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Certain species of the orders Myxobacterales and Pseudomonadales are well-known antagonists. Pseudomonas fluorescens and Pseudomonas putida have been reported to produce bacteriostatic and bactericidal substances. Among the myxobacteria there are various species which are known for their ability to produce lytic enzymes and antibiotics. Organisms from both of these groups of bacteria are known to occur in the aquatic habitat.

During studies on the microbial flora of experimental streams, it was found, at times, that myxobacteria represented a significant proportion of the bacterial population. This suggested that either the environmental conditions favored the growth of these organisms specifically or that inhibiting substances were being produced by these bacteria which prevented the growth of other bacterial species. A number of non-fruiting, fresh-water myxobacterial isolates were

found to produce antibiotic substances. This finding was of interest since the production of these substances by non-fruiting myxobacteria has not been previously reported. The antibiotic material had a fairly broad spectrum since it was capable of inhibiting the growth of a variety of different bacterial species. The inhibitory substance produced by one of the isolates was partially purified by ethanol precipitation. This antibiotic was found to be water soluble and heat stable.

An association between P. fluorescens and certain aquatic myxobacterial isolates also was suggested from the bacteriological surveys of the experimental streams. One stream had a predominance of pseudomonads and very scant myxobacterial growth in comparison with the other five streams studied. It was therefore of interest to study the interrelationship between these two groups of bacteria. Mixed culture studies showed that myxobacterial growth was markedly inhibited by the presence of certain pseudomonads. A crude toxic preparation was obtained from one of the pseudomonad isolates. This material was found to exert a bacteriostatic effect on certain myxobacteria. The substance was also inhibitory for Sarcina lutea and Bacillus cereus. The crude toxin was found to be heat stable, water soluble and dialysable.

Antagonistic Properties of Aquatic Myxobacteria and Pseudomonads

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INTRODUCTION

Antagonistic interrelationships among microorganisms have attracted attention since the early days of bacteriology. After the discovery by Pasteur that microbes were responsible for certain human, animal, and plant diseases, it was established that other organisms, later designated as antagonists, were able to combat and even destroy the disease-producing agents.

Antagonism can be defined as the phenomenon of a living organism inhibiting the growth or interfering with the activities of another living organism. This action can be caused by the creation of unfavorable growth conditions in the medium or by the production of a specific inhibitory substance, such as an antibiotic.

Three general types of microbial antagonism are recognized: bacteriostatic, bactericidal, and bacteriolytic. Bacteriostatic antagonism is demonstrated when one bacterium is inoculated into the filtrate of another and the growth of the first is either completely inhibited, or is slower than that of the control. If the growth is completely checked, it may resume later but it will not be quite normal. Bacteriolytic and bactericidal antagonisms express themselves in the destruction of other organisms present in mixed

cultures. Lysis occurs in the former type of antagonism, but not in the latter.

Many bacterial species have been shown to exhibit antagonistic properties. Included among these organisms are the pseudomonads. A number of reports indicate that certain members of this group of bacteria produce broad-spectrum antibacterial substances. Myxobacteria are also known for their antagonistic activity. Some of these organisms produce lytic substances which are capable of lysing intact bacterial cells. Antibiotic substances also have been obtained from myxobacterial culture filtrates.

During a study of the bacterial flora associated with aquatic algal populations, it was of interest to note a predominance of myxobacteria in certain habitats. An apparent inverse relationship between pseudomonads and myxobacteria was also observed in these studies. The present study was undertaken to examine the interrelationships between various aquatic myxobacteria and pseudomonads. It was also of interest to determine whether these aquatic bacteria were able to produce inhibitory substances.

HISTORICAL REVIEW

Lytic agents have been isolated from a great number of different organisms, but only a few groups of bacteria are known to exhibit lytic properties in mixed culture and to utilize living cells for their nutrients. Various myxobacteria exhibit this kind of activity. Some of these myxobacteria can also produce antibiotics, thereby demonstrating dual antibacterial activity.

The bacteriolytic property of myxobacteria was first recognized by Pinoy (1913). He noticed the lysis of a Micrococcus species when grown in association with Chondromyces crocatus on nutrient agar. Since that time it has become well-established that a great number of myxobacteria are capable of lysing a variety of eubacteria (Solntzewa, 1939; Beebe, 1941; Snieszko, McAllister, and Hitchner, 1942; Oxford, 1947; Singh, 1947; Oetker, 1953; and Noren, 1953a, 1955, 1960a, and 1962).

Many intact fruiting myxobacteria or their culture filtrates have been shown to lyse autoclaved (Singh, 1947; Oxford, 1947; Noren, 1953a, 1955, 1960a; and Bender, 1962), five percent butanol-treated (Bender, 1962), freeze-thawed (Noren, 1960a), or EDTA-treated (Gillespie and Cook, 1965) Gram-negative cells. This activity was due to the presence of secreted proteolytic enzymes (Noren, 1960a; Bender, 1962; and Margalith, 1962). Heat-treated

(Noren, 1953a, 1955; Kletter and Henis, 1963; and Gillespie and Cook, 1965) and EDTA-treated (Gillespie and Cook, 1965) Gram-positive bacteria are also lysed by myxobacteria culture filtrates. The heat-treated Gram-positive bacteria were lysed at a slower rate than were similar preparations of Gram-negative bacteria (Kletter and Henis, 1963). The resistance of heated Gram-positive bacteria to digestion by proteolytic enzymes was explained by the nature of the Gram-positive cell wall (Salton, 1953). It is well-known that the cells of Gram-positive bacteria contain less lipid and a different amino acid content than the cell walls of Gram-negative bacteria. Whether the resistance of the cell walls of Gram-positive bacteria to rupture or damage on heating was due to their low lipid content or to the nature of the protein component has not been decided.

Living cells of both Gram-positive and Gram-negative eubacteria also have been shown to be lysed by various myxobacteria (Pinoy, 1913; Solntzewa, 1939; Snieszko et al, 1942; Oetker, 1953; Kulwein, 1955; and Mathew and Dudani, 1955). In some instances it has been reported that better growth of myxobacteria resulted when they were cultivated in mixed culture with living eubacterial cells (Noren, 1960a). The mechanisms involved in the lysis of living bacterial cells by myxobacteria is still not completely clear.

In the past ten years much work has been done in isolating the myxobacterial lytic agents. The preliminary investigations by

Noren (1960b) and Bender (1962) showed that there were separate substances responsible for the lysis of dead and living bacterial cells. Noren showed that cultures of Myxococcus virescens lysed viable cells of an Aerobacter species on non-nutrient agar. However, an ammonium sulfate precipitate of a concentrated dialysed culture filtrate of this myxobacterium would lyse ruptured cells of the Aerobacter, but would not lyse the intact cells. In view of these findings, Noren stated that some factor, which could lyse intact cells of the Aerobacter sp., was produced by M. virescens, but could not be precipitated by ammonium sulfate.

Bender (1962) studied a lytic factor produced by a strain of Myxococcus xanthus. In these studies it was shown that the optimal activity against Gram-positive cells and heated Gram-negative cells was attained in the culture at different times. The proteolytic activity against heated Gram-negative cells reached a peak after about six days of cultivation and remained constant until 20 days had elapsed. The "wall-lytic" activity against intact Bacillus subtilis cells reached a peak at seven days and was completely absent at 16 days.

More recently a number of investigators have succeeded in isolating bacteriolytic enzymes from a variety of myxobacteria. Gillespie and Cook (1965) obtained a bacteriolytic enzyme from the genus Sorangium. This lytic enzyme was capable of attacking

various Gram-positive bacteria and, to a lesser extent, Gram-negative bacteria. The enzyme was also capable of lysing isolated cell walls of Arthrobacter globiformis, causing the release of substances giving the reactions of amino sugars, an indicator of cell wall lysis. The myxobacterium also produced a protease which could be separated from the lytic enzyme on hydroxapatite columns. The proteolytic enzyme did not lyse intact bacterial cells.

Hart and Zahler (1966) isolated an extracellular lytic enzyme from another strain of Myxococcus xanthus. The enzyme was capable of lysing living Micrococcus lysodeikticus cells and releasing N-acetyl amino sugars from their cell walls. This enzyme solubilized cell walls by cleaving the polysaccharide "backbone." The lytic activity spectrum was limited to Gram-positive organisms, thereby showing similarities to egg-white lysozyme. Proteolytic activity was also detected in the crude preparation and this could be separated from the lytic enzyme by gel filtration.

The first account of lytic activity in a non-fruiting myxobacterium was reported by Ensign and Wolfe (1964). The myxobacterium, which appeared to be a member of the genus Cytophaga, was shown to possess an enzyme with bacteriolytic and proteolytic activities. The extracellular enzyme was isolated from the culture filtrates of the non-fruiting myxobacterium designated myxobacter AL-1 (Ensign and Wolfe, 1965 and 1966). This enzyme hydrolysed

both proteins and cell walls of Micrococcus lysodeikticus, Arthrobacter species, Corynebacterium diphtheriae, and Staphylococcus aureus. Except for Rhodospirillum rubrum, Spirillum itersonii, and S. serpens, all of the Gram-negative organisms tested were resistant to the enzyme. The enzyme has been purified over 600-fold and all attempts to separate cell wall lytic and proteolytic activities have failed. The enzyme lyses cell walls by hydrolyzing peptide bonds in the glycosaminopeptide. The literature survey did not reveal additional reports of the lytic activity attributed to non-fruiting myxobacteria.

The bacteriolytic enzymes which have been isolated from myxobacteria have been found to be active on a number of Gram-positive bacteria. However, most Gram-negative bacteria exhibit varying degrees of resistance to these agents. It is interesting to note that although most Gram-negative bacteria are resistant to the action of the bacteriolytic enzymes, these organisms were sensitive to lysis when grown in association with myxobacteria on agar plates. This may be explained by the difference in time used in each assay, the plate assay being measured in terms of a day or longer, whereas the lytic assay was measured in minutes. Perhaps slowly acting non-enzymatic products of myxobacterial growth played a part in the lysis of resistant bacteria on plates by altering the nature of the cell walls. Whatever the cause, more intensive research will have

to be done in this area.

In addition to their lytic activity, myxobacteria have been reported to possess antibiotic activity. Oxford (1947) was the first investigator to report that cultures of myxobacteria (Myxococcus virescens) had antibiotic activity. An alcohol-soluble antibiotic produced by this organism was found to be active against Staphylococcus aureus, but inactive on Gram-negative bacteria. Kato (cited in Peterson, Gillespie and Cook, 1965) found two strains of Myxococcus fulvus which inhibited both Gram-positive and Gram-negative bacteria. A broad-spectrum antibiotic from a species of Sorangium has been found to inhibit the growth of Gram-positive and Gram-negative bacteria, various species of fungi, actinomycetes, and yeasts (Peterson et al, 1965).

Much confusion exists in regard to the production and the host range of myxobacterial antibiotics. Woods (cited by Dworkin, 1966) and Noren (1953b) were unable to repeat Oxford's observations. However, Noren found that Myxococcus virescens produced an antibiotic effective against Aerobacter aerogenes and inactive against S. aureus. Chondrococcus coralloides, on the other hand, produced an antibiotic active against S. aureus which had also a weak inhibitory effect against A. aerogenes. Later, Noren and Raper (1962) contradicted these findings. They found that the myxobacteria tested produced antibiotics against a number of

Gram-positive bacteria, but none against Gram-negative bacteria. At the same time, Kletter and Henis (1963) were unable to detect any antibiotic activity in cell-free culture filtrates of M. fulvus and M. virescens against A. aerogenes or S. aureus. It is apparent that much more work is also needed in this area.

It was suggested by Noren (1953b) that antibiotics or a "killer substance" (Imshenetsky and Kusuirina, 1951) might play a role in the initial phase of lysis of bacteria by myxobacteria. Later, Noren and Raper (1962) showed that Myxococcus virescens, M. fulvus, and M. stipitatus, M. lacteus, Chondromyces crocatus and Chondrococcus blasticus produce antibiotics that were effective against Gram-positive bacteria. However, Gram-negative bacteria which were resistant to the antibiotics were rapidly lysed, showing that no correlation exists between the antibacterial activity and the bacteriolytic capacity of these myxobacteria. Further evidence that antibiotic activity was not a prerequisite for lysis was presented by Margalith (1962), and Kletter and Henis (1963). These workers showed that myxobacteria exhibiting no detectable antibiotic activity were capable of lysing organisms.

Dworkin (1966) stated that it was entirely possible that one of the lytic factors acting against Gram-positive bacteria was a basic polypeptide antibiotic similar to bacitracin or gramicidin. A substance of this type would be expected to be heat stable, dialyzable,

and precipitable with organic solvents. It is interesting to note that Solnetzewa (1939) and Singh (1947) showed that lytic factors produced by a variety of myxobacteria against viable, intact bacteria, would pass through a cellophane membrane, and that the factor was stable to 100°C for 15 minutes.

Certain viruses also have shown some sensitivity to the products of myxobacterial cells. Hemagglutination by influenza viruses A and B and mumps virus has been shown to be inhibited by extracts prepared by grinding intact cells of M. virescens (Katzenberger, Kuhlwein, and Kausche, 1956). The active material was dialyzable and could be partially purified by paper chromatography and electrophoresis (Katzenberger and Kausche, 1957). When the dialyzable portion was added to allantoic fluid infected with influenza A or B, it reduced the LD_{50} to zero (Katzenberger, 1959). The cytopathogenic effect of influenza A or B on embryonic heart muscle was completely reversed by the addition of the partially purified myxobacterial extract (Katzenberger, 1959).

It is clear that the fruiting myxobacteria produce antibiotics against Gram-positive organisms and possibly against some Gram-negative organisms and viruses. Contradictory opinion regarding their existence and properties may have arisen because of the apparent instability of the antibiotics coupled with its production only at particular stages of the organisms life cycle. To date, no reports

have been made of non-fruiting myxobacteria possessing antibiotic activity.

Other well-known antagonists belong to the genus Pseudomonas. Pseudomonas fluorescens, P. aeruginosa, and P. putida are frequently mentioned in the literature as organisms which exert an inhibitory or bactericidal action on other species of bacteria. Garre (1887) studied the effect of P. putida on several species of bacteria. Staphylococcus aureus, Bacillus typhosus, and Klebsiella pneumoniae were inhibited while Bacillus anthracis grew normally. Olitsky (1891) showed that P. fluorescens markedly inhibited B. typhosus, Vibrio cholera, Serratia marcescens, Staphylococcus aureus, and B. anthracis. The metabolic products of both P. fluorscens and P. putida have been found to be not only bacteriostatic but also bactericidal for Bacillus typhosus (Frost, 1904).

A number of investigators have studied P. fluorscens in mixed culture. When P. fluorscens and Bacillus cereus were inoculated into sterile manured soil, it was reported that during a 15-day period, colonies of B. cereus were not obtained in the dilutions plated, while P. fluorscens multiplied enormously. The same result was reached whether a large inoculum of B. cereus was used, or unsterilized manure was used as the growth medium (Conn and Bright, 1919).

Waksman and Lomanitz (1924), using a solution of casein,

cultured B. cereus and P. fluorscens separately and in mixed culture. B. cereus was able to decompose the casein while P. fluorscens could not. In mixed culture, P. fluorscens could utilize the amino acids from the decomposed casein. Although no bacterial counts were presented, the authors state that the number of P. fluorscens cells greatly exceeded those of B. cereus in the mixed culture studies.

Recently, a Pseudomonas species which formed a green fluorescent pigment has been found to markedly inhibit the growth of Arthrobacter globiformis in mixed culture. It was suggested that the liberation of inhibitory substances from the Pseudomonas may be the reason for the predominance of Gram-negative rod-shaped organisms in the soil Rhizosphere (Chan and Katnelson, 1961).

Many toxic substances produced by Pseudomonas fluorescens have been found; whether any are identical is not known. Lewis (1929) isolated a thermostable, filterable, and dialyzable bacteriotoxin from a culture of P. fluorescens isolated from water. He found this bacteriotoxin to be both inhibitory and bactericidal to most species of bacteria, except those which produced a green fluorescent pigment.

Landenberger (1952) subjected a culture medium of P. fluorescens to paper chromatography and obtained two fractions. One fraction inhibited the growth of Bacillus subtilis and B. megatherium

when placed on agar plates seeded with the organisms. The other fraction promoted the growth of the two organisms. The growth promoting fraction was found to be a riboflavin.

Starygina (1956), using the cross-streak method, found that metabolic products from P. fluorescens were active against Escherichia coli, Bacillus subtilis, Sarcina species, Rhizobium meliloti, Rhizobium trifolii, Erwinia carotovora, and Saccharomyces cerevisiae. She also reported products of P. fluorescens which were active against the organism itself in the S and R forms. An antibiotic from P. fluorescens was isolated by Graf (1958). The substance was composed of six amino acids and an organic acid. The antibiotic was shown to be bacteriostatic for Streptococcus pyogenes, Streptococcus agalactiae, Diplococcus pneumoniae, and Corynebacterium diphtheriae (Graf and Bickel, 1961). The substance was also virucidal against influenza strains Lee and FM_1, in vitro (Bickel, 1961).

A recent report (Sieburth, 1967) showed that there was a consistent inverse relationship between the pseudomonads and arthrobacters in a rich temperate estuary during the seasonal fluctuations. Peaks in pseudomonads appeared to reflect periods of possible maximal diatom excretions, as indicated by diatom abundance and solar radiation. The author stated the pseudomonads possessed two distinct suppressing agents for arthrobacters. A concentrated broth

culture of an isolated Pseudomonas species contained both an inhibitory and an agglutinating substance against Arthrobacter cells. The inhibitory substance had a small molecular weight as indicated by the fact that it passed dialysis membranes and was retained on a G-25 Sephadex column. The agglutinating substance was not dialysable and was eluted off of the G-25 Sephadex column, thereby demonstrating a large molecular weight. The author noted that maintenance of the pseudomonads in the laboratory resulted in a progressive loss of their inhibiting and agglutinating activities. Also, concentrated broths in storage for one year at -20°C were inactive.

Pseudomonads also have been shown to produce bacteriolytic substances. A pseudomonad, which was isolated from the soil by an enrichment technique, produced a staphylolytic substance which had the properties of an enzyme. Staphylococcus aureus, S. roseus, Gaffkya tetragena, and Sarcina lutea were rapidly lysed by this substance. P. fluorescens and P. aeruginosa were also examined and were found to exhibit staphylolytic activity (Zyskind, Pattee, and Lache, 1965).

From the above discussion it is apparent that certain myxobacteria and pseudomonads both possess antagonistic properties. In view of this it would be of interest to examine the interactions between these two groups of organisms. Some information is

available on this.

Singh (1947) indicated that lytic substances produced by Myxococcus virescens, M. fulvus, and Chondrococcus coralloides were capable of lysing resting cells of P. fluorescens. Imshenetsky and Kusuirina (1951) used M. virescens to lyse living cells of P. fluorescens and P. aeruginosa. They stated there must be cell to cell contact before the myxobacteria can use the bacteria for nutrition. Noren and Raper (1962) showed the lysis of resting cells of P. fluorescens by M. virescens, M. fulvus, Chondrococcus blasticus, and Chondromyces crocatus. P. fluorescens was usually lysed at a slower rate than the other Gram-negative species tested. Noren and Raper also reported that all Gram-negative bacteria, including P. fluorescens, were resistant to the antibiotics produced by the myxobacteria. However, an antibiotic produced by a member of the genus Sorangium when present in high concentration (18 ug/disc) has been found to inhibit the growth of P. fluorescens (Peterson et al, 1965).

Some evidence also exists which indicates that various myxobacteria are susceptible to the antimicrobial activity of certain pseudomonads. Oetker (1953) found that there was no growth of M. virescens or Podangium erectum when they were placed in contact with P. aeruginosa. However, the nature of the inhibiting agent was not determined. Kulwein (1955) showed that Chondrococcus

apiculatus failed to grow in the presence of living cells of P. aeruginosa. More recently, Graf and Morhard (1966) reported a myxobacterium susceptible to an antibiotic produced by P. fluorescens. This myxobacterium was an anaerobic organism assigned to the genus Sphaerocytophaga.

The antagonistic relationships among bacteria in their natural environments, and the ecological significance thereof, are still obscure. Producers of antibiotics (including lytic agents) are found side by side with sensitive organisms and do not predominate in a mixed population under natural conditions. Therefore, the antagonistic properties observed in laboratory experiments cannot be interpreted simply as factors of selective advantage (Stolp and Starr, 1965).

Both myxobacteria and pseudomonads are known to occur in the aquatic habitat and it is possible that these organisms exert antagonistic effects on each other and on other members of the bacterial flora of lakes and streams. During studies on the ecology of myxobacteria in aquatic habitats, evidence was obtained indicating a possible mutual antagonism between these organisms. The present study was undertaken to examine the interrelationships between myxobacteria, pseudomonads, and other organisms in the aquatic environment. As indicated in the literature review, the majority of the past studies of the lytic and antibiotic properties of

myxobacteria have been carried out on the members of the group which form fruiting bodies. Since the majority of aquatic myxobacteria fail to produce fruiting bodies and are classified in the genera Cytophaga and Sporocytophaga, the present studies will contribute to our knowledge of the antagonistic properties of this group of myxobacteria.

MATERIALS AND METHODS

Isolation of Cultures

The myxobacteria were isolated from Oak Creek and Oak Creek Laboratory near Corvallis, Oregon. Cytophaga-peptonized milk (CPM) agar was used as the plating medium. This medium consists of 0.005 percent tryptone, 0.005 percent yeast extract, 0.002 percent sodium acetate, 0.002 percent beef extract, 0.05 percent peptonized milk, and 1.5 percent agar (Difco). The medium was supplemented with 7.5 ug/ml neomycin sulfate (Nutritional Biochemicals Corp., Ohio) to minimize eubacterial growth. Ten ug/ml Acti-dione (Upjohn Co., Michigan) was also added as a fungicide. Before use, the agar plates were dried at 37°C for 48 hours. Water samples (0.1 ml) were spread onto the dried CPM plates. The isolates obtained are listed in Table 1 by strain number and source of isolation.

The majority of the pseudomonads producing green fluorescent pigments were isolated from Oak Creek. An additional culture was obtained from Berry Creek. Ten-fold dilutions were made of the creek water and an inoculum of 0.1 ml was spread onto dried plates of King's medium B agar (2.0 percent proteose peptone #3, 1.0 percent glycerol, 0.15 percent K_2HPO_4, 0.15 percent $MgSO_4$, 1.5 percent agar, adjusted to a final pH of 7.2). The plates were incubated

Table 1. Myxobacterial Isolates and Sources

Myxobacterial Isolate	Source
0-10	Oak Creek
0-4	Oak Creek
M-42	Oak Creek
M-51	Oak Creek
M-53	Oak Creek
M-81	Oak Creek
M-82	Oak Creek
M-83	Oak Creek
M-84	Oak Creek
T-1	Oak Creek Laboratory
T-3	Oak Creek Laboratory
T-6	Oak Creek Laboratory
OC-745	Oak Creek
OC-748	Oak Creek
OC-759	Oak Creek
OC-7510	Oak Creek
OC-763	Oak Creek
OC-786	Oak Creek

at 18°C for three days. Colonies which produced a green fluorescent pigment that diffused into the surrounding medium were picked and restreaked onto King's medium B for purification. Eleven pure cultures of bacteria (P-81, P-82, P-83, P-84, P-85, P-86, P-87, P-821, P-822, P-823, and NP-4) were obtained from Oak Creek and isolate BP-3 was obtained from Berry Creek.

Identification of Pseudomonad Isolates

The isolates were identified using both morphological and physiological tests. The following tests were performed according to the procedures listed in the Manual of Microbiological Methods

(1957): Gram stain, motility, indol production, litmus milk reaction, growth at 4°C and 41°C. Gelatin hydrolysis was measured by the method of Frazier (1926). Utilization of trehalose was determined by the Hugh-Leifson (1953) method. Levan production was tested using 1.5 percent peptone agar enriched with 4 percent filter-sterilized sucrose (Fuchs, 1956). The media developed by King, Ward, and Raney (1954) was used to determine the production of the green fluorescent pigment, pyocyanin, and chlororaphine. Lipolysis was noted by the production of a clear zone around bacterial growth on Cytophaga agar plates containing tributyrin (0.2 percent v/v). Hemolysis was tested by overlaying a nutrient agar base plate with five ml of nutrient agar containing 4 percent washed human erythrocytes (Klinge and Graf, 1958). The egg-yolk agar reaction was carried out using the medium developed by Klinge (1957). The medium consisted of 0.2 percent glucose, 0.25 percent $Na_2HPO_4 . 2H_2O$, 0.05 percent KH_2PO_4, and 1.0 percent agar. An egg-yolk-saline suspension was added to the melted agar to make a final concentration of 10 percent. Denitrification was determined by the method of Stanier, Palleroni, and Doudoroff (1966). The modified nitrate medium consisted of 0.3 percent beef extract, 0.5 percent peptone, 0.5 percent yeast extract, 0.3 percent KNO_3, 0.5 percent glycerol, and 0.4 percent agar. This medium was overlayed after inoculation with 1.0 percent Ionagar (Oxoid).

Maintenance of Cultures

The isolates of myxobacteria and pseudomonads were maintained in Cytophaga agar deeps (0.05 percent tryptone, 0.05 percent yeast extract, 0.02 percent sodium acetate, 0.02 percent beef extract, and 0.4 percent agar). The cultures were incubated at 18°C for two to three days and stored at 4°C. Transfers into fresh medium were made at monthly intervals. The isolates also were lyophilized as soon as possible after isolation and stored in the cold.

Enumeration of Bacteria

Cytophaga-peptonized milk agar supplemented with 7.5 ug/ml neomycin and 10 ug/ml Acti-dione was used to enumerate myxobacterial populations in the artificial streams at Oak Creek Laboratory. Appropriate dilutions of the samples obtained from the experimental streams were made and 0.1 ml from each dilution was spread onto CPM agar. Each dilution was plated in triplicate and the plates were incubated at 18°C. Myxobacteria colonies were counted at 50 and 72 hours using a dissecting scope. At 15X magnification colonies of myxobacteria could be readily distinguished from eubacterial colonies by their characteristic morphology. Typical myxobacterial colonies possess a thin, spreading, rhizoid margin.

Total bacteria were enumerated by diluting the samples and making triplicate "pour-plates" at dilutions of 10^{-3} to 10^{-8}. The plating medium used was Plate Count Agar (PCA), which consisted of 0.5 percent tryptone, 0.25 percent yeast extract, 0.1 percent glucose, and 1.5 percent agar. The plates were incubated at 18°C for five days and the bacterial colonies counted.

Preparation of Bacterial Cell Substrates

Heat-killed cells were prepared from 24 hour broth cultures of Pseudomonas fluorescens, strain NP-4, Escherichia coli, Bacillus cereus, and Sarcina lutea. These organisms were grown in 250 ml of casein hydrolysate medium (0.1 percent $(NH_4)H_2PO_4$, 0.02 percent KCl, 0.02 percent $MgSO_4$, 0.2 percent casein hydrolysate, at pH 6.9). The cells were removed by centrifugation, washed once, and resuspended in 20 ml of distilled water. The suspension was heated to 90°C for ten minutes to kill the cells. A sterility check was made of the preparation in nutrient broth. Fifteen ml of the dead cell suspension was added to 500 ml of CPM agar. The turbid agar was poured into sterile Petri dishes and allowed to harden before inoculation with myxobacteria.

Resting cell suspensions were prepared according to the procedures described in the Manual of Microbiological Methods (1957). After two washings, the cells were suspended in 0.025 M NaH_2PO_4

buffer at pH 7.4 and allowed to stand for 24 hours. These cells were then added to a mineral-salts agar, which contained 0.02 percent $MgSO_4$, 0.1 percent K_2HPO_4, 0.002 percent $CaCl_2$, 0.0002 percent $MnCl_2 \cdot 4H_20$, 0.0001 percent $NaMoO_4 \cdot 2H_2O$, 0.0001 percent $FeSO_4 \cdot 7H_2O$, 0.05 percent NH_4Cl, and 1.5 percent agar.

Plates containing living cells were prepared by overlaying a Cytophaga agar base plate with five ml of soft Cytophaga agar (0.75 percent agar) inoculated with 0.1 ml of a 24-hour bacterial culture.

Assay Procedure for Lytic and Antagonistic Effects

Extracellular enzymes obtained from a myxobacterium were assayed using dead and resting cell suspensions of P. fluorescens. The activity of the enzymes were followed by measuring the decrease in optical density on the Gilford 2000 Multiple Sample Absorbance Recorder. Readings were taken at 660 mμ. Trypsin (0.2 percent) was used as a control.

The cylinder plate method (Schmidt and Moyer, 1944) was used to test the antagonistic activity of crude preparations of pseudomonad and myxobacterial inhibiting substances. King's medium B was used as the base plate with the pseudomonad preparations, and double strength Cytophaga agar was used with myxobacterial preparations. Both plates were overlayed with soft Cytophaga agar inoculated with 0.1 ml of the test organism. When the overlay had

solidified, a stainless steel hollow cylinder (7mm x 10mm) was placed on the agar surface. The cylinder was filled with the test solution and the plates were incubated.

Additional Culture Media Employed

Casein-starch agar contained 0.1 percent tryptone, 0.05 percent yeast extract, 0.05 percent beef extract, 0.05 percent sodium acetate, 0.2 percent soluble starch, 10.0 percent reconstituted skim milk, and 1.5 percent agar. For the elimination of pseudomonad growth 20-25 ug/ml of neomycin was added.

Double strength Cytophaga agar consisted of 0.1 percent tryptone, 0.1 percent yeast extract, 0.04 percent sodium acetate, 0.04 percent beef extract, and 1.5 percent agar. This medium could be supplemented with 0.01 percent brom thymol blue to minimize the myxobacterial growth or with 20-25 ug/ml neomycin to eliminate the pseudomonad growth.

Uschinsky's agar (1893) consisted of 4 percent glycerol, 0.5 percent NaCl, 0.01 percent $CaCl_2$, 0.02 percent $MgSO_4$, and 1.5 percent agar. The pH was adjusted to 7.2.

RESULTS

Oak Creek Laboratory Survey

During the course of studies on the ecology of myxobacteria in natural fresh-water streams, a series of experimental streams became available for study. The streams were designed by Dr. David McIntyre of the Oregon State University Botany Department, Corvallis, Oregon, to study the effects of light intensity and current velocity on algal populations. The arrangement consisted of six wooden troughs, with a number of metal trays (one square meter) lined with various sized rocks as the stream bed. The current velocity was varied by adjusting the speed of a paddle wheel at the end of each stream. Each stream had an exchange rate of 2.5 liters of filtered Oak Creek water per minute. The streams had been in operation nine months prior to sampling, and had well-established populations of algae. A description of the streams is given below:

Stream Number	Description
1	Low light intensity. Slight current velocity due to exchange of water. Abundant algal growth on the bed-rocks, mainly blue-greens. Some growth of Tribonema minor on surface water.
2	Low light intensity. Slow current velocity. Algal masses found only on the bed-rocks, consisting

Stream Number (continued)	Description (continued)
2	predominately of diatoms and a small number of blue-greens.
3	Low light intensity. Fast current velocity. Diatoms very predominate, and a small number of blue-greens, associated with the bed-rocks.
4	High light intensity. Slight current velocity due to exchange of water. Abundant growth of Tribonema minor on the bed-rocks with some surface growth.
5	High light intensity. Slow current velocity. Good growth of blue-green algae with a few diatoms on the bed-rocks. Some surface water growth.
6	High light intensity. Fast current velocity. Some growth of blue-green algae and diatoms on the bed-rocks.

In the initial study, the myxobacterial and total bacterial populations were enumerated in the influent and effluent waters of each stream. The results are shown in Table 2. It can be seen that the myxobacterial population in the effluent water of Stream 3 was 6.6 times greater than in the influent water. In the remaining five streams no proliferation of myxobacteria could be detected. In fact, in Streams 1, 2, and 4 a decrease in the number of myxobacteria was observed. The factors responsible for the increase in the myxobacterial population noted in Stream 3 were not investigated. However, it is interesting to note that the algal population in this stream consisted predominately of

diatoms and these organisms were prevalent in the effluent water. In view of this, it is possible that an association may exist between certain diatoms and myxobacteria in the aquatic environment. It would be interesting to investigate the interactions between these organisms in mixed cultures.

It will be noted from the data presented in Table 2 that although the myxobacterial population in Stream 3 increased markedly, the total bacterial population increased less than two-fold. Assuming the total bacterial population includes the majority of the myxobacteria present in the stream, the increase in eubacteria would be very slight if they increased at all. It would have been interesting to determine the proportion of myxobacteria which were capable of growing on the medium used for determining the total bacterial population.

Since considerable differences were noted with regard to the myxobacterial and total bacterial populations in the effluent waters of the various streams, it was of interest to study the bacterial populations associated with the algal masses. Surveys of the algal masses of each of the streams were carried out over a period of three months.

Each month one of the metal trays was taken from each stream. The algal growth on the bed-rocks was removed as was the surface algae directly above the tray. The algal masses were placed in a

known volume of water and homogenized in a Waring blender. Samples from each stream were prepared in this manner and the total bacteria and myxobacteria enumerated. Table 3 shows the results of these studies.

It can be noted that significant numbers of myxobactera are present in the algal biomass. The myxobacterial numbers in these streams ranged from 2×10^4 organisms per gram of biomass to nearly 10^8 organisms per gram of biomass. Another observation of interest is the fact that except for the significant increase of the myxobacteria in Stream 6 before the last sampling date, the myxobacterial population in Stream 3 is consistently higher than all of the other streams. This observations agrees with the initial studies carried out on the stream effluents. Again this could be due to a relationship between the myxobacteria and the predominant diatom population of the stream. The fast current velocity in Stream 3 may also contribute to the proliferation of the myxobacteria by increasing aeration. Aquatic myxobactera are predominantly strict aerobes. Since Stream 6 also had a fast current, it might be expected that an increase of myxobacteria would be present in this stream if the current velocity were influencing the proliferation of these organisms. It will be noted from Table 3 that except for Stream 3, the myxobacterial population in Stream 6 is higher than the other streams sampled. It would

have been of interest to study these streams in more detail in an effort to ascertain more precisely the factors responsible for the high incidence of myxobacteria in certain streams. Unfortunately, these experimental streams were dismantled after the third sampling and further studies could not be conducted.

Table 2. Bacterial Populations of Influent and Effluent Water of Oak Creek Experimental Streams

Source	Myxobacteria (cells/ml)	Total Bacteria (cells/ml)
Influent	120	673
Stream 1 effluent	70	3100
Stream 2 effluent	37	700
Stream 3 effluent	792	1153
Stream 4 effluent	45	700
Stream 5 effluent	120	1100
Stream 6 effluent	119	1600

The results of the studies on Stream 4 were also of interest. It will be noted that the myxobacterial population in this stream during the first two sample periods was 10 to 100 times lower than in the other streams. The eubacterial population in this stream consisted predominately of pseudomonads. These organisms did not occur to any appreciable extent in the other streams. These observations together with the fact that many pseudomonads are known to have antimicrobial activity, suggests that these organisms may have inhibited the growth of the myxobacteria in the experimental

Table 3. Bacterial Populations in Experimental Strains at Oak Creek Laboratory

Sampling Date	Stream Number	Myxobacteria (number x 10^6 per gram biomass)	Total Bacteria (number x 10^8 per gram biomass)
8/30/66	1	1.84	1.97
	2	2.35	2.51
	3	47.10	1.65
	4	0.34	----
	5	1.16	0.46
	6	6.38	3.89
10/4/66	1	7.13	9.23
	2	3.34	8.01
	3	21.50	4.58
	4	0.02	16.20
	5	0.79	9.45
	6	15.40	19.10
11/3/66	1	13.10	22.60
	2	4.31	8.30
	3	13.40	3.42
	4	7.33	15.90
	5	2.04	21.70
	6	93.70	16.20

streams. The predominant pseudomonad was isolated in pure culture from this stream and studied further to test this hypothesis.

The pseudomonad isolated from Stream 4 was designated P-4. This isolate was identified according to the criteria listed in Bergey's Manual of Determinative Bacteriology, 7th ed. (1957). Morphologically this organism was a Gram-negative rod-shaped bacterium which was actively motile. The isolate liquified gelatin, reduced nitrates, did not produce indole, produced acid from glucose, produced an alkaline reaction with peptonization in litmus milk,

produced a diffusible green pigment, and failed to grow at 37°C. On the basis of these characteristics, the organism could be identified as Pseudomonas fluorescens. Strain P-4 was employed in the subsequent studies to be reported.

Mixed Culture Experiments

Since it was possible that pseudomonads were capable of inhibiting myxobacterial populations in aquatic habitats, it was of interest to examine the interrelationships between these organisms in more detail. Preliminary experiments were carried out to test the effect of Pseudomonas fluorescens, strain P-4 against a number of myxobacterial isolates. In these studies a single streak of pseudomonad inoculum was applied to CPM agar and the myxobacterial inocula were streaked at right angles to it. Inhibition of myxobacterial growth was noted after 48 hours incubation at 18°C. The results of these studies are shown in Table 4. Of the twelve myxobacterial cultures tested the growth of six was found to be strongly inhibited by the pseudomonad, three were slightly inhibited, and the growth of the remaining three of the isolates was not affected by the pseudomonad.

These studies serve to show that antagonistic interrelationships do occur between certain myxobacteria and pseudomonads. Myxobacterial isolate M-51 showed the greatest sensitivity to

Table 4. Effect of Pseudomonad Isolate P-4 on Aquatic Myxobacteria

Myxobacteria Isolate	Inhibition
0-10	-
0-4	±
M-51	+
M-53	±
M-42	+
M-81	+
M-82	±
M-83	-
M-84	-
T-1	+
T-3	+
T-6	+

Symbols: + = inhibition of myxobacterial growth
- = no inhibition of myxobacterial growth
± = slight inhibition of myxobacterial growth

P. fluorescens, strain P-4, and was therefore used in subsequent studies.

Two-hundred and fifty ml Erlenmeyer flasks containing double strength Cytophaga broth were used in the initial mixed culture experiment. The flask was inoculated with approximately equal volumes of the test organisms and incubated at 22°C on a reciprocal shaker. Samples were taken at various intervals and both pseudomonad and myxobacterial populations enumerated. Casein-starch agar was used to enumerate the pseudomonads and the same medium

with 25 ug/ml neomycin, to eliminate the pseudomonads, was used to enumerate the myxobacteria.

The results of these studies are shown in Figure 1. Included in this figure are curves showing the growth of the pseudomonad and myxobacterium in pure culture. It can be noted that growth of P. fluorescens, strain P-4 was unaffected by the myxobacterial isolate. The growth curve for this organism in pure culture was virtually identical with that obtained in mixed culture. Growth of the myxobacterium in the presence of the pseudomonad isolate was found to be markedly inhibited after four hours of incubation. In fact, after this time the myxobacterium could no longer be detected in the culture flask. It can also be noted that the growth rate of the pseudomonad is greater than that of the myxobacterium. As a result, the pseudomonad may have been competing more effectively for nutrients in the medium than the myxobacterium. This could limit myxobacterial growth considerably. However, it should not have caused the rapid decrease observed in the myxobacterial population.

To further examine the interaction between P. fluorescens, strain P-4 and myxobacterial isolate M-51, another mixed culture experiment was carried out. In this experiment the pseudomonad was introduced into a rapidly growing culture of myxobacterium and the growth of the two organisms was followed. Casein-hydrolysate

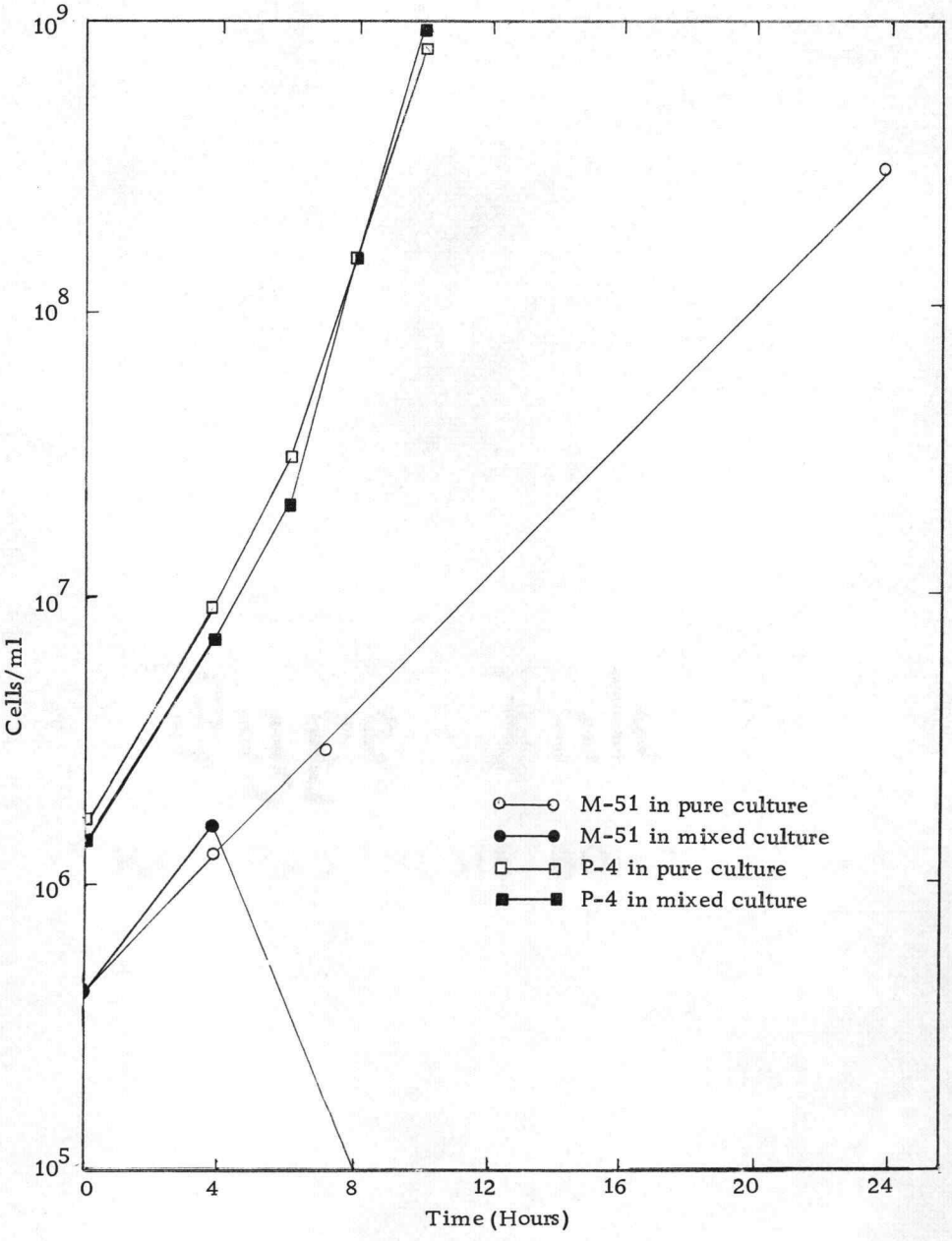

Figure 1. Growth curves of pseudomonad isolate P-4 and myxobacteria isolate M-51 in pure and mixed culture. An initial equal concentration of cells was used in the mixed culture.

broth was used instead of double strength Cytophaga broth to prevent the clumping of myxobacterial cells. The pseudomonads and myxobacteria were enumerated on 0.01 percent brom thymol blue medium and casein-starch agar with 20 ug/ml neomycin, respectively. As can be seen in Figure 2, the myxobacterial cell density was approximately 100 times greater than the pseudomonad. However, this did not appear to have any effect on pseudomonad growth. The myxobacterial population increased slowly during the first twelve hours of the experiment at which time the pseudomonad and myxobacterial cell density were nearly equivalent. While the pseudomonad population continued to increase beyond twelve hours of incubation, the myxobacterial population declined and could no longer be detected at 27 hours. These findings once again indicate that P. fluorescens is capable of inhibiting, and perhaps destroying, certain aquatic myxobacteria. It seems likely that the pseudomonad is producing an antimicrobial agent that is capable of destroying myxobacterial cells. Another possibility which could explain these findings would be rapid autolysis of the myxobacterial cells, however, this phenomenon has never been observed in even aged cultures of the organism.

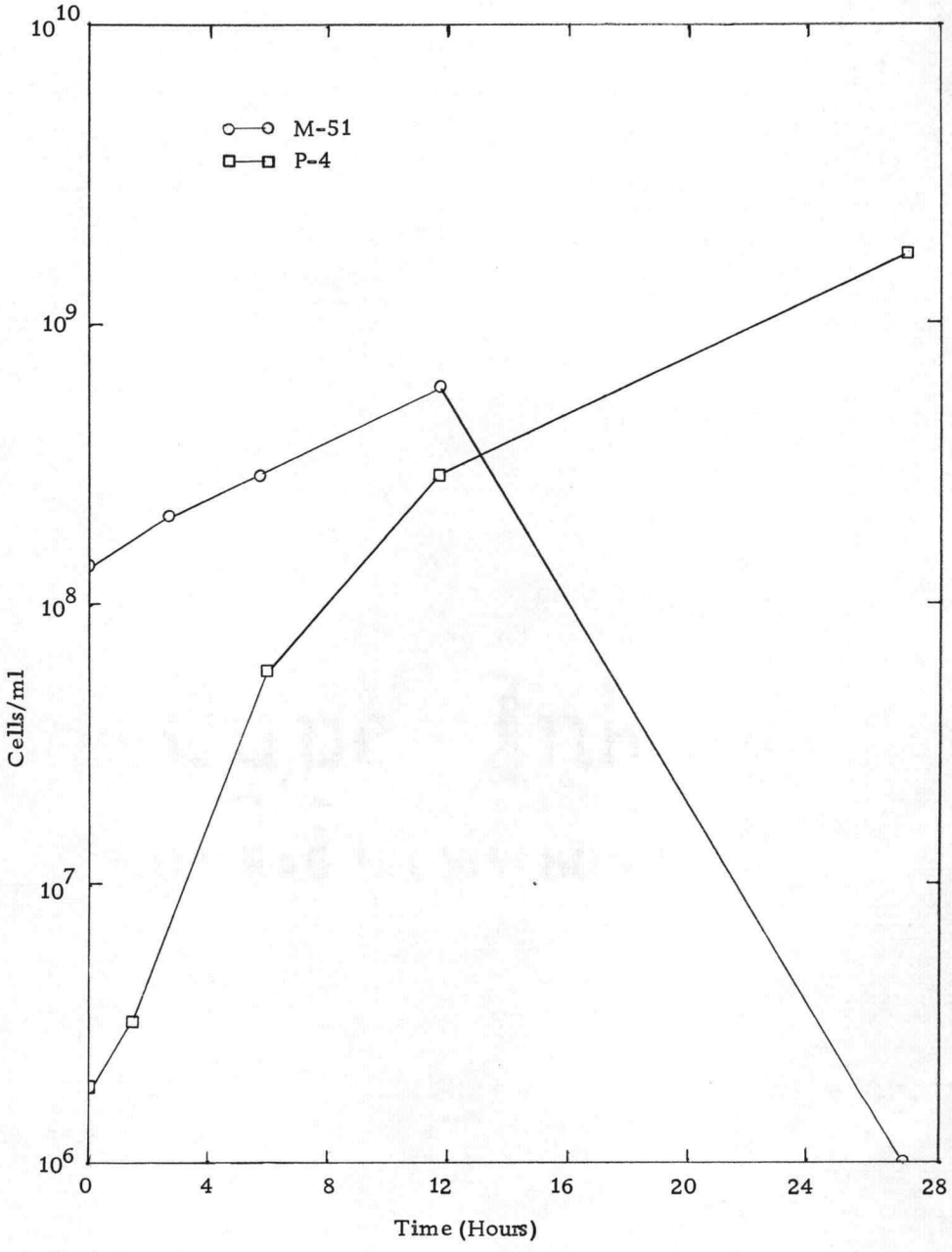

Figure 2. Growth curves of pseudomonad isolate P-4 and myxobacteria isolate M-51 in mixed culture. An unequal concentration of cells was used initially.

Antagonistic Properties of Additional Pseudomonad Isolates

Isolation and Identification of Isolates

Since it appeared that P. fluorescens, strain P-4, produced a substance which inhibited myxobacterial growth, it was of interest to see whether this property was wide-spread among other aquatic pseudomonads. For this reason a number of pseudomonad isolates were obtained from Oak Creek and Berry Creek. These organisms were identified according to the criteria listed by Bergey's Manual of Determinative Bacteriology, 7th ed. (1957) and those reported by Stanier, Palleroni, and Doudoroff (1966).

The egg-yolk agar reaction is an important characteristic in the differentiation of pseudomonads. A number of media were tried before the medium of choice was found. These included the standard egg-yolk agar described in the Manual of Microbiological Methods (1957), 5 percent egg-yolk in liver veal agar (Difco), and the two media described by Klinge (1957). The medium which gave the best results has been described in the Material and Methods section. The typical opaque halo surrounding the bacterial growth is considered to be a positive reaction and is usually evident by 48 hours incubation. Strains P-81, NP-4, and BP-3 initially produced a definite clearing effect of the surrounding medium before the opaque halo appeared. All the egg-yolk positive cultures were accompanied

by the pearly layer caused by the lipolytic activity of the organism (Willis, 1964).

The results of the various physiological tests performed on the pseudomonad isolates are shown in Table 5. Isolates P-83 and P-822 were unable to liquify gelatin and gave a negative egg-yolk reaction and were thereby identified as P. putida. Except for isolate P-82, the remaining isolates were identified as strains of P. fluorescens because of their ability to liquify gelatin, give a positive egg-yolk reaction, grow at 4°C and not at 41°C, produce an alkaline reaction in litmus milk with peptonization, and their failure to produce indole. Isolate P-82 could not be identified utilizing the limited tests employed.

An attempt was made to separate the strains of P. fluorescens into biotypes according to Stanier et al (1966). Strains P-81 and NP-4 correlated to biotype A because of their ability to produce levan, produce acid from trehalose, and their inability to denitrify. Strains P-84, P-85, P-86, and P-821 may also belong to biotype A, but they did not utilize trehalose and their levan production was questionable. Strains P-823 and BP-3 may belong to biotype B because they were able to produce levan and could denitrify. Strain P-87 was not capable of producing levan nor denitrification and therefore could not be classified in a biotype.

Table 5. Characteristics of Pseudomonad Isolates

Strain	Gram Stain	Motility	Gelatin hydrolysis	Egg Yolk Reaction	Levan Production	Denitrification	Hemolysis	Pigment on King's A Medium	Tributyrin	Indol	Litmus milk	Growth at 4°C	Growth at 41°C	Trehalose
P-81	-	+	+	C+	+	-	-	-	+	-	Alk, P	+	-	+
P-82	-	+	+	-	-	+	-	-	+	-	Alk	+	-	-
P-83	-	+	-	-	-	-	-	-	+	-	Alk	+	-	-
P-84	-	+	+	+	±	-	+	-	+	-	Alk, P	+	-	-
P-85	-	+	+	+	±	-	+	-	+	-	Alk, P	+	-	-
P-86	-	+	+	+	±	-	+	-	+	-	Alk, P	+	-	-
P-87	-	+	+	+	-	-	+	-	+	-	Alk, P	+	-	+
P-821	-	+	+	+	±	-	+	-	+	-	Alk, P	+	-	-
P-822	-	+	-	-	-	-	-	-	+	-	Alk	+	-	-
P-823	-	+	+	+	+	+	+	-	+	-	Alk, P	+	-	+
NP-4	-	+	+	C+	+	-	+	-	+	-	Alk, P	+	-	+
BP-3	-	+	+	C+	+	+	-	-	+	-	Alk, P	+	-	+

Symbols:

+ = positive reaction; - = negative reaction; ± = questionable reaction; C = definite clearing zone around pseudomonad growth on egg yolk agar; Alk = alkaline reaction in litmus milk; P = litmus milk peptonized.

Preliminary Tests for Antagonism

The antagonistic activity of the pseudomonad isolates against aquatic myxobacteria was tested by two different procedures. The cross streak method was used in one series of tests. This procedure consisted of inoculating a single streak of the pseudomonad strain on King's medium B and incubating the plate at 22°C for four days. The various myxobacteria isolates were then streaked at right angles to the pseudomonad streak. The plates were incubated for an additional two days at 18°C and the zone of myxobacterial inhibition noted. Bacillus cereus was included as a test organism in this study because it has been reported to be highly sensitive to the products of P. fluorescens (Lewis, 1929). The results of this study are recorded in Table 6. It can be seen that pseudomonad isolates P-81, P-82, P-83, NP-4, and BP-3 inhibited the majority of myxobacterial isolates as well as B. cereus. The remaining pseudomonad strains (P-84, P-85, P-86, P-87, P-821, P-822, and P-823) did not inhibit any of the myxobacterial isolates, except for isolate M-51. It will be noted that isolate M-51 was inhibited by all of the pseudomonad strains as determined by cross streaking. Since it is possible that this inhibition may have been due to a depletion of nutrients, further studies were carried out.

In order to confirm the results obtained by the cross streak method, the antagonistic properties of the pseudomonad isolates

Table 6. Preliminary Tests for Pseudomonad Antagonism

Pseudomonad Strain	OC-7510		M-51		OC-748		OC-786	OC-759	B. cereus	
	AP	CS	AP	CS	AP	CS	AP	AP	AP	CS
P-81	+	+	-	+	sl	-	+	-	sl	+
P-82	+	+	+	+	+	+	+	+	sl	+
P-83	+	+	sl	+	+	+	-	-	+	+
P-84	-	-	-	+	-	-	-	-	sl	-
P-85	-	-	-	+	-	-	-	-	sl	-
P-86	-	-	-	+	-	-	-	-	sl	-
P-87	-	-	-	+	-	-	-	-	sl	sl
P-821	-	-	-	+	-	-	-	-	sl	-
P-822	-	-	+	+	-	-	-	+	sl	-
P-823	-	-	+	+	-	-			+	+
NP-4	+	+	-	+	+	+	+	-	+	+
BP-3	sl	+	+	+	-	-	-	+	+	sl

Symbols: + = inhibition, - = no inhibition, sl = slight inhibition, AP= agar plug, CS = cross streak

were tested by another procedure. The agar plug method was utilized. In this procedure a single streak of the pseudomonad isolates was inoculated onto King's B medium. After the fourth day of incubation small agar plugs were taken near the pseudomonad streak. The plugs were then placed on King's medium B agar. An overlay consisting of soft Cytophaga agar inoculated with 0.1 ml of the test organism was poured over the plugs. A plug from King's medium B was used as a control. The plates were incubated at 18°C for 24 hours and the zones of inhibition surrounding the agar plugs were noted, as shown in Figure 3. It can be seen in Table 6 that

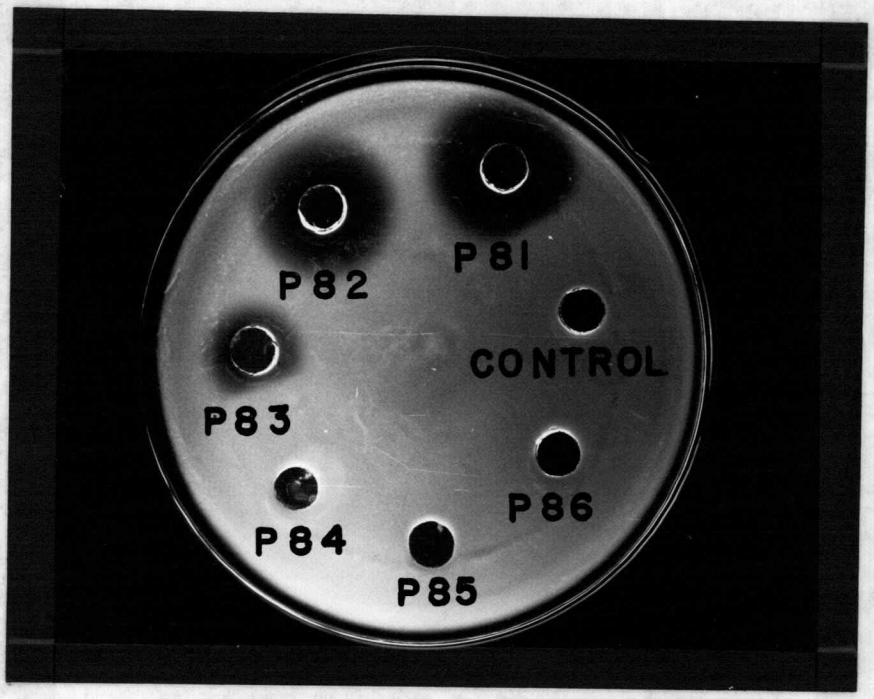

Figure 3. Inhibition of myxobacterial isolate OC-7510 by various pseudomonad isolates using the agar plug procedure.

pseudomonad isolates P-81, P-82, P-83, NP-4, and BP-3 produced detectable zones of inhibition with the majority of the myxobacterial isolates and B. cereus. These findings are in agreement with those obtained in the cross streak tests. However, the results with myxobacterial isolate M-51 differed from those obtained previously. It can be noted also that this organism was sensitive to only five of the pseudomonad isolates as determined by the agar plug procedure. Most likely, in some instances, the inhibition of isolate M-51 obtained using the cross streak method was caused by a depletion of nutrients in the growth medium. The agar plug procedure provides sufficient nutrients to support the growth of the myxobacterium and the zones of inhibition were most likely caused by the diffusible metabolic products of the pseudomonads.

The results of this study indicate that substances capable of inhibiting the growth of myxobacteria are not a universal property of P. fluorescens or P. putida. Furthermore, the host range of the toxic products of these organisms was found to differ. For example, P. fluorescens strain P-81 inhibits myxobacterial isolates OC-7510 and OC-786, but not M-51, OC-748, or OC-759. On the other hand, strain BP-3 inhibits myxobacterial isolate M-51, OC-759, OC-7510 (slightly), but does not inhibit OC-786 or OC-748. This would suggest that several different toxic agents are produced by aquatic pseudomonads.

It was very interesting to note that those strains of P. fluorescens which produced toxic agents against the majority of the myxobacteria also produced an initial clearing effect on the egg-yolk medium before demonstrating the typical opaque halo indicative of a positive reaction. As shown in Figure 4, the toxic strains, NP-4 and BP-3, produce this clearing effect while the other strains (P-87, P-821, and P-823) do not produce the effect. Of course, P. putida strains P-822 and P-83 (not shown) do not produce the effect since the organism gives a negative egg-yolk reaction.

Isolate P-81 was used in subsequent studies on the inhibitory substances produced by a pseudomonad. This strain was chosen because it had been identified as P. fluorescens and produced a good zone of inhibition. Although the toxic agent produced by pseudomonad isolate P-82 exhibited a broader host range, this organism was not investigated because it could not be identified.

Effect of P-81 Culture Filtrate on Myxobacterial Growth

An experiment was performed to demonstrate the effect of the diffusible metabolic products formed by P. fluorescens strain P-81 on the growth of myxobacterial isolate OC-7510. The pseudomonad was grown in King's medium B broth for three days, the cells removed by centrifugation at $4^{\circ}C$ and the supernatant filter-sterilized (Millipore. 0.45 μ). The pH remained at 7.2 during the pseudomonad

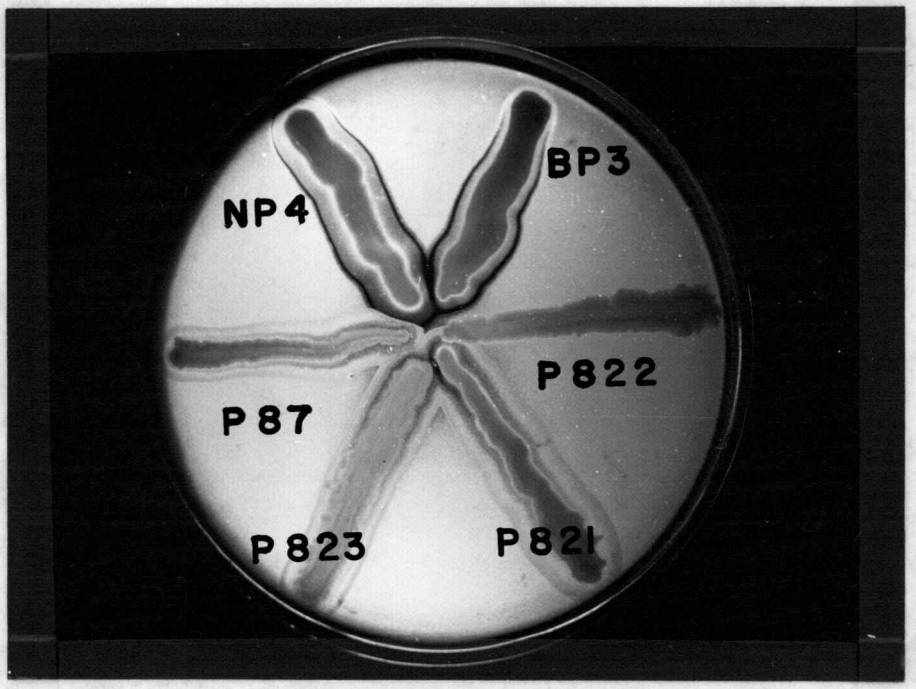

Figure 4. Egg-yolk agar reaction of various pseudomonad isolates. The clearing effect of the toxin producing strains, NP-4 and BP-3, can be noted in contrast to those strains which do not produce toxin.

growth period. A twenty-four hour culture of myxobacterial isolate OC-7510, which had been grown in King's medium B broth, was used to inoculate three flasks. The experimental flask contained 175 ml of P-81 culture filtrate and 17.5 ml of 10X Cytophaga broth. Two control flasks were included. One contained King's medium B broth plus Cytophaga broth and the other contained Cytophaga broth. After inoculation with OC-7510, the flasks were incubated at 22°C on a reciprocal shaker. Samples were taken from the flasks at various time intervals and the myxobacteria enumerated. The results of this experiment are shown in Figure 5. It can be seen that in the presence of the pseudomonad culture filtrate, the growth rate was reduced considerably compared to that of the controls. The generation time of the organisms in the experimental flask was 395 minutes whereas that of the controls ranged from 99 to 122 minutes. A lag phase was observed in the Cytophaga broth. This phenomenon was probably caused by the change in growth medium, i.e., from King's medium B to Cytophaga broth.

These studies served to show that pseudomonad strain P-81 produces a substance (s) which is bacteriostatic to myxobacterial isolate OC-7510. This is indicated by the fact that the growth rate of the myxobacterium is markedly reduced but was not completely inhibited in the presence of the pseudomonad culture filtrate.

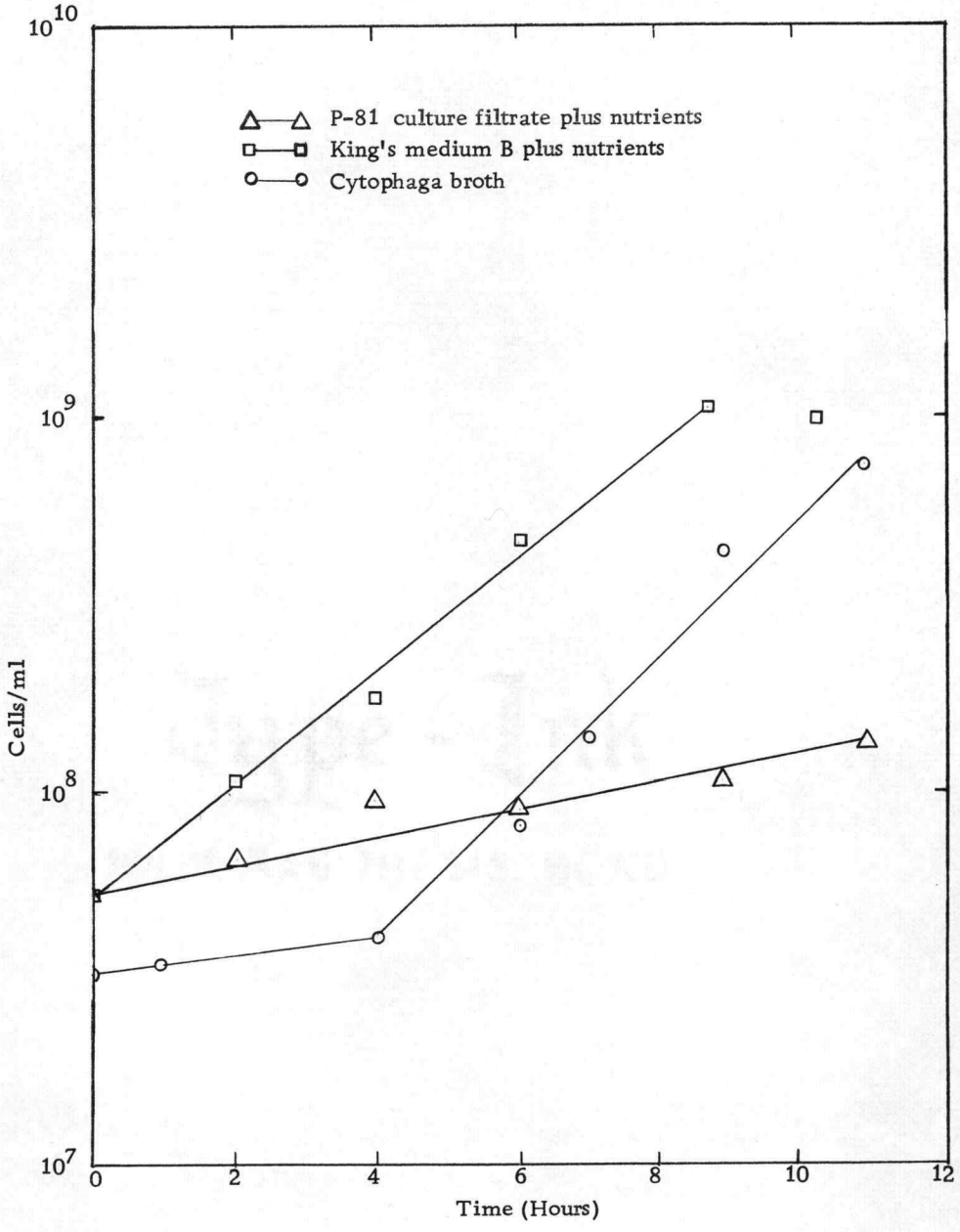

Figure 5. Effect of P-81 culture filtrate on the growth of myxobacterial isolate OC-7510.

Isolation and Properties of Crude P-81 Toxin

Isolation. To obtain a crude toxin preparation, an extract was made from the agar on which strain P-81 had grown. King's medium B or Uschinsky's agar was poured into an aluminum metal tray (8-3/4" x 22" x 5"). Sterile cellophane was placed over the agar surface and inoculated with 20 ml of an overnight culture of P. fluorescens, strain P-81. The tray was then incubated at room temperature for three days. At this time the cellophane sheet was removed together with the abundant pseudomonad growth. The underlying agar was homogenized in a Waring blender and centrifuged. The supernatant was decanted, brought to pH 7.1 and filter-sterilized. This extract was designated "crude P-81 toxin."

Inhibitory Properties of Crude Toxin. The crude toxin was tested against various myxobacterial isolates, Sarcina lutea, and Bacillus cereus. The cylinder plate method (Schmidt and Moyer, 1944) was used to test the antagonistic activity of the crude toxin. King's medium B agar was used as the base plate medium. The plates were overlayed with soft Cytophaga agar inoculated with 0.1 ml of the test organism. When the overlay had solidified, a stainless steel hollow cylinder (7mm x 10mm) was placed on the agar surface. The cylinder was then filled with the crude toxin and the plates were incubated at 18°C or room temperature. Uninoculated medium which had undergone the same extraction procedure as the

crude toxin was used as the control. The results of this study are shown in Table 7. The zone of inhibition became less apparent after 24 hours, due to the fact that the test organisms began to grow in the zone. This same phenomenon was observed using the agar plug preliminary test and the P-81 culture filtrate. Apparently the toxin is either not stable or is inactivated by the test organisms.

Table 7. Inhibitory Activity of P-81 Toxin

Test Organism	Inhibition
Myxobacterial isolates:	
OC-7510	+
O-10	-
O-4	-
M-51	-
M-53	+
M-81	-
M-82	+
M-83	+
M-84	-
T-1	+
T-6	-
Sarcina lutea	+
Bacillus cereus	+

Symbols: + = inhibition - = no inhibition

Physical Properties of Crude Toxin. Heat stability of the crude toxin was tested by placing the preparation in a boiling water bath for 15 minutes. Samples were taken at various times, cooled, and tested for activity using the cylinder plate method. The preparation was also autoclaved for 15 minutes. Uninoculated medium was used as a control. The toxin proved to be heat stable by

withstanding 100°C for 15 minutes. However, the toxin was inactivated by autoclaving for 15 minutes. The control did not have any activity before or after similar heat treatments.

Dialysability of the toxin against distilled water was tested. The crude preparation was dialyzed for 48 hours at 4°C. At this time five ml samples were taken from inside and outside the dialysis tubing and lyophilized. The residues were resuspended in one ml of water and tested for activity. The substances present inside and outside the dialysis tubing inhibited the growth of myxobacterial isolate OC-7510 when tested by the cylinder plate method. Uninoculated Uschinsky's broth was used as a control and a lyophilized five ml portion did not affect the growth of OC-7510. Therefore, it was concluded that the inhibitory substance(s) were dialysable.

Solubility of the crude toxin was tested by lyophilizing five ml portions of the crude preparation, and extracting the dry films with 10 ml of the following organic solvents: 95 percent ethanol, acetone, chloroform, and petroleum ether. The suspensions were thoroughly shaken and the non-soluble residues were removed by filtration (Whatman #1). The organic solvents were removed from the extracts by evaporation. The extracts were resuspended in one ml of water. The residues were air-dried and suspended in two ml of water. A water extract of the lyophilized toxin was used as a control. The solutions were tested for activity against OC-7510 using the cylinder

plate method. The P-81 toxin was very water soluble and was insoluble in the organic solvents tested (Table 8).

Table 8. Solubility of P-81 Toxin

Solvent	Inhibition of Myxobacterial Isolate OC-7510	
	Extract	Residue
95 percent ethanol	-	+
Acetone	-	+
Chloroform	-	+
Ether	-	+
Water	+	....

Symbols: + = inhibition - = no inhibition

Antagonistic Properties of Aquatic Myxobacteria

Myxobacteria are also well-known antagonists. This antagonism results from the production of bacteriolytic enzymes and/or antibiotics by the myxobacteria. These substances have been reported to be produced only by fruiting myxobacteria. It was of interest to determine whether the nonfruiting aquatic myxobacteria isolated from Oak Creek produced any antagonistic substances. If these substances are produced they may play a role in the ecology of these myxobacteria, and could have contributed to the predominance of myxobacteria in Stream 3 in the Oak Creek Laboratory survey.

Bacteriolysis

The bacteriolytic activity of four aquatic myxobacterial

isolates was tested using heat-killed, resting, and living cells of various eubacteria as substrates. The isolates were spot-inoculated onto agar suspensions of the prepared cells. All plates were incubated at 18°C. Lytic activity was indicated by a clearing zone surrounding the myxobacterial growth. The results (Table 9) were recorded at 48 hours. Further incubation up to two weeks did not change the initial results. It can be seen that all the myxobacterial

Table 9. Myxobacterial Attack on Dead, Resting, and Living Eubacteria

Myxobacterial Isolate	*Ps. fluorescens*			*S. lutea*			*B. cereus*			*E. coli*		
	D	R	L	D	R	L	D	R	L	D	R	L
OC-759	+	-	-	sl	-	-	+	-	-	+	-	-
OC-7510	+	-	-	sl	-	sl	+	-	sl	+	-	-
OC-763	+	-	+	sl	-	++	+	-	+	+	-	+
OC-786	+	-	+	sl	-	++	+	-	+	+	-	+

Symbols:
+ = clear zone around myxobacteria growth
- = no clear zone
sl = slight clear zone
D = dead (heat-killed) cells
R = resting cells
L = living cells

isolates were able to attack the heat-killed Gram-negative cells, and to a lesser extent the heat-killed Gram-positive cells. The difference in degree of lysis may be explained by the reports of Salton (1953). Salton has shown that Gram-negative bacteria rupture more readily when heated because of the nature of their cell walls. The

heat will rupture the Gram-negative cells either because of the high lipid content or the different amino acid constitution of their cell walls, as compared to the less sensitive Gram-positive cell walls. As a result the greater sensitivity of Gram-negative cells to the lytic activity of myxobacteria may be due to their increased susceptibility to heat.

The intact resting cells were resistant to myxobacterial attack. This shows that the myxobacterial isolates do not possess bacteriolytic enzymes. If bacteriolytic enzymes were present, the resting cells would have been lysed. Even though no bacteriolytic enzymes were detected, myxobacterial isolates OC-763 and OC-786 were capable of inhibiting the growth of certain eubacterial cells when grown in association with these organisms. Since the resting cells were resistant to lysis by the myxobacteria, the clear zone surrounding the myxobacterial growth must be due to inhibition of the eubacteria by an antibacterial substance produced by these myxobacteria. The factors causing the lysis of heat-ruptured cells and the inhibition of living eubacteria are studied in subsequent sections.

Proteolysis

The fact that the myxobacterial isolates tested exhibited lytic activity on dead eubacterial cells, but not on resting cells suggested

that these organisms possessed proteolytic enzymes capable of lysing ruptured cells. Therefore, it was of interest to examine these isolates in more detail in an effort to demonstrate proteolytic enzymes. Myxobacterial isolate OC-7510 was selected for these studies because it did not inhibit living cells of P. fluorescens, strain NP-4, but it exhibited a marked ability to lyse the heat-killed cells of strain NP-4.

Isolate OC-7510 was grown in one percent yeast extract broth for 30 hours on a rotary shaker at 25°C. The cells were removed by centrifugation at 4°C and the supernatant filter-sterilized. The filtrate was placed in a dialysis tubing and immersed in Carbowax 4000 (Union Carbide, N. Y.) for 20 hours at 4°C. A twenty-fold concentration of the culture filtrate was thus obtained. This crude preparation was used as the extracellular enzyme preparation. The activity of the preparation was determined using heat-killed and resting cells of P. fluorescens, strain NP-4, as substrates. The decrease in optical density of the suspensions served as the criterion for proteolytic activity. The protocol for this experiment is shown below:

Cuvette	Dead Cells	Resting Cells	Buffer	Crude Enzyme
1	0.5 ml	----	2.0 ml	1.0 ml
2	0.5 ml	----	3.0 ml	----
3	----	0.2 ml	2.0 ml	1.0 ml
4	----	0.2 ml	3.0 ml	----

A control experiment was conducted in a similar manner using 0. 2 percent trypsin, a known proteolytic enzyme. The results of the activity assays are compiled in Figure 6. It can be seen that the proteolytic action of the culture filtrate is comparable with that of trypsin. The greater activity of the trypsin as compared to the crude enzyme preparation is most likely due to differences in enzyme concentrations. Since resting cells were not lysed and the heat-killed cells were lysed, it can again be concluded that the myxobacterium studied possessed a proteolytic enzyme which attacks the protein materials exposed by the heat-rupture of the cell walls. Furthermore it does not appear that the myxobacterium possesses a bacteriolytic enzyme which is capable of decomposing cell walls. These findings are in agreement with those of Noren (1960a), Bender (1962), and Margalith (1962).

The proteolytic activity of the crude enzyme preparation could be precipitated by acetone. This was done by slowly adding one volume of acetone (-15°C) to the concentrated culture filtrate with constant stirring. The precipitate which formed was removed by centrifugation at 4°C. A second acetone precipitate was collected in the same manner. Both precipitates were resuspended in 0. 25 M NaH_2PO_4 buffer and extensively dialysed against the same buffer at 4°C. These two fractions were then assayed for proteolytic activity using heat-killed P. fluorescens cells as the substrate.

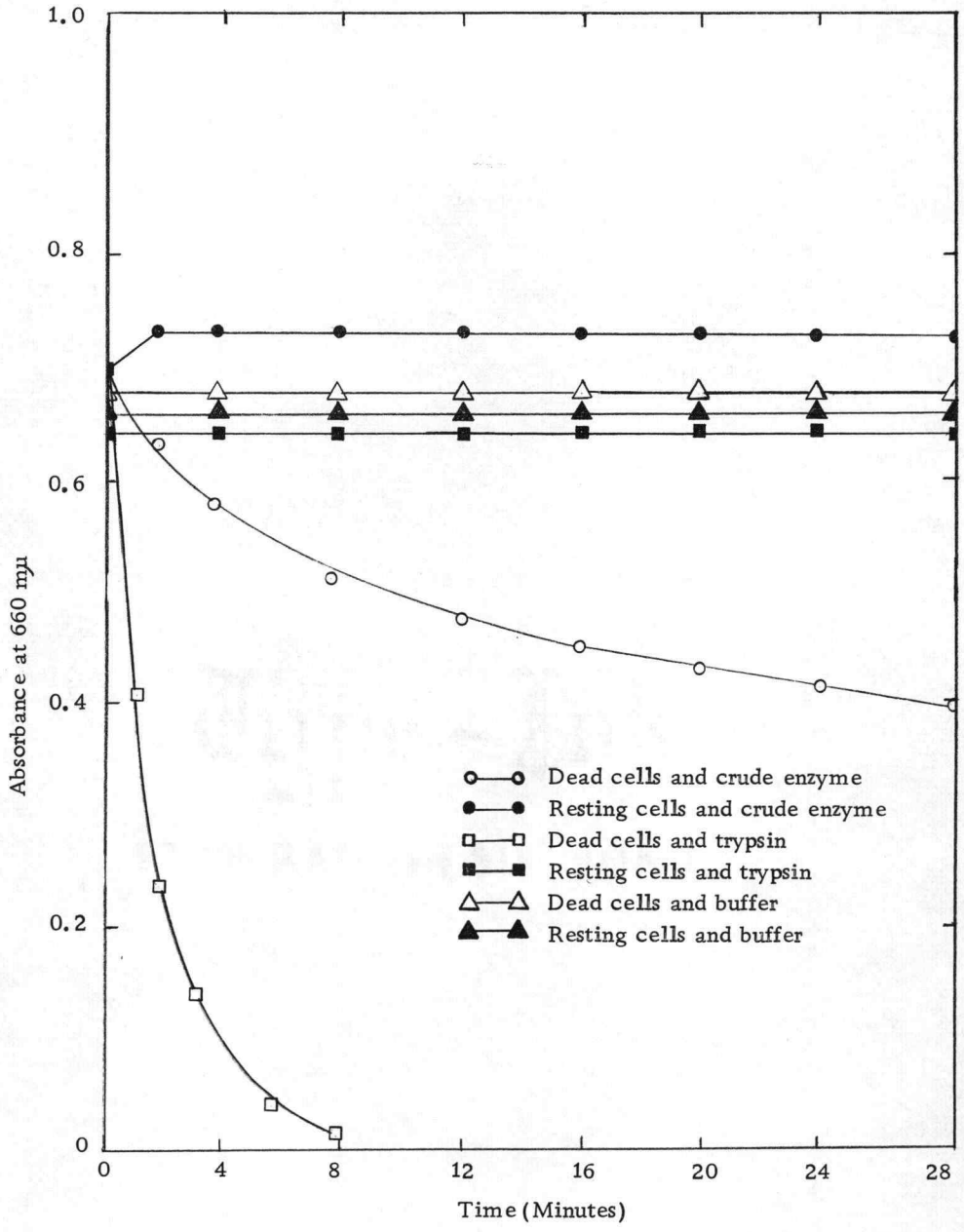

Figure 6. Activity of crude extracellular enzymes from myxobacterial isolate OC-7510 and trypsin on dead and resting cells of P. fluorescens, strain NP-4.

Figure 7 shows all the proteolytic activity in the first volume acetone precipitate and no activity in the second precipitate.

Myxobacterial Antibiotic

The inhibition of eubacteria by certain aquatic myxobacteria (Table 9) indicated a possible production of an antibiotic substance. Further studies were carried out to determine the nature of the inhibition.

Various aquatic myxobacterial isolates were spot-inoculated onto double strength Cytophaga agar plates which had been dried at 37°C for 24 hours. The plates were then incubated at 18°C for one to two days. At this time, the plates were exposed to chloroform vapors for 30 minutes and aerated for one hour at 37°C. Five ml of soft Cytophaga agar inoculated with 0.1 ml of the test organism were used to overlay the plates. The plates were then reincubated at room temperature for eubacteria and 18°C for myxobacteria. Table 10 shows that some of the myxobacterial isolates (OC-748, OC-763, and OC-786) produced a substance capable of inhibiting all of the test organisms. Other isolates (M-42 and T-3) produced a substance, which only effected other myxobacteria. Isolate OC-745 did not produce any inhibitory substance.

The production of proteolytic enzymes by the myxobacteria may be the cause of the inhibition. The proteolytic activity of all

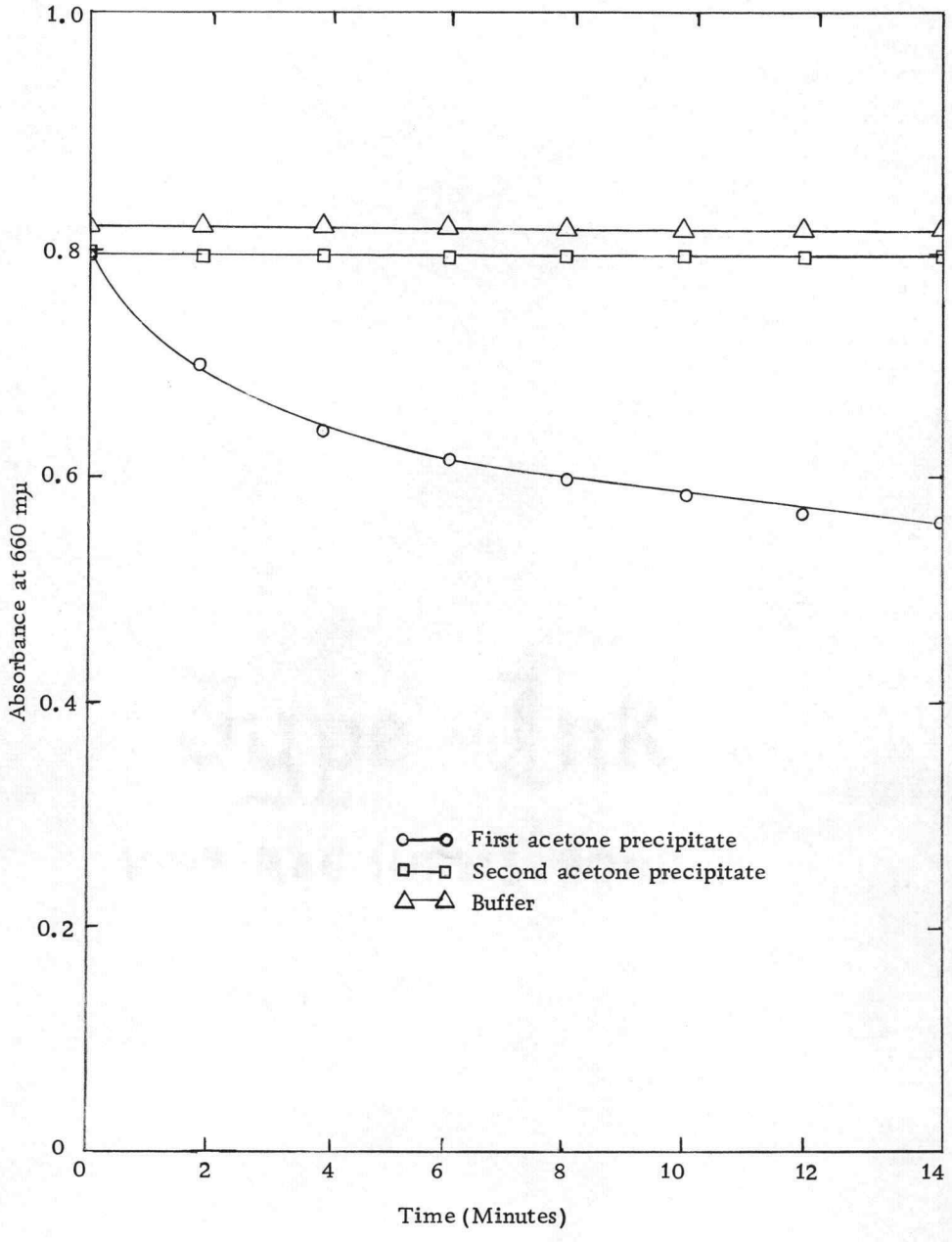

Figure 7. Proteolytic action of acetone precipitates of OC-7510 crude enzyme preparation on dead cells of P. fluorescens, strain NP-4.

Table 10. Inhibition of Eubacteria and Myxobacteria by Aquatic Myxobacteria Isolated from Oak Creek

Test Organisms	Myxobacterial Isolates					
	OC-748	OC-763	OC-786	OC-745	M-42	T-3
Chondrococcus columnaris, strain						
225	+	+	+	-	+	+
234	+	+	+	-	+	+
235	+	+	+	-	+	+
238	+	+	+	-	+	+
244	+	+	+	-	+	+
2-B58-5b(1d)	+	+	+	-	+	+
Myxobacterial Isolates						
OC-759	+	+	+	-	+	+
OC-745	+	+	+	-	+	+
OC-748	+	+	+	-	+	+
Eubacteria						
E. coli	+	+	sl	-	-	-
A. aerogenes	sl	sl	sl	-	-	-
S. lutea	+	+	+	-	-	-
B. cereus	sl	+	+	-	-	-
B. subtilis	sl	+	+	-	-	-
P. fluorescens, strain NP-4	+	+	+			

Symbols: + = inhibition - = no inhibition
sl = slight inhibition

of the myxobacterial isolates used in the above study was determined by the method previously described using heat-killed cells of P. fluorescens, strain NP-4. All of the isolates were shown to possess proteolytic activity. If the inhibitory substance was a proteolytic enzyme, isolate OC-745 would be expected to inhibit the test organisms as did isolates OC-748, OC-763, and OC-786. However, isolate OC-745 did not exhibit inhibitory activity. Furthermore, it has been

shown that proteolytic enzymes do not attack intact bacterial cells. Trypsin (0.2 percent) was tested for inhibitory activity against Chondrococcus columnaris by the cylinder plate method. The enzyme failed to show any activity. In view of these findings, it was concluded that the inhibition exhibited by certain myxobacterial isolates was caused by the secretion of an antibiotic substance.

Myxobacterial isolate OC-748 produced strong zones of inhibition when sensitive organisms were tested against its metabolic products. Figure 8 illustrates the inhibition of Sarcina lutea by isolate OC-748. The first zone, which was present at 24 hours of incubation, was caused by the inhibition of S. lutea directly above the area of chloroformed myxobacterial growth. The second zone of inhibition was caused by the subsequent diffusion of the antibiotic into the surrounding medium. Isolate OC-748 was also tested against all the pseudomonad isolates obtained from Oak and Berry Creeks. It inhibited all these organisms to some extent except for isolate P-87. Myxobacterial isolate OC-748 was used in subsequent studies to be reported.

Preparation of Crude Antibiotic. A cell-free preparation of myxobacterial isolate OC-748 was obtained by inoculating the organism onto cellophane-covered double strength Cytophaga agar. After 48 hours of incubation at 18°C, the cellophane was removed together with the cell growth and the underlying agar was homogenized

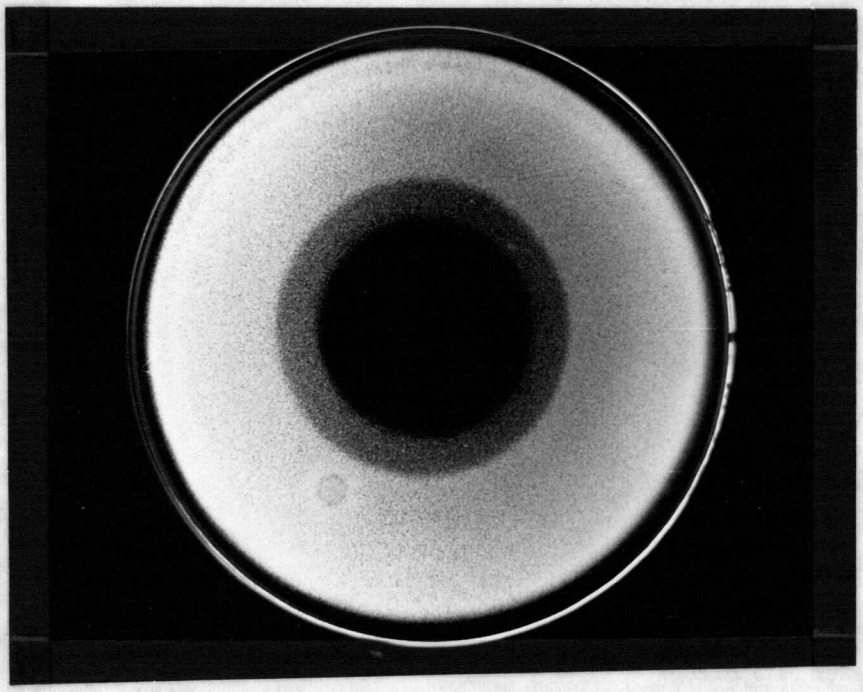

Figure 8. Inhibition of Sarcina lutea by myxobacterial isolate OC-748. First zone observed at 24 hours. Secondary zone formed by the diffusion of antibiotic into the surrounding medium. Photograph taken at 48 hours.

and centrifuged to obtain the "crude antibiotic." This preparation was used to determine the physical properties of the antibiotic. It was later found that the antibiotic was also produced in broth cultures of isolate OC-748.

Inhibitory Properties of the Crude Antibiotic. The crude antibiotic was assayed by the cylinder plate method against a few different species of bacteria (Table 11). It can be seen that, in most cases, these results are comparable to those obtained in the preliminary tests (Table 10). One very important exception between

Table 11. Activity of Myxobacterial Isolate OC-748 Crude Antibiotic Against Various Bacteria

Test Organism	Inhibition
C. columnaris, strain 2-B58-5b(1d)	+
E. coli	+
A. aerogenes	-
S. lutea	+
B. cereus	+
All pseudomonad isolates	-

Symbols: + = inhibition - = no inhibition

the crude antibiotic and the metabolic products present in the spot-inoculated agar is that the crude antibiotic does not have any activity against the pseudomonad isolates. The factor responsible for the inhibition of the pseudomonads on the plate survey has not been determined.

Physical Properties of the Crude Antibiotic. Heat stability, solubility, and dialysability were determined by the methods described for the crude pseudomonad toxin.

The crude antibiotic was heat stable when subjected to 100°C for 15 minutes, but it was inactivated by autoclaving for 15 minutes.

The antibiotic was able to pass through cellophane, as evidenced by the fact that antibiotic activity was present in the underlying agar when strain OC-748 was inoculated onto the cellophane-cover double strength Cytophaga agar. Furthermore, the antibiotic activity in 24-hour broth cultures of strain OC-748 was able to pass through a dialysis membrane. However, the antibiotic activity was non-dialysable in six-day old cultures. This variability in dialysability may be explained by assuming that the antibiotic was trapped somehow by the abundant myxobacterial slime which was present by the sixth day. On the other hand, the antibiotic may be the slime material. Perhaps the slime in the 24-hour culture is present in small units, but in older cultures the slime becomes so copious that it will not pass through the dialysis membrane.

The crude antibiotic, like the pseudomonad toxin, was very water soluble. As shown in Table 12, nearly all of the activity could be removed from the residue with water, but none appeared in the extracts prepared with organic solvents. It was interesting to note that amount of activity recovered from the organic solvent

Table 12. Solubility of Myxobacterial Isolate OC-748 Crude Antibiotic

Solvent	Inhibition	
	OC-748 Antibiotic Extract	OC-748 Antibiotic Residue
95% ethanol	-	+
Acetone	-	+
Chloroform	-	+
Ether	-	+
Water	+	slight

Symbols: + = inhibition - = no inhibition

treated residues by water was essentially equivalent to that obtained by the water treated residue.

Partial Purification of the Antibiotic. The antibiotic activity could be precipitated from the crude preparation by the addition of ethanol. Six volumes of cold (-5°C) 95 percent ethanol was slowly added to the crude antibiotic preparation with constant stirring. The flocculent white precipitate formed was allowed to settle at 4°C for one hour and collected by centrifugation. The precipitate had a waxy appearance. This material was resuspended in one-fifth the original volume of water and reprecipitated with six volumes of 95 percent ethanol. If no precipitate formed, a few crystals of NaCl were added and the precipitate again formed upon standing and could be collected by centrifugation. An alcohol precipitation of the uninoculated culture medium was used as a control. Both precipitates were assayed for antibiotic activity against C. columnaris, strain 2-B58-5b (1d) using the cylinder plate method. The alcohol

precipitate obtained from the OC-748 culture filtrate was very active against the test organism, while the control showed no activity.

Antibiotic activity could be readily precipitated from broth cultures of myxobacterial isolate OC-748. The organism was grown in casein hydrolysate broth enriched with 0.1 percent filter-sterilized glucose. The culture was incubated on a rotary shaker at 22°C for 48 hours. The antibiotic activity was precipitated by the procedure described above. An alcohol precipitate of the uninoculated broth did not exhibit inhibitory activity.

Two methods of drying the partially purified antibiotic were tried. One method involved resuspending the precipitate in a small amount of water followed by lyophilization. It was found that the antibiotic was inactivated by this procedure. However, lyophilization of the broth culture filtrate or the agar supernatant did not inactivate the inhibitory substance. A second method consisted of successively washing the precipitate in 95 percent ethanol, 100 percent ethanol, and anhydrous ether, followed by air-drying. The activity of the precipitate was retained using this method.

The heat sensitivity of the antibiotic was repeated using the dried partially purified preparation. The results were the same as those obtained using the crude antibiotic. Although not repeated with the purified substance, the solubility and dialysability of the antibiotic is most likely to be the same as demonstrated by the crude antibiotic preparation.

DISCUSSION

The present study served to show that under certain conditions myxobacteria make up a significant proportion of the cultivated microflora of aquatic habitats. In the survey carried out in the Oak Creek Laboratory experimental streams, in one instance, nearly 29 percent of the cultivable bacterial population associated with the algal biomass was found to be composed of myxobacteria. This occurred in Stream 3 where the myxobacterial population was composed primarily of one myxobacterial type based on colony morphology. The proportion of myxobacteria among the bacterial population in the effluent of Stream 3 was also high, consisting of nearly 69 percent myxobacteria. Several factors could have contributed to the high incidence of myxobacteria noted. The fact that the current velocity in this stream was rapid would tend to provide ample aeration and thus maintain a high dissolved oxygen content of the water. Since aquatic myxobacteria are predominately aerobic organisms, these conditions should favor their proliferation. However, ample oxygenation should also favor the growth of other aerobic bacteria as well. For this reason additional factors undoubtedly contributed to the predominance of myxobacteria in this stream.

The algal population of Stream 3 was composed

predominately of diatoms. Hence it is possible that an association exists between these organisms which favors myxobacteria growth. Perhaps myxobacteria are capable of utilizing excretory products of certain diatoms more readily than other bacteria. According to Gessner (cited in Brock, 1966) algae excrete primarily amino acids. Since myxobacteria have been shown to utilize amino acids and short peptides as sole sources of nutrient (Dworkin, 1962), it may be possible that amino acids excreted by diatoms could stimulate the growth of these organisms. Additional studies on this interaction between these two groups of organisms would be of interest.

The production of antagonistic substances by the myxobacterial population in Stream 3 also could have contributed to their predominance. Myxobacteria are well-known for their antagonistic properties. The production of bacteriolytic enzymes appears to be the primary mode of this antagonism. These enzymes are capable of lysing intact bacterial cells and are not to be confused with proteolytic enzymes which lyse ruptured bacterial cells. The presence of bacteriolytic enzymes have been reported mainly in the fruiting myxobacteria. To date, only one species of Cytophaga has been found to produce this type of enzyme. In the present study, efforts were made to demonstrate the production of bacteriolytic enzymes by various isolates of aquatic myxobacteria. While

proteolytic enzymes were produced by these organisms, no evidence for the presence of bacteriolytic enzymes was obtained. In view of this, it is unlikely that these substances could have contributed to the high incidence of myxobacteria observed in Stream 3.

Several fruiting myxobacteria isolated from soil have been shown to produce antibiotic substances. In some instances the products produced by these organisms were found to inhibit only Gram-positive bacteria. However, substances active against both Gram-positive and Gram-negative bacteria also have been obtained from other soil myxobacteria. If antibiotics were produced by aquatic myxobacteria, these substances might play a role in the development of populations of these organisms in natural habitats. The only report of antibiotic activity among the fresh-water myxobacteria was provided by Anacker and Ordal (1959). These workers reported on the production of intraspecific antibiotics known as bacteriocins by the fruiting aquatic myxobacterium, C. columnaris.

As a result of the present study, antibiotic substances were found to be produced by some of the fresh-water cytophagas isolated from Oak Creek. This finding was of interest since antibiotic production by members of this genus has not been reported. The inhibitory material produced by one of these organisms was studied and found to possess a wide antimicrobial spectrum. The substance

inhibited the growth of both Gram-positive and Gram-negative organisms. The antibiotic also was found to be heat stable, and highly water soluble. Whether this inhibitory material is produced and active in the microenvironment could not be ascertained. However, since the substance was highly water soluble, it would seem that it is ideally suited for activity in an aquatic environment. Additional studies are needed on these antibiotic materials in order to determine their precise role in natural habitats.

The predominating myxobacterium obtained from the third stream at Oak Creek Laboratory (isolate T-3) produced an antibiotic substance. This substance was only active against other myxobacteria and did not affect the growth of any of the eubacteria tested. Since the myxobacterial flora of Stream 3 consisted predominately of one type of organism, it is possible that the growth of the other myxobacterial species was inhibited by this antibiotic. It would be of interest to study the interaction between the myxobacterium isolated from the stream and other myxobacteria using mixed culture procedures.

Another observation of interest obtained from the surveys carried out in the Oak Creek Laboratory experimental streams was with regard to the predominance of pseudomonads in Stream 4 and their relationship to the low numbers of myxobacteria observed in the stream. The predominant pseudomonad was isolated from

the stream and identified as Pseudomonas fluorescens. This isolate was designated P-4. It was of interest to study the affect of the presence of this pseudomonad on aquatic myxobacteria. A preliminary investigation showed that the pseudomonad inhibited the majority of the myxobacteria tested. In mixed culture with one of the more sensitive myxobacteria, strain P-4 was able to inhibit, and perhaps destroy, the myxobacterium. The inhibition may have been caused by exhaustion of an essential nutrient by the pseudomonad, pH effect, or by the release of an extrametabolite by the pseudomonad. Further studies were then undertaken to determine what factor or factors contributed to the inhibition. However, after prolonged laboratory cultivation, strain P-4 lost its antagonistic properties. This phenomenon was not without precedence. Sieburth (1967) reported that the pseudomonads isolated during his investigation lost their inhibitory powers after prolonged cultivation. Liu (1961) stated that other properties such as lecithinase, hemolysin, and pigment production can also be lost from strains of P. fluorescens, as well as from other members of the fluorescent pseudomonad group. Additional isolates had been obtained from Oak Creek to determine whether inhibitory activity was wide-spread among aquatic pseudomonads and one of these strains was used to further investigate the inhibitory properties of P. fluorescens.

The pseudomonad isolates obtained from Oak Creek were

identified as either P. fluorescens or P. putida, both well-known antagonists. The antagonistic properties of the pseudomonad isolates were determined by the agar plug method. The two strains of P. putida produced substances inhibitory to certain myxobacterial isolates. Some of the strains of P. fluorescens produced inhibitory substances against the majority of the myxobacteria tested, however, this was not characteristic of all of the isolates of the organism. The toxin-producing strains of P. fluorescens could be detected by their reactions on egg-yolk agar. These strains produced a wide clearing zone in the medium preceding the appearance of the typical opaque halo caused by lecithinase. Those strains which did not produce a toxin did not have a wide clear zone. This observation has not been reported by other workers. The strains of P. fluorescens which produce the toxin described by Graf (1958) do not exhibit different egg-yolk reactions from the nontoxigenic strains (Klinge and Graf, 1958). However, Klinge and Graf reported that the strains which produce the toxin hemolyse washed human erythrocyctes. Hemolysis was determined on all the pseudomonad isolates obtained from Oak Creek and the production of inhibitory substances was found to be independent of hemolysis.

P. fluorescens, strain P-81 was used to further investigate the properties of the inhibitory substance. A myxobacterium, shown to be sensitive to the metabolic products of strain P-81, was

inoculated into the culture filtrate of the pseudomonad. The reduced growth rate which was observed showed that the pseudomonad toxin exhibited weak bacteriostatic activity on the myxobacterium. This experiment also showed that the inhibition was not caused by a pH effect, because the pH of the filtrate remained at 7.4 throughout the pseudomonad growth period. Exhaustion of nutrients was also eliminated as a cause of the inhibition, since additional nutrients were added to the culture filtrate.

Many attempts were made to isolate the specific inhibitory substance without success. However, the toxin present in crude preparations was found to be highly water soluble and insoluble in common organic solvents. It was heat stable and able to pass through a dialysis membrane.

The insolubility of the P-81 toxin in alcohol is contrary to the alcohol solubility of the toxic substances from P. fluorescens described by Lewis (1929) and Graf (1958). In view of this finding together with the differing results obtained in the egg-yolk agar and hemolysis reactions, it is necessary to conclude that the P-81 toxin is different than those inhibitory substances previously described. The exact chemical nature of the P-81 toxin cannot be determined without purification.

Although the pseudomonad toxin described in this study is a relatively weak toxin, there is a very important practical aspect

which must be emphasized. In enumerating the myxobacterial population of natural habitats, presence of pseudomonads on the plates could greatly influence the numbers of myxobacteria obtained. The pseudomonads have rapid growth rates and the toxin which some of them produce could inhibit the growth of nearby myxobacterial cells. The addition of neomycin to the medium used for enumerating myxobacteria minimizes the eubacterial growth but does not eliminate it.

It was very unfortunate that studies on the bacterial populations in the experimental streams at Oak Creek Laboratory could not be continued for a longer period of time. It would have been interesting to continue the studies of the interrelationships between the microorganisms occurring in these streams. Additional studies on the chemical nature, mode of action and role of the inhibitory substances described in this report would also be of interest. The precise role which these substances may play in a natural environment is hard to ascertain. The phenomena which are observed under laboratory conditions may not even exist in nature. However, as previously indicated, these highly water soluble substances are ideal if they do play a role in an aquatic environment. In the microenvironments which do exist in nature, these substances could be of great importance.

SUMMARY

A bacteriological survey of experimental streams has shown that, under certain conditions, myxobacteria compose a high proportion of the bacterial population associated with the algal biomass. A number of factors may have caused this increase of myxobacteria. One of the factors could be the production of substances by the myxobacteria capable of inhibiting the growth of other bacteria. Certain aquatic myxobacterial isolates were shown to produce antibiotic substances capable of inhibiting various bacteria. Production of an antibiotic by a non-fruiting myxobacteria has not previously been reported. This antibiotic could be partially purified from myxobacterial culture filtrates by ethanol precipitation. The antibiotic was found to be heat stable and very water soluble.

Another observation made during the study of the experimental streams was an apparent inverse relationship between aquatic myxobacteria and pseudomonads. The pseudomonad isolated from the noted stream was identified as Pseudomonas fluorescens. This strain and other strains isolated from Oak Creek and Berry Creek were shown to produce toxic substances against certain aquatic myxobacteria and Bacillus cereus. This was not a universal characteristic of all strains of P. fluorescens. The strains which did produce this toxin could be detected by their reaction on egg-yolk

agar. They produced a clear zone in the surrounding medium preceding the opaque halo typical of a positive egg-yolk agar reaction. This toxin was obtained in crude form and was found to be heat stable, dialysable and very water soluble.

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