taken from agar plates.

The dimensions of the cells were found to vary with the age of the culture. As the age of the culture increased, the organisms became shorter and increased in width. The dimensions of the older cells averaged 2 to 4 μ in length and 0.5 to 0.8 μ in width. The degree of flexibility of the rods decreased with age of cultures and in older cultures the organisms appeared to be as rigid as many of the eubacteria. Changes in cell morphology similar to those described in this study were noted by Stanier (1947).

In addition to the effect of age on cell size, cell form or shape was also affected by the age of the culture. Numerous involution forms were noted in older cultures. These bizarre structures consisted of branched, curved, coiled and ring or oval forms, which vary considerably in shape or size. Involution forms developed more

readily in broth cultures than on agar plates. Nutrient concentration also seemed to have an effect on the appearance of these structures in cultures. In rich media, such as litmus milk, tryptone broth or nutrient broth many more involution forms were noted than in more dilute media such as Cytophaga medium and peptonized milk medium. These bizarre forms began to appear in rich media after 24 hours incubation; after one month, virtually all the cells had been converted to involution forms. In contrast, only 10 to 30% of the cells grown in Cytophaga broth had been converted to involution forms after a one month period of incubation. Apparently, development of these degenerated stages was an irreversible process since involution forms could not be converted back to vegetative cells.

The development of these bizarre degenerative stages in myxobacterial cultures is not a new observation. Garnjobst (1945) described a degenerative process, which was characterized by the coiling of the rods into simple or complex rings which finally disintegrated. Borg (1960) also described the development of bizarre structures in broth cultures as a degenerative process. According to Borg, cells first became granular, then extruded a barely visible bubble at one point in the cell. This process was followed by complete disintegration. Borg indicated that lack of oxygen may be the factor responsible for the development of these involution forms since the restriction of oxygen by the addition of a cover glass

increased the number of ring forms in a preparation.

Motility

Stanier (1940) described myxobacterial motion as follows: "It consists of slow, even gliding in one direction with none of the side to side movements or rapid directional changes associated with the motility of the flagellated organisms." Typical myxobacterial motility was easily detected by examining young vegetative cells by means of phase contrast microscopy. All the organisms studied in this investigation showed typical active gliding motility. The rate of forward motion has been measured by a number of investigators and has been found to be as much as 15 μ per minute (Stanier, 1940). As cultures aged the cells became less active with regard to gliding motility but still maintained the characteristic flexing or swinging movements noted in myxobacterial cultures. This latter type of motility is characterized by one end of the vegetative cell attaching to the surface of the glass and the other end flipping up and oscillating back and forth.

Although myxobacterial motility has been described on numerous occasions, there is still no adequate explanation for this type of motion. The organisms lack flagella, characteristic of the true bacteria, and pseudopodial and cilia modes of locomotion characteristic of the protozoa. No attempt was made in the study to determine

mechanisms of locomotion of these organisms.

Colony Morphology

On Cytophaga agar the majority of organisms studied in the investigation produced typical swarming colonies of myxobacteria. The colonies produced were yellow-orange in color, thin, flat, and spreading with irregular edges (Figures 1, 2 and 3). When viewed by reflected light, the colonies appeared to be iridescent. The edges of very young colonies were composed of a thin layer of cells. These edges were invisible to the naked eye and only apparent when viewed with reflected light under the microscope. This characteristic could be demonstrated very well by examining colonies with phase contrast microscopy, or in a habit pattern prepared by fixation through the agar with Bouin's fixative and staining the cells either with giemsa stain or 0.05% crystal violet.

A few of the test organisms showed considerable variation in the colony types produced. In some instances the organisms produced flat, spreading colonies characteristic of the myxobacteria, and in other instances deeply colored colonies with entire edges were produced. Figure 4 is a photograph of one of these non-spreading colony types. The fact that colony type varied considerably with some of the organisms suggested that either the culture medium or environmental factors might have a considerable influence on colony

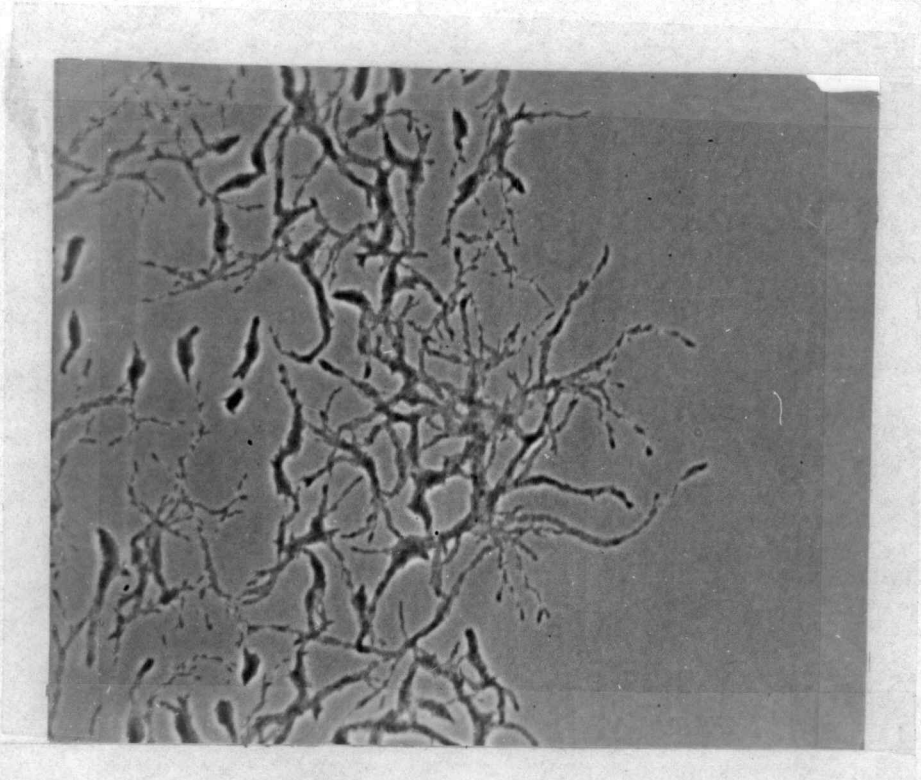

Figure 1. Phase contrast micrograph of the margin of 12 hour old colony strain 38 on Cytophaga agar. 450x.

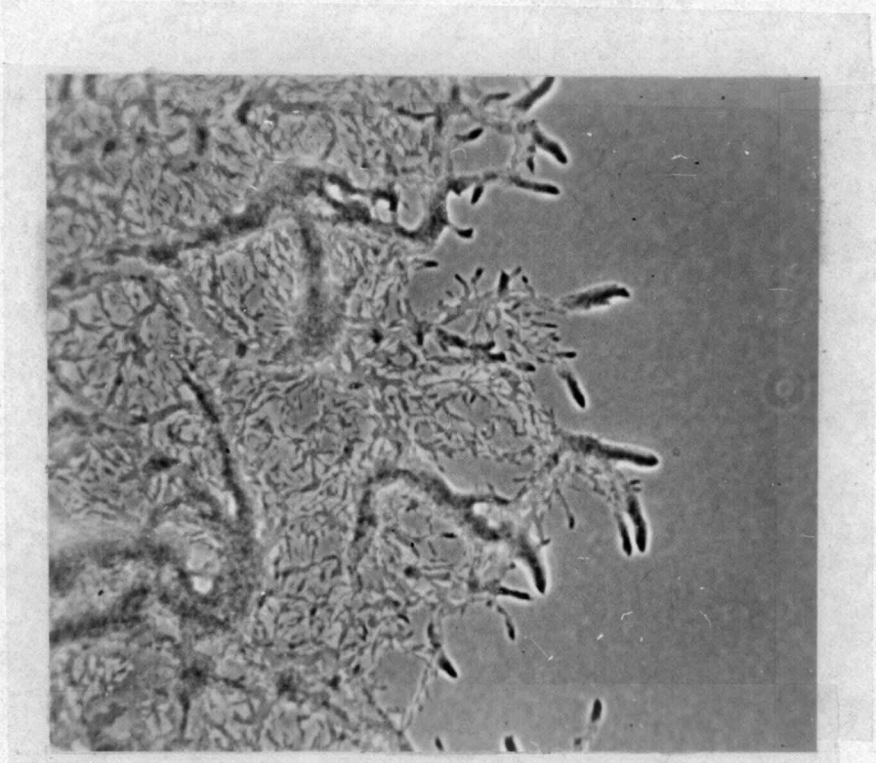

Figure 2. Phase contrast micrograph of the margin of 12 hour old colony strain 39 on Cytophaga agar. 450x.

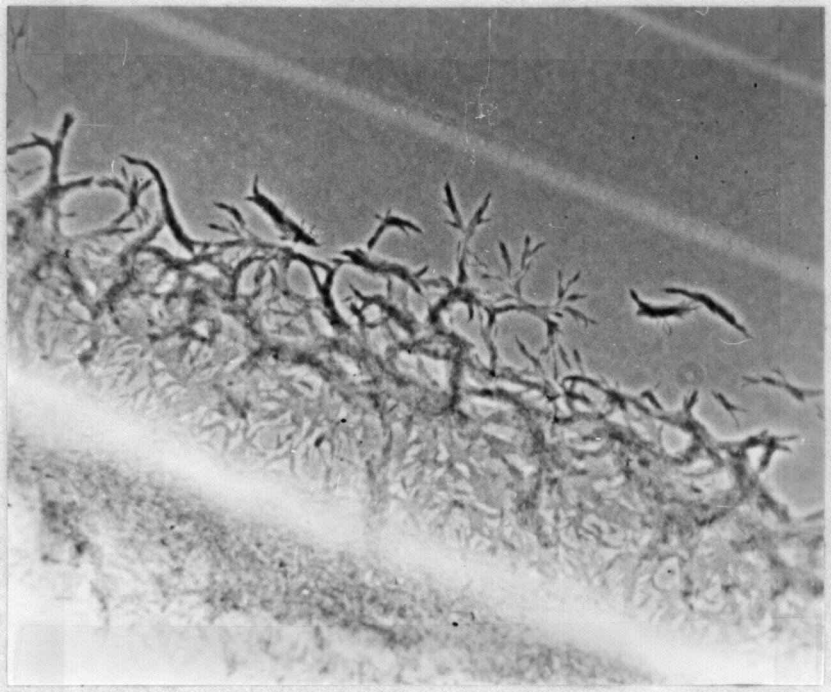

Figure 3. Phase contrast micrograph of the margin of 12 hour old colony strain 40 on Cytophaga agar. Magnification. 450x.

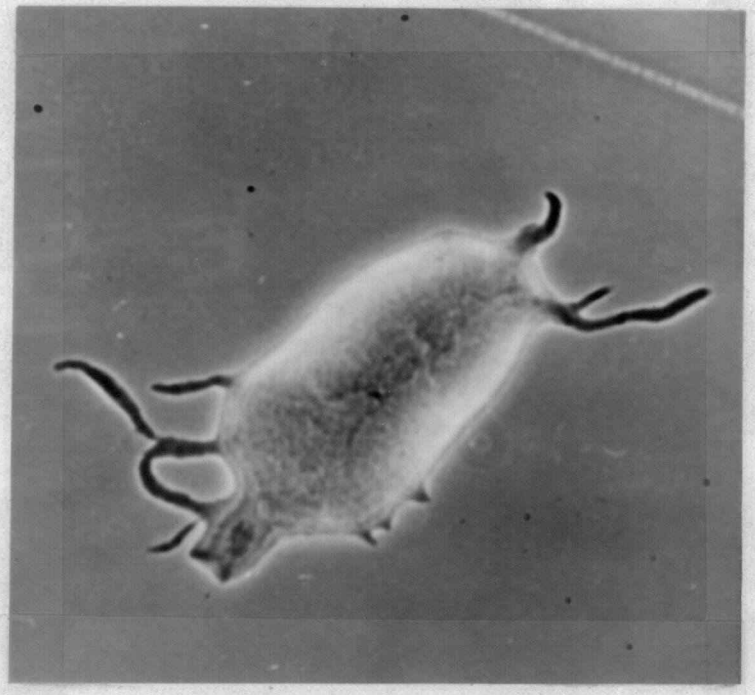

Figure 4. Phase contrast micrograph of the margin of nonspreading 24 hour colony strain 24 on Cytophaga agar. Magnification. 450x.

morphology. For this reason studies were carried out to determine the effect of a variety of media on colony appearance.

To test the effect of the medium on colony morphology, the test organisms were inoculated onto a variety of rich media and onto media containing very dilute nutrients. On comparatively rich medium such as nutrient agar, starch agar, gelatin agar or blood agar, the myxobacterial colonies failed to spread and were indistinguishable from typical eubacterial colonies. On a more dilute medium such as Cytophaga agar, the colonies became broad, flat and spread rapidly over the surface of the medium. On highly dilute medium such as CPM, the colonies were extremely flat and the growth was nearly invisible in many cases. Coloration of colonies on the more dilute media is also less intense than on richer media. As a result of these findings it appears that nutrient concentration has a decided effect on colony morphology. Low nutrient concentration seems to favor spreading and the production of colonies which are typical of the myxobacteria.

To test the effect of agar concentration on colony morphology, Cytophaga medium was modified by using agar concentration of 0.6%, 0.9%, 1.2%, 1.5%, and 1.8%. All of the organisms produced spreading types of colonies on a medium containing 0.6% agar. In fact on this medium the organisms tended to penetrate the agar and creep through the medium. On agar concentration of 0.9% and 1.2%

most of the organisms produced colonies which were typical of the myxobacteria. At high agar concentrations such as 1.8%, many of the organisms produced smooth, more compact colonies with a limited amount of spreading.

It thus appears that in some cases at least, colony morphology can be varied at will by adjusting the nutrient concentrations and the agar concentrations of the media employed. Low concentrations of nutrient and agar in a medium tend to favor the development of typical myxobacterial colonies. These findings tend to confirm many of the observations of Stanier (1947) on various aspects of myxobacterial colony development.

Fruiting Bodies and Microcysts

Since fruiting bodies and microcysts are of importance in the classification of myxobacteria, considerable effort was made to detect these structures in the organisms studied. The presence of microcysts and the absence of fruiting bodies serves to differentiate the genus Sporocytophaga from other members of the order Myxobacterales. Members of the genus Cytophaga form neither microcysts nor fruiting bodies.

Of the 41 cultures studied, only one was found to produce structures similar to fruiting bodies. These structures were produced by a strain of Chondrococcus columnaris, the etiological agent

of columnaris disease, on bits of fish tissue submerged in water. This organism was included in this study in order to compare the saprophytic species of myxobacteria with pathogenic forms. On litmus milk and tryptone broth, this strain of Chondrococcus columnaris also produced columnar structures which were similar to those noted on pieces of fish tissue. These columnar masses of cells were produced on the sides of the tubes near the top of the culture medium.

The resemblence of microcysts to the involution forms frequently encountered in myxobacterial cultures has led to conflicting opinions with regard to the presence or absence of microcysts. According to Dworkin (1966), microcysts should fulfill the following criteria: (1) They must be able to germinate; (2) They should be more resistant than vegetative cells; (3) They must appear refractile under the phase contrast microscope; (4) The morphological changes leading to formation of microcysts must be clearly distinguishable from those characteristic of spheroplast formation. On numerous occasions, cultures of various ages were examined by phase contrast microscopy for the presence of microcysts. Although involution forms were frequently present in the cultures, microcysts were never observed. These observations together with the fact that fruiting bodies were never observed, except in one instance, indicate that the majority of the organisms studied in this investigation

are members of the genus Cytophaga.

It is interesting to note that although cultures of Chrondrococcus columnaris have been reported to produce both fruiting bodies and microcysts, (Ordal and Rucker, 1944; Borg, 1960), microcysts were never observed in cultures of the organism studied in this investigation. Perhaps the cultures of Chondrococcus columnaris used in this study had lost the ability to produce microcysts as a result of mutation. Mutants of Myxococcus xanthus which have lost the ability to form microcysts have been reported by McVittie, Messik and Zahler (1962).

Cultural Characteristics

Temperature Range of Growth

The ability of strains to grow in Cytophaga broth at temperatures of 5°C, 18°C, 25°C, 30°C and 37°C was recorded after periods of incubation of one day to two weeks depending on the incubation temperature used. All strains tested grew readily at 18°C and 5°C. However, as would be expected, growth at 5°C was much slower than at higher temperatures. At temperatures above 18°C the number of organisms capable of growing was inversely proportional to increase in temperature. At 25°C all but 11 of the strains tested were able to grow and at 30°C all but 15 of the test organisms grew.

However, at 37°C, only two organisms were able to grow. A summary of these results can be seen in Table 2.

As a result of the studies on temperature range of growth, it is possible to divide the myxobacterial isolates into two groups. These two groups are defined as follows:

Psychrophilic group: Those organisms which fail to grow at 25°C.

Mesophilic group: Those organisms which grow readily at 25°C and above.

Sodium Chloride Tolerance

The tolerance to NaCl was determined by inoculating Cytophaga broth containing various amounts of NaCl and examining for growth within two weeks. The salt concentrations used were 0.0%, 0.1%, 0.25%, 0.5%, 1%, 2%, 3% and 4%. All of the organisms were able to grow in a medium containing either no salt or 0.1% salt. This indicates that NaCl is not required for the growth of these myxobacterial cultures. As salt concentration was increased above 0.1%, the number of organisms capable of growing gradually diminished. For example, in 1% NaCl only 19 of the isolated were able to grow. Only 4 of the organisms grew in 3% salt and none grew in salt at a concentration of 4%. A summary of these results is shown in Table 2.

Table 2. Temperature range of growth and NaCl tolerance of the test isolates.

	Temperature range of growth					NaCl Tolerance					
Strain	5°C	18°C	25°C	30°C	37°C	0.1%	0.25%	0.5%	1%	2%	3%
1	Gr	Gr	NG	NG	NG	Gr	NG	NG	NG	NG	NG
2	Gr	Gr	NG	NG	NG	Gr	NG	NG	NG	NG	NG
3	Gr	Gr	NG	NG	NG	Gr	NG	NG	NG	NG	NG
4	Gr	Gr	NG	NG	NG	Gr	NG	NG	NG	NG	NG
5	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	Gr
6	Gr	Gr	NG	NG	NG	Gr	NG	NG	NG	NG	NG
7	Gr	Gr	NG	NG	NG	Gr	Gr	PG	NG	NG	NG
8	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	Gr
9	Gr	Gr	NG	NG	NG	Gr	NG	NG	NG	NG	NG
10	Gr	Gr	NG	NG	NG	Gr	NG	NG	NG	NG	NG
11	Gr	Gr	Gr	Gr	Gr	Gr	Gr	NG	NG	NG	NG
12	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	NG	NG	NG
13	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	NG	NG
14	Gr	Gr	PG	NG	NG	Gr	Gr	NG	NG	NG	NG
15	Gr	Gr	NG	NG	NG	Gr	NG	NG	NG	NG	NG
16	Gr	Gr	Gr	NG	NG	Gr	Gr	Gr	Gr	Gr	NG
17	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	Gr
18	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	NG	NG
19	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	NG
20	Gr	Gr	Gr	NG	NG	Gr	Gr	Gr	Gr	Gr	NG
21	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	NG	NG	NG
22	Gr	Gr	Gr	Gr	Gr	Gr	Gr	Gr	NG	NG	NG
23	Gr	Gr	NG	NG	NG	Gr	Gr	NG	NG	NG	NG
24	Gr	Gr	Gr	NG	NG	Gr	NG	NG	NG	NG	NG
25	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	NG
26	Gr	Gr	NG	NG	NG	Gr	Gr	NG	NG	NG	NG
27	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	PG
28	Gr	Gr	Gr	Gr	NG	Gr	Gr	NG	NG	NG	NG
29	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	NG
30	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	NG
31	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	NG
32	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	PG	NG
33	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	NG
34	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	NG
35	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	Gr	NG
36	Gr	Gr	NG	NG	NG	Gr	NG	NG	NG	NG	NG
37	Gr	Gr	Gr	Gr	NG	Gr	Gr	NG	NG	NG	NG
38	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	NG	NG
39	Gr	Gr	Gr	Gr	NG	Gr	Gr	Gr	Gr	NG	NG
40	Gr	Gr	Gr	Gr	NG	Gr	Gr	NG	NG	NG	NG
41	Gr	Gr	Gr	NG	NG	Gr	PG	NG	NG	NG	NG

Gr: Growth
NG: No Growth
PG: Poor Growth

Anaerobic Growth

The ability of the isolates to grow under anaerobic conditions was tested using the procedure of Anderson and Ordal (1961). As shown in Table 3, seven of the isolates were capable of growing anaerobically. This finding indicates that these seven organisms are facultative anaerobes, since they grow both in the presence and absence of oxygen.

pH Range of Growth

Cytophaga broth containing 0.0006 M KH_2PO_4 and 0.0006 M K_2HPO_4 was used to determine the effect of pH on growth. The broth was adjusted to pH values ranging from 5 to 9. The cultures were incubated at 18°C and examined at intervals for the presence or absence of growth. The results of these studies are presented in Table 3.

It can be seen from the data presented in Table 3 that all of the test isolates were able to grow in a medium adjusted to pH 7. The organisms appear to be more sensitive to acid conditions than to basic conditions, since nearly half of the organisms were able to grow at a pH of 9 while none of the organisms grew at a pH of 5. The psychrophilic group of isolates were found to be extremely sensitive to pH and most of these organisms failed to grow at a pH of 6 or less.

Table 3. Effect of pH and anaerobic conditions on growth of the test organisms.

Strain	pH6	pH6.5	pH7	pH8	pH9	Anaerobic Growth
1	NG	NG	Gr	NG	NG	A
2	NG	Gr	Gr	NG	NG	A
3	NG	Gr	Gr	NG	NG	A
4	NG	Gr	Gr	NG	NG	A
5	Gr	Gr	Gr	Gr	Gr	FA
6	PG	Gr	Gr	NG	NG	A
7	NG	NG	Gr	NG	NG	A
8	Gr	Gr	Gr	Gr	Gr	A
9	NG	Gr	Gr	NG	NG	A
10	NG	Gr	Gr	NG	NG	A
11	NG	NG	Gr	NG	NG	A
12	PG	Gr	Gr	Gr	Gr	FA
13	Gr	Gr	Gr	Gr	Gr	A
14	NG	NG	Gr	NG	NG	A
15	NG	NG	Gr	NG	NG	A
16	PG	Gr	Gr	Gr	Gr	A
17	Gr	Gr	Gr	Gr	Gr	A
18	Gr	Gr	Gr	Gr	Gr	A
19	Gr	Gr	Gr	Gr	Gr	A
20	Gr	Gr	Gr	Gr	Gr	A
21	NG	NG	Gr	NG	NG	FA
22	NG	NG	Gr	NG	NG	A
23	NG	NG	Gr	NG	NG	A
24	PG	Gr	Gr	NG	NG	A
25	Gr	Gr	Gr	Gr	Gr	A
26	NG	NG	Gr	NG	NG	A
27	Gr	Gr	Gr	Gr	Gr	A
28	NG	NG	Gr	NG	NG	A
29	NG	Gr	Gr	Gr	Gr	FA
30	PG	Gr	Gr	Gr	Gr	A
31	Gr	Gr	Gr	Gr	Gr	FA
32	Gr	Gr	Gr	Gr	Gr	A
33	Gr	Gr	Gr	Gr	Gr	A
34	Gr	Gr	Gr	Gr	Gr	FA
35	Gr	Gr	Gr	Gr	Gr	FA
36	NG	NG	Gr	NG	NG	A
37	NG	NG	Gr	NG	NG	A
38	Gr	Gr	Gr	Gr	Gr	A
39	NG	NG	Gr	Gr	Gr	A
40	NG	NG	Gr	Gr	NG	A
41	Gr	Gr	Gr	Gr	NG	A

Gr: Growth
NG: No growth
A: Aerobic Growth
FA: Facultative Anaerobic Growth

Sensitivity to Antibacterial Agents

As shown by the data presented in Table 4, the majority of the isolates were sensitive to dihydrostreptomycin and tetracycline. About half of the organisms were sensitive to erythromycin, bacitracin, novobiocin and penicillin whereas only 5 strains were sensitive to neomycin and 5 strains to polymyxin B.

Heat Resistance

Survival in Cytophaga broth after 5 to 15 minutes exposure to temperatures of 37°C and 55°C was tested. All cultures remained viable after 5 minutes exposure at 37°C and all but two strains survived exposure to 37°C for 15 minutes. Exposure to a temperature of 55°C for 5 minutes was lethal to all but 19 strains, while exposure to this temperature for 15 minutes was lethal to all but 8 strains. From these results it is concluded that the majority of the myxobacterial isolates used in this study are not thermoduric.

As indicated by Dworkin (1966) there are a number of reports in the literature indicating that microcysts are considerably more heat resistant than vegetative cells. To test for the presence of resistance cells in the cultures of myxobacteria studied in this investigation, 30 day old cultures were exposed to a temperature of 70°C for 15 minutes. None of the organisms survived this heat

Table 4. Sensitivity of test isolates to selected antibiotics.

Strain	Antibiotic							
	erythro-mycin 2 mg	poly-myxin 50 units	baci-tracin 2 units	dihydro-streptomy-cin 2 mg	neo-mycin 5 mg	novo-biocin 5 mg	penicil-lin 2 mg	tetracy-line 5 mg
1	S	R	S	S	R	S	S	R
2	S	R	S	S	R	S	S	S
3	S	R	S	S	R	S	S	S
4	S	R	S	S	R	S	S	S
5	R	R	R	S	R	S	R	S
6	S	R	S	S	R	S	S	S
7	S	R	S	S	R	S	S	S
8	S	R	R	S	R	S	S	S
9	S	R	S	S	R	S	S	S
10	S	R	S	S	R	S	S	S
11	S	R	R	S	R	S	S	S
12	S	R	R	S	S	S	S	S
13	S	R	R	S	R	S	R	S
14	S	R	S	S	R	S	S	S
15	S	R	S	S	S	S	S	S
16	S	R	R	S	S	S	R	S
17	S	R	R	S	R	S	R	R
18	R	R	R	S	R	R	R	S
19	S	R	R	S	S	S	S	S
20	R	R	R	S	R	S	S	S
21	S	R	R	S	S	S	R	S
22	R	R	R	S	R	R	R	R
23	S	R	R	S	R	R	R	R
24	S	R	R	S	R	R	R	S
25	S	R	R	S	R	R	R	S
26	S	R	R	S	R	R	S	S
27	S	R	R	S	R	R	R	S
28	S	R	R	S	R	R	R	S
29	S	R	S	R	R	R	S	S
30	R	R	R	S	R	S	R	R
31	R	R	R	S	R	S	S	R
32	R	S	R	S	R	R	R	R
33	R	S*	R	R	R	R	S	S
34	R	R*	R	S	R	S	R	S
35	R	R	R	S	R	S	S	S
36	R	S	S	S	R	R	S	S
37	S	S	S	S	R	R	S	S
38	R	R	R	S	R	R	S	S
39	R	R	R	S	R	S	R	S
40	R	R	S	S	R	S	S	S
41	R	S*	S	S	R	R	S	S

R: Resistant
S: Sensitive
* 500 unit

treatment, indicating that resistant cells are not present. A summary of the data obtained in this portion of the study is shown in Table 5.

Biochemical Characteristics

Carbohydrate Utilization

Most of the isolates are non-oxidizers and non-fermenters of carbohydrates as indicated by the fact that the majority of the organisms produce no acid when grown in the presence of these substances. Of the 41 cultures examined, 14 were found to oxidize glucose and seven were capable of fermenting this carbohydrate. Using the procedure of Anderson and Ordal, strain 34 was found to require CO_2 in order to ferment glucose. The fact that some fermentative isolates were found is of interest since there have been very few reports of these types of myxobacteria being isolated from the aquatic habitat.

The organisms which were able to ferment glucose were also tested for the ability to oxidize and ferment a number of other sugars. The carbohydrates selected for this study were galactose, lactose, maltose, mannitol, and sucrose. As shown in Table 6, four of the organisms fermented galactose, four maltose, three lactose and one organism fermented sucrose. None of the organisms tested

Table 5. Heat resistance of young and old cells of test isolates.

	Young				Old	
	37°C		55°C		50°C	70°C
	5 min.	15 min.	5 min.	15 min.	10 min.	5 min.
1	Gr	Gr	NG	NG	NG	NG
2	Gr	Gr	NG	NG	NG	NG
3	Gr	Gr	NG	NG	NG	NG
4	Gr	Gr	NG	NG	NG	NG
5	Gr	Gr	Gr	PG	Gr	NG
6	Gr	Gr	NG	NG	NG	NG
7	Gr	Gr	NG	NG	NG	NG
8	Gr	Gr	Gr	PG	NG	NG
9	Gr	Gr	NG	NG	NG	NG
10	Gr	Gr	NG	NG	NG	NG
11	Gr	Gr	PG	NG	NG	NG
12	Gr	Gr	Gr	NG	Gr	NG
13	Gr	Gr	NG	NG	Gr	NG
14	Gr	Gr	NG	NG	NG	NG
15	Gr	Gr	NG	NG	NG	NG
16	Gr	Gr	PG	NG	Gr	NG
17	Gr	Gr	Gr	Gr	Gr	NG
18	Gr	Gr	Gr	PG	Gr	NG
19	Gr	Gr	Gr	PG	Gr	NG
20	Gr	Gr	Gr	NG	NG	NG
21	Gr	PG	NG	NG	NG	NG
22	Gr	Gr	PG	NG	NG	NG
23	Gr	PG	NG	NG	NG	NG
24	Gr	PG	NG	NG	NG	NG
25	Gr	Gr	Gr	Gr	Gr	NG
26	Gr	PG	NG	NG	NG	NG
27	Gr	Gr	Gr	Gr	Gr	PG
28	Gr	Gr	NG	NG	NG	NG
29	Gr	Gr	NG	NG	NG	NG
30	Gr	Gr	PG	NG	NG	NG
31	Gr	Gr	Gr	NG	NG	NG
32	Gr	Gr	PG	NG	NG	NG
33	Gr	Gr	Gr	PG	NG	NG
34	Gr	Gr	NG	NG	NG	NG
35	Gr	Gr	Gr	NG	Gr	NG
36	PG	NG	NG	NG	NG	NG
37	Gr	Gr	NG	NG	NG	NG
38	Gr	Gr	PG	NG	NG	NG
39	Gr	Gr	Gr	NG	NG	NG
40	Gr	NG	NG	NG	NG	NG
41	Gr	PG	NG	NG	NG	NG

Gr: Growth
NG: No growth
PG: Poor growth

Table 6. Ability of test isolates to ferment or oxidize various carbohydrates.

Strain	Substrate						
	Glucose	Galactose	Lactose	Maltose	Mannitol	Sucrose	CO_2 Dependent Glucose Fermentation
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-
5	F, A	F, A	A	F, A	-	-	-
6	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-
12	F, A	F, A	F, A	F, A	-	-	-
13	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-
18	A	-	-	-	-	-	-
19	A	-	-	-	-	-	-
20	A	-	-	-	-	-	-
21	F, A	F, A	F, A	F, A	-	-	-
22	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-
25	A	-	-	-	-	-	-
26	-	-	-	-	-	-	-
27	A	-	-	-	-	-	-
28	-	-	-	-	-	-	-
29	F, A	F, A	F, A	F, A	-	F, A	-
30	-	-	-	-	-	-	-
31	F, A	A	A	A	-	-	-
32	A	-	-	-	-	-	-
33	A	-	-	-	-	-	-
34	A	-	-	-	-	-	F
35	F, A	A	A	A	-	-	-
36	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-
39	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-
41	-	-	-	-	-	-	-

F: Acid produced anaerobically

A: Acid produced aerobically

-: Growth but no acid production

was able to ferment mannitol.

No gas production was noted from any of the carbohydrates tested.

Degradation of Macromolecules

Starch hydrolysis was detected by observation of a clear zone around colonies after flooding with Gram's iodine solution. Eleven of the test organisms were negative for starch hydrolysis, the remaining organisms were positive.

Hydrolysis of tributyrin using the method of Davis and Park (1962), was checked by detecting clearing of 0.2% (v/v) tributyrin in Cytophaga agar after 48 hours to two weeks. All but five of the isolates were positive for hydrolysis of tributyrin as indicated by clearing around the colonies. Two organisms, strains number 15 and 40, grew very poorly on the tributyrin test medium.

The organisms studied in this investigation were found to be actively proteolyticly. All were capable of hydrolyzing gelatin and casein.

The ability to split aesculin was observed by the formation of a black precipitate around the colonies in the presence of ferric ammonium citrate on aesculin agar plates. Eleven of the test cultures were negative and the remainder were positive.

The decomposition of chitin was observed by examining zones

of clearing around the colonies indicated hydrolysis of the chitin. All of the organisms grew on the chitin medium and 19 of the isolates were capable of degrading this material.

The method of Emerson and Weiser (1963) was used to demonstrate cellulose degradation. A positive reaction for the digestion of carboxymethyl cellulose was indicated by the presence of a depressed area surrounding colonies after four to five days incubation. The results of these studies indicated that none of the isolates were capable of digesting carboxy-methyl cellulose. A cellulose decomposing Sporocytophaga isolated from soil was used as a positive control in this test. The degradation of macromolecules is summarized in Table 7.

Miscellaneous Physiological Studies

In an attempt to characterize the organisms further, a number of additional physiological studies were carried out. These tests included the breakdown of urea, decarboxylation of lysine and ammonia production from arginine. The entire group of test isolates were found to be inactive with regard to these tests. None of the organisms were able to produce urease, to decompose lysine to cadaverine, or to deaminate arginine.

Thirty-two of the organisms were found to decompose tyrosine. Of these, twenty three, produced a black to light brown, diffusable,

Table 7. Ability of test isolates to degrade selected macromolecules.

	Substrate						
Strain	Starch	Casein	Gelatin	Cellulose	Chitin	Aesculin	Tributyrin
1	-	-	+	-	-	-	-
2	-	+	+	-	-	-	+
3	-	+	+	-	-	-	+
4	-	+	+	-	-	-	+
5	+	+	+	-	+	+	+
6	-	+	+	-	-	-	+
7	-	+	+	-	-	-	+
8	+	+	+	-	+	+	+
9	-	+	+	-	-	-	+
10	-	+	+	-	-	-	+
11	-	+	+	-	-	-	-
12	+	+	+	-	-	+	+
13	+	+	+	-	+	+	+
14	+	+	+	-	-	+	+
15	+	+	+	-	-	-	NG
16	+	+	+	-	-	+	+
17	+	+	+	-	+	+	+
18	+	+	+	-	+	+	+
19	+	+	+	-	+	+	+
20	+	+	+	-	+	+	+
21	+	+	+	-	+	+	+
22	+	+	+	-	-	+	+
23	+	+	+	-	-	+	+
24	+	+	+	-	-	+	-
25	+	+	+	-	+	+	+
26	+	+	+	-	-	+	+
27	+	+	+	-	+	+	+
28	+	+	+	-	-	+	+
29	-	+	+	-	-	+	+
30	+	+	+	-	+	+	+
31	+	+	+	-	+	+	+
32	+	+	+	-	+	+	+
33	+	+	+	-	+	+	+
34	+	+	+	-	+	+	+
35	+	+	+	-	+	+	+
36	-	+	+	-	-	-	-
37	+	+	+	-	+	+	+
38	+	+	+	-	+	+	+
39	+	+	+	-	+	+	+
40	+	+	+	-	-	+	-
41	+	+	+	-	-	+	NG

\+ hydrolysis of macromolecule

\- growth but no hydrolysis

NG: No growth

melanin-like pigment from tyrosine. Nine of the organisms hydrolyzed tyrosine without pigment production. Evidence of hydrolysis in these cases was noted by the disappearance of tyrosine around the colonies.

Eleven of the test cultures were able to reduce nitrate and three of these organisms produced large amounts of gas after 24 hours incubation. It is of interest to note that strains #17, 25 and 27 were able to grow anaerobically in broth containing KNO_3 at a concentration of 0.1%.

None of the organisms gave positive indol on Voges Proskauer or methyl red reactions.

Hydrogen sulfide was produced from 0.4% tryptone broth by 23 of the isolates as determined using lead acetate filter paper test strips. The remainder of the isolates were not able to produce hydrogen sulfide.

Nearly all test cultures were positive for catalase. A few of the psychrophilic organisms gave a very weak or negative reaction in this test.

Organisms were also tested for cytochrome oxidase. This enzyme oxidizes dimethyl-phenylene-diamine in the presence of molecular oxygen and cytochrome C, and upon the addition of alpha naphthol, indolphenol blue is formed. Twelve organisms were cytochrome oxidase positive and the remainder were negative.

The results of the physiological studies just described are summarized in Table 8.

Nutritional Studies

The ability of the organisms to use glucose as a sole source of carbon was tested using the mineral basal medium described by Stanier (1942). None of the test isolates was able to grow in this medium indicating that the nutritional requirements could not be satisfied by the simple nutrients provided. Upon the addition of more complex nutrients such as peptone or casein hydrolysate to the basal medium, excellent growth was obtained. This indicates that the mineral salts medium employed was not toxic to the organisms.

The ability of the organisms to utilize the amino acids, glycine, alanine, arginine, asparagine, glutamic acid, leucine, histidine, methionine and tyrosine as a sole source of carbon and nitrogen was also tested. None of these amino acids supported growth of any of the organisms and thus could not satisfy the nutritional requirement of this group of aquatic myxobacteria.

When 0.1% peptone was added to the basal medium containing the amino acids, it was found that some of the media failed to support the growth of the organisms. This suggests that, at the concentration employed, certain of the amino acids have an inhibitory effect on growth. Those amino acids which supressed the growth

Table 8. Miscellaneous physiological reaction of the test isolates.

Strain	Tyrosine	Nitrate	H_2S	Catalase	Cytochrome Oxidase	Citrate
1	P	-	S	±	-	-
2	P	-	-	±	-	-
3	P	-	S	±	-	-
4	P	-	-	±	-	-
5	-	R	S	+	-	-
6	P	-	-	±	-	-
7	P	-	S	±	-	-
8	T, P	-	S	+	-	-
9	P	-	S	+	-	-
10	P	-	S	+	-	-
11	-	-	S	+	+	-
12	T	R	S	±	-	-
13	P	-	S	+	-	-
14	T, P	-	-	+	+	-
15	T, P	-	-	+	+	-
16	T, P	-	-	+	-	-
17	T, P	R, AR	S	+	-	C
18	T	-	S	+	-	-
19	T	R	S	+	-	-
20	T	R	S	+	-	-
21	-	-	S	+	-	-
22	T, P	-	-	+	+	-
23	T	-	-	+	-	-
24	T, P	-	-	+	+	-
25	-	R, AR	S	+	+	-
26	T, P	-	-	+	+	-
27	-	R, AR	S	+	+	-
28	T, P	-	S	+	+	-
29	T	-	S	+	+	-
30	T, P	-	S	+	-	-
31	-	R	S	+	-	-
32	T, P	-	S	+	-	-
33	T	-	-	+	-	-
34	-	R	S	+	-	C
35	-	R	-	+	-	-
36	T	-	-	+	+	-
37	T	-	-	+	-	C
38	-	R	-	+	-	-
39	P	-	-	+	-	-
40	T, P	-	-	+	+	-
41	T, P	-	-	+	+	-

T: hydrolysis of tyrosine
P: Black pigment produced from tyrosine
R: Nitrate reduced aerobically
AR: Nitrate reduced anaerobically
S: H_2S produced from tryptone
+: Catalase or cytochrome oxidase produced
-: Negative reaction, but growth is present. For citrate media, no growth.
C: citrate utilized

of the myxobacteria include arginine, asparagine, histidine, methionine and tryptophan.

Citrate utilization by test organisms was detected by examining tubes of Koser citrate medium for turbidity. Three organisms were able to grow in this medium and thus are able to use citrate as a sole carbon source and ammonia as a nitrogen source.

It is interesting to note that the majority of the test isolates were able to grow on 1.5% non-nutrient agar for several transfers. As would be expected the amount of growth obtained was limited. Apparently the organisms were using impurities in the agar as nutrient. No evidence of agar digestion was noted. The results of nutritional studies are given in Table 9.

Lytic Activity

A number of reports indicate that various myxobacteria are capable of lysing cells of a variety of bacteria and fungi. The ability of the myxobacteria studied in the investigation to lyse dead cells of a number of gram negative and gram positive bacteria was examined. Fourteen organisms were selected for the study and bacterial agar was prepared according to the procedures described in the section on materials and methods. A list of the organisms employed and the results of those studies are shown in Table 10.

It can be seen that all of the myxobacteria were able to lyse

Table 9. The effect of selected amino acids on growth of the test isolates.

	Substrate				
Strain	L-arginine	L-asparagine	L-histidine	Methionine	L-tryptophan
1	Gr	I	I	I	Gr
2	Gr	I	I	I	Gr
3	I	I	I	I	Gr
4	Gr	I	I	I	Gr
5	Gr	Gr	Gr	Gr	Gr
6	Gr	I	Gr	I	Gr
7	I	I	Gr	I	Gr
8	Gr	Gr	Gr	Gr	Gr
9	I	I	I	I	Gr
10	I	I	I	I	Gr
11	I	I	PG	I	I
12	I	I	Gr	I	Gr
13	Gr	Gr	Gr	I	Gr
14	Gr	I	Gr	I	Gr
15	Gr	I	Gr	I	Gr
16	Gr	I	Gr	Gr	Gr
17	Gr	Gr	Gr	Gr	Gr
18	Gr	Gr	Gr	Gr	Gr
19	Gr	I	Gr	Gr	Gr
20	Gr	Gr	Gr	Gr	Gr
21	Gr	Gr	Gr	I	Gr
22	PG	I	I	I	I
23	PG	I	I	I	I
24	Gr	I	I	I	Gr
25	Gr	I	Gr	Gr	Gr
26	Gr	Gr	I	I	Gr
27	Gr	I	Gr	Gr	Gr
28	Gr	Gr	Gr	I	Gr
29	Gr	I	Gr	Gr	Gr
30	Gr	I	Gr	Gr	Gr
31	Gr	Gr	Gr	Gr	Gr
32	Gr	Gr	Gr	Gr	Gr
33	Gr	I	Gr	I	Gr
34	Gr	Gr	Gr	PG	Gr
35	Gr	Gr	Gr	Gr	Gr
36	Gr	I	I	I	I
37	Gr	I	Gr	I	Gr
38	Gr	Gr	Gr	I	Gr
39	Gr	Gr	Gr	I	Gr
40	I	I	I	I	I
41	Gr	I	Gr	I	Gr

Gr: Growth
I: Inhibited growth
PG: Poor growth

dead cells of Serretia marcescens. The majority of the test isolates also lysed cells of Alcaligenes viscolactis, Proteus vulgaris, Pseudomonas fluorescens, Pseudomonas aeruginosa, Aerobacter aerogenes, Escherichia coli, Streptococcus feacalis, Staphylococcus aureus, Sarcina urea and Bacillus subtilis. The other bacteria used in this study were lysed by varying numbers of the myxobacteria isolates. It is of interest to note that none of the myxobacteria appear to be selective for either gram negative or gram positive organisms.

Numerical Analysis

Using 81 morphological, cultural and biochemical characteristics, the degree of similarity between the 41 test isolates was determined according to the method of Sneath (1957) and Beer et al. (1962), Smith et al. (1965).

Percentage similarity coefficients (S) were calculated for each possible pair of organisms by laying the record paper strips side by side, counting the number of times positive signs coincide (Ns) and the number of times positive and negative signs coincide (Nd), and substituting in the following formula:

$$S = \frac{Ns\ (100)}{Ns + Nd}$$

The strains were sorted manually at falling similarity levels.

Code for Test Organisms

A. *Corynebacterium hoffmanii*
B. *Alcaligenes viscolactis*
C. *Proteus vulgaris*
D. *Serretia marcescens*
E. *Pseudomonas fluorescens*
F. *Pseudomonas aeruginosa*
G. *Aerobacter aerogenes*
H. *Escherichia coli*
I. *Streptococcus faecalis*
J. *Staphylococcus aureus*
K. *Sarcina urea*
L. *Mycobacterium smegmatis*
M. *Bacillus megatherium*
N. *Bacillus subtilis*

Table 10. Ability of test isolates to lyse selected dead bacteria.

	Test Organisms													
Strain	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	-	+	+	+	+	+	+	-	+	-	+	-	+	+
2	-	+	+	+	+	+	+	-	+	-	+	-	+	+
3	-	+	+	+	+	+	+	-	+	+	+	-	+	+
4	-	+	+	+	+	+	+	-	+	-	+	-	+	+
5	-	+	+	+	+	+	+	+	+	-	+	-	-	+
6	-	+	+	+	+	+	+	+	-	-	+	-	+	+
7	-	+	+	+	+	+	+	+	+	+	+	-	+	+
8	+	+	+	+	+	+	+	+	+	-	+	-	-	+
9	-	+	+	+	+	+	+	+	+	-	+	-	+	+
10	-	+	+	+	+	+	+	+	+	-	+	-	+	+
11	-	+	+	+	+	+	+	+	+	-	+	+	+	+
12	-	+	+	+	+	+	+	+	-	-	+	-	+	+
13	+	+	+	+	+	+	+	+	+	-	+	+	+	+
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	-	+	+
16	-	-	+	+	+	+	+	+	+	-	-	-	+	+
17	-	+	+	+	+	+	+	+	+	-	+	+	+	+
18	-	+	+	+	+	+	+	+	+	+	+	+	+	+
19	-	+	+	+	+	+	+	+	+	+	+	-	+	+
20	-	+	+	+	+	+	+	+	+	+	+	-	+	+
21	-	+	+	+	-	-	-	+	-	-	-	-	-	-
22	+	+	-	+	+	+	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	-	-	+	+	+	+	+	+	+	-	+	+	+	+
26	+	+	+	+	-	+	+	+	+	+	+	-	+	+
27	-	-	+	+	+	+	+	+	+	-	+	+	+	+
28	-	+	+	+	+	+	+	+	+	+	+	+	+	+
29	-	+	+	+	+	+	+	+	+	+	-	-	-	+
30	-	+	+	+	-	+	+	+	+	-	+	+	+	+
31	-	+	+	+	+	+	+	+	+	+	+	-	+	+
32	+	+	+	+	+	+	+	+	+	+	+	-	-	+
33	-	+	+	+	+	+	+	+	+	+	+	-	+	+
34	-	+	+	+	+	+	-	+	+	-	+	-	-	+
35	-	+	+	+	+	+	+	+	+	+	+	+	-	+
36	-	+	+	+	+	+	+	+	+	+	-	-	+	+
37	+	+	+	+	+	+	+	+	+	+	+	-	+	+
38	-	+	+	+	+	+	+	+	+	-	-	-	+	+
39	+	+	+	+	+	+	+	+	+	+	-	+	+	+
40	-	+	+	+	+	+	+	+	+	-	+	+	+	+
41	+	+	+	+	+	+	+	+	+	+	-	+	+	+

+: Lysis of autoclaved bacterial cells

-: No lysis but growth

Figure 5 shows a rearranged list of the organisms together with the corresponding S values. It can be seen that the calculated values of similarity between separate organisms range from 34% to 100%.

Five main groups can be distinguished. These five groups together with the strains contained in each is listed below.

Group 1 - contains strains 1, 7, 3, 2, 9, 10, 4, 6, 15, and 14

Group 2 - contains strains 23, 24, 28, 22, 26, 41 and 37

Group 3 - contains strains 39, 38, 31, 35, 5, 34, 32, 33, 20, 19, 18 and 13

Group 4 - contains strains 12, 16, 8, 30, 17, 25, and 27

Group 5 - This is a diverse group composed of organisms with very little similarity. Contained in this group are strains 36, 40, 11, 29 and 21

The organisms in the first four groups are linked at 70% similarity or greater.

1	100																																								
7	82	100																																							
3	88	91	100																																						
2	84	85	87	100																																					
9	88	88	97	97	100																																				
10	88	88	97	97	100	100																																			
4	69	81	76	82	80	80	100																																		
6	76	75	80	88	87	87	72	100																																	
15	76	79	89	83	81	81	76	74	100																																
14	65	71	74	74	71	71	70	64	83	100																															
23	60	66	69	63	61	61	60	58	73	90	100																														
24	60	63	57	63	61	61	57	58	58	75	84	100																													
28	58	64	44	59	63	63	61	57	68	77	85	76	100																												
22	51	60	57	52	52	52	58	50	52	70	80	68	77	100																											
26	54	60	63	60	60	60	64	52	73	79	77	82	70	65	100																										
41	56	46	51	58	56	56	55	60	68	77	73	83	70	63	77	100																									
37	52	58	61	58	60	60	59	55	64	75	70	34	71	67	72	73	100																								
36	58	56	65	61	61	61	53	61	64	65	58	62	52	49	58	68	61	100																							
40	53	59	59	63	61	61	65	60	62	72	64	59	69	63	58	66	59	51	100																						
11	60	57	57	56	60	60	54	50	53	63	63	58	63	52	56	52	60	58	59	100																					
39	49	57	57	56	53	53	60	51	51	72	65	62	67	70	57	39	65	44	66	60	100																				
38	46	51	51	50	50	50	59	51	58	67	55	49	61	60	54	38	65	47	60	58	80	100																			
31	48	56	55	47	49	49	51	47	56	58	54	46	58	61	50	55	57	40	55	53	72	80	100																		
35	38	47	45	43	42	42	46	43	46	55	50	53	54	51	53	54	57	39	53	54	69	73	84	100																	
5	39	43	44	40	42	42	44	40	58	58	47	47	53	48	45	49	46	33	50	53	65	60	60	87	100																
34	40	47	45	42	45	45	46	42	45	52	46	44	54	48	47	47	54	35	50	48	63	72	78	75	74	100															
32	44	52	49	43	44	44	49	47	49	55	59	56	64	66	60	62	67	41	56	49	25	72	78	70	66	71	100														
33	40	48	48	49	52	54	51	49	48	57	56	52	60	60	54	56	65	42	51	51	67	76	78	75	67	78	81	100													
20	48	55	54	48	51	51	53	50	52	60	56	56	60	57	58	61	60	46	57	57	71	75	82	82	79	73	75	84	100												
19	48	56	56	50	52	52	56	47	54	63	56	54	64	58	55	55	62	41	51	58	70	75	82	78	77	73	79	83	90	100											
18	45	53	53	47	49	49	54	47	50	63	63	58	71	65	52	58	60	43	61	56	79	77	76	74	76	68	74	82	82	85	100										
13	51	56	56	55	55	55	57	50	57	61	60	63	69	63	57	63	61	38	61	65	60	72	71	75	76	68	76	63	75	78	80	100									
12	43	50	47	49	60	60	54	47	57	53	47	45	55	49	48	47	48	38	55	51	56	68	69	70	72	61	58	59	68	74	57	79	100								
16	50	57	52	56	51	51	57	49	44	58	60	58	61	63	58	57	55	39	58	55	72	70	65	64	64	62	69	69	77	76	68	76	61	100							
8	51	56	54	60	54	54	57	49	58	58	51	56	62	58	56	58	58	40	56	61	71	68	69	69	75	67	75	72	80	80	72	82	63	73	100						
30	50	55	52	47	51	51	53	46	49	53	59	53	64	61	52	54	51	37	63	56	79	60	73	64	69	67	73	71	73	74	80	76	58	74	75	100					
17	49	53	50	47	49	49	50	45	58	55	55	58	65	58	47	50	49	37	60	57	70	68	71	66	74	68	67	62	75	79	78	78	65	75	78	84	100				
25	40	44	44	42	44	44	52	38	45	52	54	48	43	53	43	57	51	36	55	56	67	73	70	67	73	65	67	68	78	76	80	72	60	70	65	73	78	100			
27	40	47	46	44	46	46	49	40	47	52	55	49	39	54	44	48	50	34	56	55	68	70	70	68	73	65	64	69	74	78	80	75	60	60	60	70	78	96	100		
29	43	51	51	45	47	47	52	40	54	45	52	46	57	48	50	53	60	44	53	64	60	62	65	64	63	56	62	64	58	60	65	56	64	53	58	54	53	54	54	100	
21	39	44	45	41	43	43	54	38	44	45	45	38	53	47	46	34	50	34	40	36	48	48	57	54	56	48	49	41	46	53	50	50	62	41	46	49	45	45	46	57	100
Strain Number	1	7	3	2	9	10	4	6	15	14	23	24	28	22	26	41	37	36	40	11	39	38	31	35	5	34	32	33	20	19	18	13	12	16	8	30	17	25	27	29	21

Figure 5. Similarity matrix: 41 myxobacterial isolates.

DISCUSSION

Myxobacteria isolated from the fresh water environment are particularly difficult to identify, since very few descriptions of these organisms are available in the literature. The present study partially fills this gap in our knowledge by providing taxonomic information on a number of myxobacteria isolated from fish. Based on the results of this work, it is possible to propose a new taxonomic scheme for aquatic cytophagas.

That the organisms studied in this investigation are myxobacteria is indicated by their cellular morphology. Individual vegetative cells were gram negative, unicellular, flexible rods with round ends. Typical myxobacterial gliding or creeping motility and low refractility was noted upon direct phase contrast microscopic examination of living, young, vegetative cells. Young vegetative cells were long and thin but after aging became shorter, thicker and appeared to be as rigid as those of many eubacteria. As cultures aged the cells lost their gliding motility. Involution forms appeared after further aging, especially in rich media and in broth cultures. Once cells had reached these degenerate stages, they would not revert to the vegetative form. All these characteristics of individual cells have been reported by others to be typical of myxobacteria (Thaxter, 1892; Stanier, 1942, 1947; Dworkin, 1966).

Table 11. Number and percentage of strains giving positive results to tests.

Characteristic	Number Positive*	Percent Positive
Gram negative rods	41/41	100
Gliding motility	41/41	100
Fruiting bodies formed	1/41	2.4
Involution forms	41/41	100
Growth at:		
5°C	41/41	100
18°C	41/41	100
25°C	30/41	73.17
30°C	25/41	60.98
37°C	2/41	4.88
NaCl tolerance:		
No. tolerating 0%	41/41	100
No. tolerating 0.1%	41/41	100
No. tolerating 0.25%	31/41	75.61
No. tolerating 0.5%	22/41	53.66
No. tolerating 1%	19/41	46.34
No. tolerating 2%	15/41	36.59
No. tolerating 3%	4/41	9.76
No. tolerating 4%	0/41	0
Growth at:		
pH 5	0/41	0
pH 6	20/41	48.78
pH 6.5	27/41	65.85
pH 7	41/41	100
pH 8	22/41	53.66
pH 9	20/41	48.78
Anaerobic growth:	107/41	17.07
Sensitivity to:		
Erythromycin	26/41	63.41
Polymyxin B	5/41	12.20
Bacitracin	15/41	36.59
Dihydrostreptomycin	39/41	95.12
Neomycin	5/41	12.20
Novobiocin	26/41	63.41
Penicillin	16/41	39.02
Tetracycline	34/41	82.93
Heat Resistance		
a) Young cells		
37°C No. killed in 5 min.	0/41	0
No. killed in 15 min.	2/41	4.88
55°C No. killed in 5 min.	22/41	53.66
No. killed in 15 min.	33/41	80.93
b) Old cells		
50°C No. killed in 10 min.	31/41	75.61
70°C No. killed in 5 min.	40/41	97.56
No. killed in 10 min.	41/41	100

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Table 11. Cont.

Characteristic	Number Positive*	Percent Positive
Carbohydrate utilization:		
Glucose oxidized	14/41	34.19
Glucose fermented	6/41	14.63
Galactose oxidized	6/6	100
Galactose fermented	4/41	9.76
Lactose oxidized	6/6	100
Lactose fermented	3/41	7.32
Maltose oxidized	6/6	100
Maltose fermented	4/41	9.76
Mannitol oxidized	0/6	0
Mannitol fermented	0/41	0
CO_2 dependent glucose fermentation	1/41	2.44
Degradation of Macromolecules:		
Casein hydrolyzed	41/41	100
Gelatin hydrolyzed	41/41	100
Starch hydrolyzed	30/41	73.17
Cellulose hydrolyzed	0/41	0
Chitin hydrolyzed	19/41	46.34
Tributyrin hydrolyzed	34/38	82.93
Aesculin hydrolyzed	30/41	73.17
Miscellaneous physiological reaction:		
Urease produced	0/41	0
Lysine dearboxylase produced	0/41	0
Arginine decaboxylase produced	0/41	0
Tyrosine decomposed	32/41	78.05
Hydrogen sulfide produced	23/41	56.10
Indol produced	0/41	0
Methyl red positive	0/41	0
Voges-Proskauer positive	0/41	0
Nitrate reduced aerobically	11/41	26.83
Nitrate reduced anaerobically	3/41	7.32
Catalase produced	34/34	97.56
Cytochrome oxidase	12/41	29.27
Nutritional studies:		
Glucose utilization as sole carbon source	0/41	0
Citrate utilization as sole carbon source	3/41	7.32
Amino acid as sole carbon and nitrogen source:		
Glycine	0/41	0
Alanine	0/41	0
Arginine	0/41	0
Glutamic acid	0/41	0
Histidine	0/41	0
Leucine	0/41	0
Methionine	0/41	0
Tyrosine	0/41	0

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Table 11. Cont.

Characteristic	Number Positive*	Percent Positive
Amino acids inhibiting growth:		
Arginine	7/41	17.07
Asparagine	26/41	63.41
Histidine	12/41	29.27
Methionine	26/41	63.41
Tryptophan	5/41	12.20
Lysis of dead bacterial cells:		
Corynebacterium hoffmanii	12/41	29.27
Alcaligenes viscolactis	38/41	92.68
Proteus vulgaris	40/41	97.56
Serratia marcescens	41/41	100
Pseudomonas fluorescens	38/41	92.68
Pseudomonas aeruginosa	40/41	97.56
Aerobacter aerogenes	39/41	95.12
Escherichia coli	37/41	90.24
Streptococcus faecalis	38/41	92.68
Staphylococcus aureus	21/41	51.22
Sarcina urea	33/41	80.49
Mycobacterium smegmatis	16/41	39.02
Bacillus megatherium	34/41	82.93
Bacillus subtilis	40/41	97.56

* the numerator indicates the number of strains positive for the test and the denominator indicates the total number of cultures tested.

Colony morphology was found to be influenced by cultural conditions. Less coloration and a greater degree of spreading occurred on dilute media as compared to rich media. Agar concentration also affected colony morphology. At higher agar concentrations growth was generally more compact. Results similar to these have been reported by Stanier (1942, 1947).

Summarized in Table 11 are the results of the cultural and physiological tests performed on the myxobacterial isolates. Included in this table are number and percentage of strains giving positive results in each test. As can be seen, the majority of organisms are strictly aerobic, do not require sodium chloride and grow best at a pH in the vicinity of 7.0.

On the basis of observations on temperature range of growth, the organisms could be separated into two groups: a psychrophilic group which grew at 18°C but not at room temperature, and a mesophilic group which grew readily at room temperature and above. Of the 41 test isolates, 13 organisms were found in the psychrophilic group. Most of the organisms in this group were more sensitive to sodium chloride and to acid or alkaline conditions than were the isolates in the mesophilic group. Also, the strains in the psychrophilic group were strictly aerobic while ten organisms in the mesophilic group were found to grow anaerobically. It appears from these results that the psychrophilic group is not as adaptable as the

mesophilic group to environmental changes.

Fourteen of the isolates were found to utilize glucose. Of these seven were capable of fermenting the sugar. The occurrence of fermentative Cytophaga have been reported only recently. In 1955 Bachman (1955) succeeded in isolating a Cytophaga which was capable of growing anaerobically at the expense of simple sugars such as glucose, sucrose and lactose. Since this time several other facultative anaerobic cytophagas have been described (Anderson and Ordal, 1961; Veldkamp, 1961). Cytophaga succinicans, the organism described by Anderson and Ordal (1961), differed from the other fermentative cytophagas described in that CO_2 was required for fermentation and in addition the organism was isolated from a fresh water source rather than marine mud.

All the organisms investigated in this study showed some ability to degrade macromolecules. This feature has been reported for myxobacteria and appears to be a characteristic of the group (Dworkin, 1966). Of the organisms tested, all were able to hydrolyze gelatin and casein and nearly all hydrolyzed tributyrin and aesculin. Only 11 of the isolates failed to hydrolyze starch. Nineteen of the 41 isolates also decomposed chitin.

In general, the psychrophilic group of organisms was not as active in the decomposition of macromolecules as the mesophilic group. This is evidenced by the fact that the majority of the

organisms in the former group failed to utilize starch, aesculin and tributyrin. None of the group was able to break down chitin. These findings further emphasize the differences between these two groups of myxobacteria. Whether these observations can be extended to include all psychrophilic fresh water myxobacteria cannot be concluded from these studies because of the small sample size.

For the most part, the myxobacteria studied in this investigation did not appear to be overly fastidious with regard to their nutritional requirements. The majority of the organisms were able to grow on 1.5% non-nutrient agar for several transfers. Growth on this medium, however, was quite sparse, as would be expected. While no attempt was made to determine the precise nutritional requirements of the organisms, it was found that none of the isolates were able to grow using glucose as a carbon source and ammonia as a nitrogen source. However, three of the test isolates were able to use citrate as a sole carbon source. Dworkin (1966) in reviewing the nutritional requirements of myxobacteria pointed out that mono and disaccarides were probably not important as energy sources for the genus Myxococcus. Conflicting information was available for other groups of myxobacteria but, in general, glucose does not seem to be utilized to any great extent, and in some cases, autoclaved glucose may be inhibitory.

The ability of single amino acids to satisfy the carbon and

nitrogen requirements of the myxobacteria was tested, however, none of the isolates were able to grow on these simple compounds. When added to a complete medium, at a concentration of 0.5%, it was found that the amino acids arginine, asparagine, histidine, methionine and tryptophan actually inhibited the growth of many of the isolates. Observations similar to these have been reported by Noren (Dworkin, 1966). On the basis of their effects on the growth of Myxococcus virescens, Noren was able to divide amino acids into three groups: those that were required, those that were stimulatory and those that were inhibitory.

The heat resistance of old cultures was used in an effort to demonstrate the presence of resistant cells in the isolates. Microcysts of Myxococcus ruber were reported by Bauer (Dworkin, 1966) to be resistant to 70°C for 30 minutes, while vegetative cells were killed at 50°C. None of the organisms studied in this investigation were able to survive 70°C for a period of 15 minutes. As a result, no evidence for the presence of microcysts or resistant cells was obtained. Furthermore, structures similar in appearance to microcysts were never observed in the cultures upon examination with phase contrast microscopy.

On the basis of morphological appearances, using the present system of classification, all test isolates except strain 11 can be placed in the family Cytophagaceae. This family is distinguished

from the four other myxobacterial families now listed in Bergey's Manual of Determinative Bacteriology, 7th ed. by its lack of microcysts or fruiting bodies. The family Cytophagaceae contains only one genus, Cytophaga, under which 40 of the test organisms may be placed.

The remaining organism, strain 11, forms fruiting bodies or columnar masses of cells, but no microcysts. This organism is difficult to classify under the present system. Garnjobst (1945) placed an organism with similar morphological features in the genus Cytophaga. Dworkin (1966) however, feels the ability to form microcysts may be lost as a result of repeated laboratory cultivation and suggests that the organism described by Garnjobst is in reality identical to the species Chondrococcus columnaris isolated by Ordal and Rucker (1944). This reasoning could account for the lack of microcysts in strain 11. If it is true that strain 11 lost the ability to produce microcysts as a result of subculture, the organism should be classified in the family Myxococcus. It would have been desirable to test the pathogenicity of strain 11 to determine if the organism could produce typical lesions of columnaris disease. However, facilities for these tests were not available. Evidence for a close relationship between strain 11 and the organism Chondrococcus columnaris is supported by the presence of common atigens in the two strains (Pacha, 1966). In view of this relationship, culture 11 is most likely

a strain of Chondrococcus columnaris. These findings indicate the desirability of using only characteristics of reasonable stability for the classification of these organisms.

There is some conflict in the literature concerning the physiological activities of Chondrococcus columnaris. Ordal and Rucker (1944) reported that the organism was able to oxidize glucose whereas neither Nigrelli and Hutner (1945) nor Garnjobst (1945) were able to demonstrate this characteristic. The results of the present study support the findings of Nigrelli and Hutner and of Garnjobst in that strain 11 neither fermented nor oxidized glucose. It would seem from these findings that the ability to oxidize glucose is either a variable characteristic of this organism or that the results of Ordal and Rucker are invalid.

Five groups of organisms were recognized on the basis of the numerical analysis of the data and an attempt was made to identify the major characteristics which would serve to identify each group. Group 1 was composed predominately of psychrophilic organisms and could be separated on this basis. However, the cultural and physiological properties which would serve to differentiate the other groups were not obvious. Perhaps if these data were analyzed by a computer more clear-cut distinctions between the groups would become apparent.

Since it was not possible to differentiate all five groups of

myxobacteria on the basis of cultural and physiological properties, it seemed advisable for purposes of classification to separate the organisms initially on the basis of temperature range of growth. Using this criterion, two groups of organisms could be recognized. The psychrophilic group contained strains 1, 2, 3, 4, 6, 7, 9, 10, 14, 15, 23, 26 and 36. Four of the organisms on this group hydrolyze starch and can be separated from the other members of the psychrophilic group by this characteristic. It is possible to further separate these four organisms by the presence or absence of the enzyme cytochrome oxidase and their ability to hydrolyze aesculine. Those organisms which do not hydrolyze starch can be further subdivided on the basis hydrogen sulfide production in tryptone broth and whether or not cytochrome oxidase is present. Of the isolates in the psychrophilic group, strains 2, 4, and 6 appear to be clearly related to the species Cytophaga psychrophila as described by Borg (1960).

The mesophilic group of isolates contains the remaining 27 strains. These organisms are grouped on the basis of their ability to grow at room temperature and above. Two of the organisms in this group produced a water soluble black or dark brown pigment. This characteristic could be used to distinguish these strains since none of the other organisms exhibited this property. One of these organisms, strain 22, produced the pigment in all broth medium

tested. When grown on Cytophaga agar this organism produced a yellow colony which began to darken after four or five days incubation. At this time a water soluble pigment diffused into the medium surrounding the colonies. The other organism, strain 24, produced black pigment only when grown in tryptone broth. The latter organism grew more slowly than strain 22 and formed no water soluble pigment on agar medium. Organisms among the myxobacteria capable of producing water soluble pigments have been reported by other workers (Borg, 1960; Stanier, 1940). Borg isolated a Cytophaga from fish which produced a diffusable brown pigment on tryptone broth and this organism had an optimum temperature of 18°C. A marine species, Cytophaga krzemieniewskae, which produced a soluble black pigment was described by Stanier.

The remainder of the organisms in the mesophilic group can be separated into two categories depending upon whether or not they are capable of growing anaerobically. Ten of the isolates were strictly aerobic. Of those which were facultatively anaerobic, three required nitrate as an oxidizing agent for anaerobic growth. These findings are similar to results reported by Stanier (1947) in which a myxobacterium from soil, was described. This organism was named Cytophaga johnsonas var. denitrificans and was found to be a strict aerobe except in the presence of nitrate. However, there have been no reports of similar organisms isolated from the fresh

water environment.

Seven of the facultative anaerobes were able to ferment glucose. Included in this group are strains 5, 12, 21, 29, 31, 34 and 35. One of these organisms, strain 34 was found to require CO_2 for glucose fermentation. It is likely that this organism is similar to Cytophaga succinicans (Anderson and Ordal, 1961).

On the basis of the results obtained in this study, a new taxonomic scheme can be devised for the genus Cytophaga. This scheme is presented in Table 12. The characteristics used in setting up the major categories involved source, sodium chloride requirement and temperature range of growth. Each of the major categories of myxobacteria isolated from water was further subdivided on the basis of additional cultural and physiological tests.

While the taxonomic scheme proposed for the genus Cytophaga is more complete than that provided by Stanier (1957), additional studies are needed. It would be highly desirable to use criteria other than source of isolation as a means for separation of species. By examining the cultural and physiological properties of soil isolates in the same manner as has been done for the fresh water forms, perhaps a more useful key could be devised. A serological investigation of the genus Cytophaga as well as studies on the DNA homology among this group would undoubtably be useful approaches for studying the interrelationships between members of this genus.

Table 12. Key to the species of genus Cytophaga.

I. From soil. Not obligately halophilic.
See Bergey's Manual of Determinative Bacteriology, 7th edition, for the classification of this group.

II. From water
A. Obligately halophilic, not growing in medium without at least 0.5% sodium chloride.
1. Produced a diffusible black to brown pigment
1. Cytophaga krzemiewskae
2. Do not produce a diffusible black to brown pigment.
a. Growth not inhibited by the presence of 0.2% glucose.
b. Aerobic.
c. Growth with inorganic source of nitrogen.
d. Produces yellow pigment
e. Nitrate reduced.
2. Cytophaga haloflava
ee. Nitrate not reduced.
3. Cytophaga haloflava var. nonreductans
dd. Pink pigment produced.
4. Cytophaga rosea.
cc. No growth with inorganic source of nitrogen.
5. Cytophaga diffluens
bb. Facultativelt anaerobic.
c. Produces yellow pigment.
6. Cytophaga fermentans
cc. Produces red pigment.
7. Cytophaga salmonicolor
aa. Growth inhibited by the presence of 0.2% glucose.
b. Aerobic
8. Cytophaga sensitiva
bb. Facultatively anaerobic.
c. Colonies on agar plate cream to yellow.
9. Cytophaga fermentans var. agarovorans
cc. Colonies on agar plate salmon-colored.
10. Cytophaga salmonicolor var. agarovorans.
B. Not obiligately halophilic.
1. Psychrophilic, negligible or no growth at 25°C.
a. Starch hydrolyzed.
b. Cytochrome oxidase positive.
c. Aesculin hydrolyzed.
11. Strains 14 and 26
cc. Aesculin not hydrolyzed.
12. Strain 15
bb. Cytochrome oxidase negative.
aa. Starch not hydrolyzed.
13. Strain 23
b. Hydrogen sulfide produced.
14. Strains 1, 3, 7, 9, 10
bb. Hydrogen sulfide not produced.
c. Cytochrome oxidase weak positive
15. Strain 36

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cc. Cytochrome oxidase negative

16. Strains 2, 4 and 6

Cytophaga psychrophila

2. Mesophilic, grow readily at 25°C.

a. Water soluble black to brown pigment produced.

17. Strains 22 and 24

aa. Do not produce a water soluble black or brown pigment.

b. Aerobic.

c. Glucose oxidized.

d. Tyrosine hydrolyzed.

e. Hydrogen sulfide produced.

f. Nitrite produced from nitrate.

18. Strains 19 and 20

ff. Nitrite not produced from nitrate.

19. Strains 18 and 32

ee. Hydrogen sulfide not produced.

20. Strain 33

cc. Glucose not oxidized.

d. Chitin utilized.

e. Citrate utilized.

21. Strain 37

ee. Citrate not utilized

f. Hydrogen sulfide not produced.

g. Nitrate reduced.

23. Strain 38

gg. Nitrate not reduced.

24. Strain 39

dd. Chitin not utilized.

e. Tyrosine hydrolyzed.

f. Cytochrome oxidase positive.

25. Strains 28, 40 and 41

ff. Cytochrome oxidase negative.

26. Strain 16

bb. Facultatively anaerobic.

c. Nitrate required for anaerobic growth.

d. Glucose oxidized

27. Strains 25 and 27

dd. Glucose not oxidized.

28. Strain 17

c. Nitrate not required for anaerobic growth; glucose fermented.

d. CO_2 required for fermentation.

29. Strain 34

Cytophaga succinicans

dd. CO_2 not required for fermentation.

e. Maltose fermented.

f. Chitin utilized.

g. Lactose fermented.

30. Strain 21

gg. Lactose not utilized.

ff. Chitin not utilized.

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g. Sucrose fermented.

32. Strain 29

gg. Sucrose not fermented.

33. Strain 12

ee. Maltose not fermented.

f. Hydrogen sulfide produced

34. Strain 31

ff. Hydrogen sulfide not produced.

35. Strain 35

III. From animal source.

A. Anaerobiosis

1. Isolated from human oral cavity.

36. Sphaerocytophaga

SUMMARY

Forty one myxobacteria isolated from the surface of fish were examined for morphological, cultural, and physiological characteristics. Phase contrast microscopic examination showed all to have typical myxobacterial morphology. One organism formed fruiting bodies on fish tissue and columnar masses of cells on tryptone broth and on litmus milk, but did not form microcysts. The remaining forty organisms formed neither fruiting bodies nor microcysts and consequently can be considered members of the genus Cytophaga. These organisms could be separated into two groups, a psychrophilic group and a mesophilic group. On the basis of a variety of cultural and physiological characteristics, a new taxonomic scheme of the genus Cytophaga has been proposed.

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