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Occurrence and Characteristics of Nonfruiting Myxobacteria Isolated from Pine Forest Soils

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OCCURRENCE AND CHARACTERISTICS OF NONFRUITING MYXOBACTERIA ISOLATED FROM PINE FOREST SOILS

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by

Paul M. Kulvi

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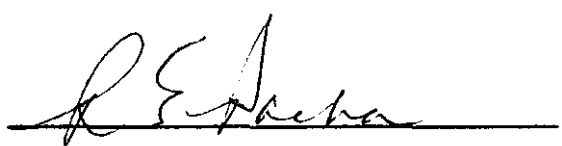

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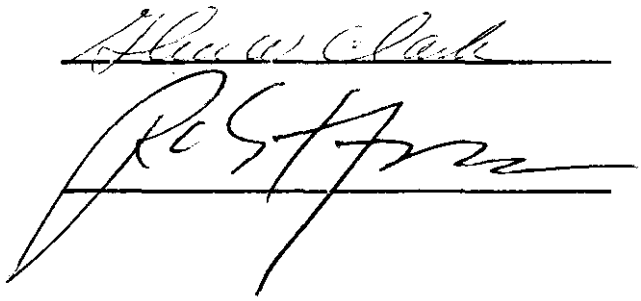

OCCURRENCE AND CHARACTERISTICS OF NONFRUITING MYXOBACTERIA ISOLATED FROM PINE FOREST SOILS

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A study was conducted to contribute to the taxonomy of the nonfruiting myxobacteria and to determine the effects of fire on the occurrence and characteristics of nonfruiting myxobacteria isolated from pine forest soil

Three years after a prescribed burn, the population of nonfruiting myxobacteria was decreased in the burn site. The organisms isolated were distinct from any of the known species of nonfruiting myxobacteria, and apparently are new species.

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INTRODUCTION

The nonfruiting myxobacteria are widespread in soil and natural waters and are important to the ecology of these habitats. These organisms possess degradative properties which fit them for an essential role in the recycling of organic materials which are often relatively resistant to decomposition by other microorganisms (Christensen, 1977). Their role in the humification of plant and animal remains in soil has been demonstrated by Formisano (1947), Kononova (1949), and Christensen (1977). Much of the work has centered around cellulytic activities, however several other complex organic molecules are also known to be degraded and recycled by these organisms.

The eighth edition of *Bergey's Manual of Determative Bacteriology* (1974) lists six genera within the nonfruiting myxobacteria. The most prominent are *Cytophaga* and *Flexibacter*. These two genera are distinguished by the ability of *Cytophaga* members to degrade agar, cellulose, or chitin. Species of *Cytophaga* and *Flexibacter* are characterized by their ability to utilize a variety of carbohydrates and by various other physiological tests.

Stanier (1942) and Christensen (1977) have studied *Cytophaga* while Lewin and Lounsbery (1969) have examined the

genus *Flexibacter*. These investigations established the basis for the separation of the two genera. However, descriptions of the species within the two genera are often incomplete. Data on the ability of many of the species to utilize or produce certain compounds is not available. This prevents differentiation of many non-fruiting myxobacteria. Therefore, the primary purpose of this project was to develop methods which would add to the taxonomy of the nonfruiting myxobacteria.

A secondary purpose was to determine the effects of fire on the occurrence and characteristics of soil nonfruiting myxobacteria. Prescribed burning has been used as a forest management procedure in the preparation of sites for seeding and planting and in increasing the livestock carrying capacity of summer range (Tarrant, 1956; Woodard, 1977). Since microorganisms play a key role in mineralization and decomposition in forest soils, environmental manipulations which affect these organisms could have an effect on soil fertility. Ahlgren and Ahlgren (1960), Neal (1963), Ahlgren (1974), and Husby (1978) have all examined the effects of prescribed burning on microbial populations.

Although studies on this subject were conducted under a variety of conditions and locations, they all indicate an immediate decrease in total bacterial populations following a fire and then an increase to a higher

level than before the burn (Husby, 1978; Neal, 1963; Wright and Tarrant, 1957). Husby (1978) conducted plate counts for total bacteria using soil extract agar (Fred and Waksman, 1928). Since growth requirements for soil bacteria vary greatly, a single medium is inadequate for detecting all species present (Alexander, 1961). Direct counts of numbers of bacteria conducted by Skinner, Jones, and Mollison (1952) gave higher counts than corresponding plate counts. Husby's (1978) results, therefore, underestimated the total number of bacteria present in the sampling sites. Wright and Tarrant (1957) and Husby (1978) noted the greatest effect of burning occurred in the uppermost part of the soil. These data indicate that burning results in increases in soil bacterial populations. Therefore, a secondary purpose of the present investigation was to determine the effects of prescribed burning on the occurrence and characteristics of soil nonfruiting myxobacteria.

The present investigation is a follow-up of the study performed by Husby (1978), in which he examined a variety of factors including the effects of fire on bacterial numbers. These results are contrasted to those of the present investigation.

MATERIALS AND METHODS

Study Area

The study area was located 13 kilometers southeast of Swauk Pass in the Table Mountain region of Kittitas County, Washington at an elevation of 1,730 meters.

The study area was originally a site selected for a prescribed burn study which began in 1975. For the present investigation a control site and a burn site were selected. These sites corresponded to sites three and four used by Husby (1978). The control site was approximately 300 meters from the burn site and each site measured about 64 square meters. The vegetation at both the burn and control sites was described by Woodard (1978).

Bacterial Isolations

The first set of samples was taken on August 9, 1978. These samples were then screened and mixed according to the procedure of Husby (1978). A second set of samples was taken on August 16, 1978 and processed in the same manner.

Ten grams of soil were weighed and serial dilutions were made in sterile, buffered distilled water blanks. A 0.1 ml inoculum of each dilution was aseptically

inoculated onto plates of 0.1% *Cytophaga* Peptonized Milk agar (Carlson and Pacha, 1968). Dilutions of 10^{-2} through 10^{-5} were prepared in this manner. Each dilution was plated in duplicate. In order to inhibit fungal growth, filter sterilized cycloheximide was added to the agar at a final concentration of 100 ug/ml. The plates were incubated at room temperature until growth occurred.

A second method of isolation involved making a single streak of a thick, autoclaved suspension of *Escherichia coli* on the surface of a non-nutrient agar plate containing cycloheximide at a concentration of 100 ug/ml. A gram of soil from each of the soil layers was placed on one end of each streak. The plates were incubated at room temperature until growth occurred.

Colony counts were performed and selected colonies of gram negative organisms were transferred to plates of 1.5% *Cytophaga* agar (Pacha and Porter, 1968). Stock cultures were maintained on 0.4% *Cytophaga* agar deeps stored at 4° C after growth occurred. The stock cultures were transferred to new *Cytophaga* agar deeps at 3-month intervals. Isolates were grouped according to their characteristics, and representatives of these groups were stored in a 50:50 mixture of fetal calf serum and trypticase soy broth at a temperature of -70° C.

All the isolates were labeled by the use of codes, based on the sampling site, the layer of soil, and the

type of plate from which the isolate was taken. The first number in the sequence indicated a particular sampling trip. The location of the sampling site and the soil layer were next, followed by the year, and finally, the actual number of the isolate. The sequence 1-4F-78-1, for example, indicates that the isolate was obtained on the first sampling trip, and was taken from the control site's fermentative layer. The year was 1978 and it was the first organism isolated. If the sequence contained an "A" between the year and the number, it indicated the organism was isolated using an *Escherichia coli* concentrate. A total of 70 isolates were obtained and their code numbers, the sources, and the isolation method used are listed in Table 1.

Cultural Characteristics of Isolates

Temperature Range

The ability of the strains to grow in *Cytophaga* broth at 37, 24, 10, and 4° C was tested. The cultures were observed at regular intervals for growth for 3 weeks.

Anaerobic Growth

The ability of the isolates to grow anaerobically, involved inoculating each isolate onto 1.5% *Cytophaga* agar plates, which were then placed in an anaerobe jar. After 4 days, the plates were examined for growth.

Table 1

Source of Isolates

Stain	Source	Isolation Method
1-4F-78-1 through 1-4F-78-40	Control Site, Fermentative Layer	0.1% *Cytophaga* Peptonized Milk Agar
1-4F-78-A-41 through 1-4F-78-A-56	Control Site, Fermentative Layer	*Escherichia coli* concentrate
1-4M-78-A-1 through 1-4M-78-A-6	Control Site, Mineral Layer	*Escherichia coli* concentrate
1-3F-78-A-1 through 1-3F-78-A-6	Burn Site, Fermentative Layer	*Escherichia coli* concentrate
1-3M-78-A-1 through 1-3M-78-A-2	Burn Site, Mineral Layer	*Escherichia coli* concentrate

Penicillin Resistance

The isolate's resistance to penicillin was tested by adjusting the absorbance of broth cultures of the isolates with *Cytophaga* broth to 0.15 at 525 nm as measured on a Coleman Junior Spectrophotometer (Coleman Instruments Corporation, Maywood, Illinois). Sterile cotton swabs were then used to spread the cultures over the surface of 1.5% *Ctyophaga* agar plates. A 10 mg penicillin disc was placed on the plate and the cultures incubated at room temperature for 3 days. The lack of a clear zone around the disc indicated resistance to penicillin.

Biochemical Activities of Isolates

Hydrogen Sulfide Formation

Hydrogen sulfide formation was detected by inoculating each test organism into a tube of 1% tryptone broth. Strips of lead acetate paper were suspended above the broth in the tube. The tubes were examined after 2 weeks' incubation. A blackening of the strip was indicative of H_2S production.

Catalase Production

Catalase production was tested on 1.5% *Cytophaga* agar slants. After growth had occurred, the slants were flooded with hydrogen peroxide. The presence of catalase was indicated by the production of gas.

Nitrate Reduction

The ability to reduce nitrate was examined using procedures described in the *Manual of Microbiological Methods* (1957). Zinc powder was used to detect false negatives.

Glucose Degradation

The ability of the isolates to use glucose, both aerobically and anaerobically, was tested by using a modification of the Hugh-Leifson procedure. The basal medium consisted of 0.2% peptone, 0.5% NaCl, .03% K_2HPO_4, 0.3% Difco agar, and 0.03% Bromthymol Blue. Glucose was filter sterilized and added at a final concentration of 0.5%. A plug of vaspar overlayed with non-nutrient agar was used to create anerobic conditions. A change in the color of the medium from blue-green to yellow was indicative of the utilization of glucose.

Starch Hydrolysis

The medium used to detect starch hydrolysis contained 0.3% beef extract, 0.5% tryptone, 0.2% soluble starch, and 1.5% agar. The plates were incubated for 3 days and then flooded with Gram's iodine. Colonies with the ability to hydrolize starch produced clear areas in an otherwise blue medium.

Gelatin Liquefaction

Gelatin liquefaction was tested using 0.4% Difco nutrient gelatin. The tubes were incubated for 3 days. The tubes were then refrigerated for 30 minutes and observed for liquefaction.

Tyrosine Degradation

The medium used to test for tyrosine degradation contained 0.5% tyrosine and 1.5% Difco nutrient agar. The plates were incubated for 2 weeks and then examined for the disappearance of tyrosine under the areas of growth.

Carboxymethylcellulose (CMC) Degradation

The medium used to test for CMC degradation contained 0.1% peptone, 1.5% CMC, and 0.7% Difco agar. This is a modification of the method used by Emerson and Wiser (1963). The plates were spot inoculated with broth cultures of each isolate and examined at regular intervals for 1 month for depressions in the agar surrounding the colonies.

Chitin Degradation

The ability to decompose chitin was tested with a chitin suspension prepared according to the procedure of Stanier (1947). A solution containing 0.1% peptone, 0.5% chitin, and 1.0% Difco agar was overlayed onto non-nutrient agar plates and allowed to dry at 37° C for 1 day. Broth cultures of the isolates were spot inoculated onto

the overlay agar and incubated for 3 days. Hydrolysis was detected by the dissolution of chitin around the areas of growth.

Utilization of Dead Bacterial Cells

Bacterial cell agar was prepared with washed cell suspensions of *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas fluorescens*. The growth obtained from each of these organisms in 250 ml of nutrient broth was washed three times in distilled water, suspended in 80 ml of distilled water containing 1.5% Difco agar and autoclaved. To test for lytic activity, non-nutrient agar plates were overlayed with 5 ml of bacterial cell agar and spot inoculated with broth cultures of each isolate. Evidence of lysis was indicated by the appearance of clear zones around the areas of growth.

Utilization of Living Bacterial Cells

The procedure for determining the ability to lyse living cells was essentially the same as for dead cells, except that the cells were washed and sedimented in buffered distilled water and the cell pellet suspended in 5 ml of water to make a thick cell suspension. Streaks of the cell preparations were made on non-nutrient agar plates and each isolate was inoculated at one end of the streak. The plates were incubated for 1 week and observed for growth

of the isolates. The isolates were unable to grow on non-nutrient agar.

Casein Degradation

The medium used to test for casein degradation contained 0.5% tryptone, 0.37% beef extract, 1.5% agar, and 0.2% skim milk. The plates were incubated at room temperature for 48 hours and examined for areas of hydrolysis in an otherwise opaque medium.

Carbon Source Utilization

The ability of the isolates to use various carbon sources was studied using a variation of the mineral base of Stanier (1942). The mineral base consisted of 1.0% $(NH_4)_2SO_4$, 1.0% K_2HPO_4, and 0.2% $MgSO_4$. Sterile *Cytophaga* broth was used to adjust the absorbance of the isolates to 0.15 at 525 nm as measured on a Coleman Junior Spectrophotometer. Single streaks of the isolates were plated onto 1.0% mineral base agar plates and Difco Carbohydrate Differentiation Discs (Difco Laboratories, Detroit, Michigan), each containing 20 milligrams of carbohydrate were placed on the plates.

Carbon sources not available on discs were added to the mineral base at a final concentration of 0.01%.

After a week's incubation at room temperature the plates were examined for growth.

The experiment was controlled by inoculating the isolates onto plates containing 0.01% glucose and onto plates with no carbon source. It had been previously demonstrated that all the isolates were able to use glucose at this concentration. Also, any growth observed that was equivalent to growth observed on the plates without a carbon source, would be attributed to nutrient carryover.

Pigment Production

Pigments present in the isolates were determined using *Cytophaga* broth cultures. The growth obtained from 250 ml of *Cytophaga* broth cultures was sedimented and carotenoid pigments extracted by adding 0.2 grams of anhydrous sodium sulfate and 5 milliliters of a 50:50 mixture of absolute ethanol and ethyl ether. The ether-ethanol mixture was allowed to evaporate and the pigments were resuspended in .45 milliliters of ether. This mixture was then applied to Eastman Kodak Chromatography Paper (#13181) (Eastman Kodak Company, Rochester, New York). The paper was then inserted into a solvent consisting of 93% hexane and 7% abolute ethanol. The solvent was allowed to migrate for 90 minutes after which the R_F values were determined.

RESULTS

Occurrence of Nonfruiting Myxobacteria in Soil

Gliding organisms were recognized by their characteristic colony morphology. Colonies produced were yellow-orange in color, thin, flat, and spreading with irregular edges. Plate counts indicated a higher density of nonfruiting myxobacteria in the control site than in the burn site. Furthermore, the nonfruiting myxobacteria were also more abundant in the fermentative layer of the control site than in the mineral layer. Densities of the organisms at each sample site are in Table 2.

Descriptions of Isolates

The organisms studied in this investigation corresponded to the classical description of myxobacteria. All the isolates were found to be gram negative, rod shaped bacteria which exhibited gliding motility. Gliding motility was verified by using phase contrast microscopy to examine the isolates for their ability to glide over 1.5% *Cytophaga* agar plates.

There were a number of characteristics which the isolates shared in common. All of the organisms demonstrated

Table 2

Density of Myxobacteria in Each Layer of the Sampling Sites

	# of Organisms/Gram of Dry Wt. of Soil			
	Burn Site		Control Site	
Sample	Fermentative Layer	Mineral Layer	Fermentative Layer	Mineral Layer
1	$< 10^2$	$< 10^2$	1.2×10^5	$< 10^2$
2	$< 10^2$	$< 10^2$	1.4×10^5	$< 10^2$

an ability to grow between 4 and 24° C, but were unable to grow at 37° C. This indicates that they are mesophilic in nature. They were also unable to grow on prereduced *Cytophaga* agar plates in an anaerobic environment, indicating they were strict aerobes. However, failure to prereduce the plates, enabled the organisms to produce slight amounts of growth when incubated anaerobically. This suggests that the isolates are capable of limited growth in a microaerophilic environment.

The isolates were able to oxidize, but unable to ferment glucose. This is consistent with the finding that the isolates are strict aerobes. The organisms were all catalase positive and demonstrated an ability to hydrolize both starch and casein and liquefy gelatin.

The isolates demonstrated varying abilities for the remainder of the characteristics, however. Only 4% were able to degrade CMC, 11% were able to degrade tyrosine, and 15% were able to degrade chitin. Thirteen percent were able to grow in the presence of 10 mg of penicillin.

A higher percentage of the isolates, 67%, demonstrated an ability to produce hydrogen sulfide and 87% were able to reduce nitrate to nitrite. The organisms were also able to lyse a variety of living and nonliving bacterial cells. The bacterial cells and the percentage of the isolates that were able to use them are listed in Table 3.

Table 3

Bacterial Cells Utilized by Isolates

Bacterial Cells	Percentage of Isolates Utilizing Dead Cells	Percentage of Isolates Utilizing Dead Cells
Pseudomonas fluorescens	94%	99%
Escherichia coli	94%	91%
Staphylococcus aureus	94%	96%
Bacillus subtilis	87%	97%

The characteristics of each of the isolates are listed in Table 4.

Upon examination of the results, it became evident that the isolates could be tentatively divided into 14 different groups based on the following five tests: H_2S production, nitrate reduction, tyrosine degradation, chitin degradation, and carboxymethylcellulose degradation These groups, their members, and their characteristics are listed in Table 5. A summary of the distinguishing characteristics of the groups is provided below.

Groups 1-4: Organisms in these groups shared the common characteristics of being unable to degrade tyrosine, chitin, and CMC and were differentiated from one another by their abilities to produce H_2S and reduce nitrate.

Table 4

Characteristics of Isolates

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
H_2S	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	-	-	-
NO_2	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Glucose Oxidation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin Liquifaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose Fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine Degradation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth																		
37^{o} C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24^{o} C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{o} C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4^{o} C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chitin Degradation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Table 4 (continued)

	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
H_2S	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NO_2	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+
Glucose Oxidation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin Liquifaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose Fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine Degradation	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
Growth																		
37° C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chitin Degradation	+	+	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-

Table 4 (continued)

	37	38	39	40	$4M_1$	M_2	3	4	5	6	41	42	43	44	45	46	47	48
H_2S	+	+	+	-	-	-	+	-	-	+	-	-	+	+	+	+	-	-
NO_2	+	+	+	+	+ weak	-	+	+	-	+	+	+	+	+	+	+	+	+
Glucose Oxidation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin Liquification	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose Fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine Degradation	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth																		
37° C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chitin Degradation	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4 (continued)

	49	50	51	52	53	54	55	56	$3F_1$	$3F_2$	3	4	5	3F6	$3M_1$	$3M_2$
H_2S	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+
NO_2	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+
Glucose Oxidation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin Liquifaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose Fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine Degradation	+	-	-	-	-	+	-	-	-	-	-	-	+ weak	-	-	+
Growth																
37° C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chitin Degradation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4 (continued)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
CMC Degradation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacterial Cell Lysis																		
Extract:																		
P. fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E. coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. subtilis	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Live:																		
P. fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E. coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B. subtilis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Casein Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Resistance to Penicillin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4 (continued)

	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
CMC Degradation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacterial Cell Lysis																		
Extract:																		
P. fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E. coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B. subtilis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Live:																		
P. fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E. coli	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. subtilis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Resistance to Penicillin	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+

Table 4 (continued)

	37	38	39	40	4M1	M2	3	4	5	6	41	42	43	44	45	46	47	48
CMC Degradation	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Bacterial Cell Lysis																		
Extract:																		
P. fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
E. coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
S. aureus	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
B. subtilis	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
Live:																		
P. fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E. coli	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
B. subtilis	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Casein Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Resistance to Penicillin	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-

Table 4 (continued)

	49	50	51	52	53	54	55	56	$3F_1$	$3F_2$	3	4	5	$3F_6$	$3M_1$	$3M_2$
CMC Degradation	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-
Bacterial Cell Lysis																
Extract:																
P. fluorescens	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E. coli	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. subtilis	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
Live:																
P. fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
E. coli	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+
S. aureus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. subtilis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Resistance to Penicillin	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+

Table 5

Grouping of Isolates Based on Testing

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Class 1 4F22	-	-	+	+	+	+	-	-	-	-	+	+	+	-	-
Class 2 4F03, 4F15	+	-	+	+	+	+	-	-	-	-	+	+	+	-	-
Class 3 4F17, 4F05, 4F06, 4F16, 4F46, 4F10, 4F41B, 4F12, 4M04, 4F50, 4F47, 4M01, 4F41A, 4F49B	-	+	+	+	+	+	-	-	-	-	+	+	+	-	-
Class 4 4F04, 4F09, 4F14, 4F07, 4F01, 4F23, 4F48B, 4F43, 4F44, 4F21, 4F29, 4F30, 4F52B, 4F02, 4F42, 4F34, 4F35, 4F36, 4F36, 4F45, 4F08, 4M06, 4F52A, 4F26, 4F11, 4M03, 4F13, 4F28, 4F27, 3F03, 3F02, 3F04, 4F49A	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-
Class 5 4F32	+	-	+	+	+	+	-	-	+	-	+	+	+	-	-

Table 5 (continued)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Class 6 4F51	-	+	+	+	+	+	-	-	+	-	+	+	+	-	-
Class 7 4F48A 3F05, 4F31, 3M02	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-
Class 8 3M01, 4F18, 4F20	-	+	+	+	+	+	-	-	-	-	+	+	+	+	-
Class 9 4F25, 4F24, 4F39, 4F37, 4F19, 4F38	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-
Class 10 4F33	+	-	+	+	+	+	-	-	+	-	+	+	+	+	-
Class 11 4F10	-	+	+	+	+	+	-	-	+	-	+	+	+	+	-
Class 12 3F06	-	-	+	+	+	+	-	-	-	-	+	+	+	+	+
Class 13 3F01	+	-	+	+	+	+	-	-	-	-	+	+	+	-	+
Class 14 4M05	-	-	+	+	+	+	-	-	+	-	+	+	+	-	+

Table 5 (continued)

Tests
1. H_2S Production
2. NO_2 Reduction
3. Glucose Oxidation
4. Starch Hydrolysis
5. Gelatin Liquifaction
6. Presence of Catalase
7. Anaerobic Growth
8. Glucose Fermentation
9. Tyrosine Degradation
10. Growth at 37^{o} C
11. Growth at 24^{o} C
12. Growth at 10^{o} C
13. Growth at 4^{o} C
14. Chitin Degradation
15. CMC Degradation

Groups 5-7: Organisms in these groups shared the ability to degrade tyrosine, but not chitin or CMC and were differentiated from one another by their abilities to produce H_2S and reduce nitrate.

Groups 8-9: Organisms in these groups possessed the ability to degrade chitin, but were unable to act upon tyrosine or CMC. Organisms in group 9 were able to produce H_2S and reduce nitrate, while group 8 failed to produce H_2S.

Groups 10-11: The organisms in these two groups were able to degrade both chitin and tyrosine, but demonstrated varying abilities to produce H_2S and reduce nitrate.

Groups 12-14: Organisms in these groups were all able to degrade CMC and 14 also was able to degrade tyrosine. Again, these groups were differentiated by their abilities to produce H_2S and reduce nitrate.

Pigment Extraction

In order to further characterize the isolates, representatives of each of the groups of isolates were selected, their pigments extracted, and the R_f values of the pigments determined. The R_f values indicated a wide variety of pigments present in the isolates. Most of the isolates contained two different pigments, while some

contained as many as three pigments and some as few as one. The R_f values ranged from as low as .111 to as high as .588.

The isolates used and the R_f values of their pigments are listed in Table 6.

Carbon Source Utilization Patterns

In order to develop a carbon source utilization pattern, representatives of each of the tentative groups of isolates were tested for their ability to use a variety of carbon sources. Examination of the results indicated that the carbon sources could be divided into three groups based on the number of organisms that were able to use them. Arabinose, Xylose, Mannose, Dulcitol, Sucrose, Maltose, Galactose, Acetic Acid, and Pyruvic Acid were all used by more than 71% of the isolates. Levulose, Inulin, Lactose, Rhamnose, Inositol, Salicin, and Citric Acid were used by between 7 and 57% of the isolates. Mannitol, Sorbitol, Adonitol, Benzoic Acid, D-L Malic Acid, Propionic Acid, and Succinic Acid were not used by any of the isolates. The isolates and the carbon sources used are listed in Table 7.

Table 6

R_f Values of Pigments Extracted

Isolates	R_f Values		
1-4F-78-22	.293	.457	.574
1-4F-78-15	.111	.222	
1-4F-78-06	.054	.190	
1-4F-78-04	.110	.194	
1-4F-78-32	.108	.215	
1-4F-78-51	.111	.222	
1-4F-78-24	.268	.463	
1-4F-78-33	.215	.300	.432
1-4F-78-31	.273		
1-4F-78-40	.155	.244	.447
1-3F-78-A-6	.522		
1-3F-78-A-1	.194		
1-4M-78-A-5	.211	.341	.588

Table 7

Carbon Source Utilization Pattern

Isolates	Arabinose	Adonitol	Dulcitol	Galactose	Inositol	Inulin	Lactose	Levulose
Flexibacter								
1-4F-78-22	++	-	+	++	-	-	-	-
1-4F-78-15	++	-	+	++	-	-	+	-
1-4F-78-06	++	-	+	++	-	-	-	+
1-4F-78-04	++	-	+	++	-	+	-	+
1-4F-78-32	++	-	-	++	-	-	-	+
1-4F-78-51	-	-	-	++	-	+	-	+
1-4F-78-31	+	-	+	++	-	-	-	-
Cytophaga								
Category 1								
1-4F-78-18	+	-	-	++	-	+	-	+
1-4F-78-24	+	-	+	++	-	-	-	-
1-4F-78-33	++	-	+	++	-	-	-	-
1-4F-78-40	+	-	+	++	-	-	-	+
Category 2								
1-4F-78-A6	++	-	+	++	-	-	-	+
Category 3								
1-3F-78-A-1	-	-	+	+	+	+	-	+
1-4M-78-A-5	-	-	-	+	+	-	-	+

++ = heavy growth

+ = light growth

- = no growth

Table 7 (continued)

Isolates	Maltose	Mannitol	Mannose	Rhamnose	Salicin	Soritol	Sucrose	Xylose
Flexibacter								
1-4F-78-22	++	-	++	-	-	-	-	++
1-4F-78-15	++	-	++	-	-	-	-	++
1-4F-78-06	++	-	++	-	-	-	+	++
1-4F-78-04	++	-	++	-	-	-	+	++
1-4F-78-32	++	-	++	-	-	-	-	++
1-4F-78-51	++	-	++	-	-	-	+	++
1-4F-78-31	++	-	++	-	-	-	+	++
Cytophaga								
Category 1								
1-4F-78-18	++	-	++	-	-	-	+	++
1-4F-78-24	++	-	++	-	-	-	+	++
1-4F-78-33	++	-	++	-	-	-	+(weak)	++
1-4F-78-40	++	-	++	-	-	-	+	-
Category 2								
1-4F-78-A-6	++	-	++	-	+	-	+	++
Category 3								
1-3F-78-A-1	++	-	+	+	+	-	+	+
1-4M-78-A-5	++	-	-	+	+	-	-	+

Table 7 (continued)

Isolates	Acetic* Acid	Benzoic* Acid	Citric* Acid	DL-Malic* Acid	Proprionic* Acid	Pyruvic* Acid	Succinic* Acid
Flexibacter							
1-4F-78-22	++	-	+	-	-	+	-
1-4F-78-15	++	-	+	-	-	+	-
1-4F-78-06	++	-	+	-	-	+	-
1-4F-78-04	++	-	+	-	-	+	-
1-4F-78-32	++	-	-	-	-	+	-
1-4F-78-51	++	-	+	-	-	+	-
1-4F-78-31	++	-	+	-	-	+	-
Cytophaga							
Category 1							
1-4F-78-18	++	-	-	-	-	-	-
1-4F-78-24	++	-	-	-	-	+	-
1-4F-78-33	++	-	+	-	-	+	-
1-4F-78-40	++	-	-	-	-	-	-
Category 2							
1-4F-78-A-6	++	-	+	-	-	-	-
Category 3							
1-3F-78-A-1	++	-	+	-	-	+	-
1-4M-78-A-5	+	-	-	-	-	+	-

*Acetic, Benzoic, Citric, Malic, Proprionic, Pyruvic, and Succinic acids were analyzed at concentrations of 0.01%

DISCUSSION

A study was conducted to further contribute to the taxonomy of the nonfruiting myxobacteria and to determine the effects of fire on the occurrence and characteristics of these organisms. These studies were conducted 3 years after a prescribed burn.

Previous studies have demonstrated increases in soil bacterial populations following prescribed burns (Ahlgren, 1974; Husby, 1978; Neal, Wright, and Bollen, 1965; and Sims, 1976). Wright and Tarrant (1957) observed that the most significant increases occurred in the uppermost layers. Neal (1963) noted an increase in 2 days. In the present investigation, relatively few nonfruiting myxobacteria were demonstrated in the burn site, whereas large numbers of these organisms were demonstrated in the control site. This decrease is probably due to the destruction of litter by fire. The isolates obtained in the present investigation are capable of degrading a wide range of macromolecules and utilizing a variety of carbon sources. It is likely that the litter, which is formed from fallen vegetation, plays a large role in supplying nutrients to the nonfruiting myxobacteria. Thus, when the litter and vegetation in the burn site were destroyed by fire, the nonfruiting myxobacteria were deprived of their main source of nutrients.

Husby (1978), in his investigation of the sampling sites used in the present investigation, noted an increase in the total bacterial population following the burn. Since the isolates were able to utilize a variety of bacterial cells, it was of interest to note that the population of the nonfruiting myxobacteria decreased, whereas the total bacterial population increased. A possible explanation is that the organisms proliferating in the burn site are not a sufficient source of food for the nonfruiting myxobacteria. The disparity in numbers between the two layers of the control site might be the result of the nutrients in the litter being utilized before they are able to diffuse into the lower layer. However, a more complete investigation of these results needs to be conducted.

Due to the limited number of tests used in the characterization of myxobacteria and the paucity of information concerning the tests results, the identification of nonfruiting myxobacteria is difficult. The eighth edition of *Bergey's Manual of Determinative Bacteriology* (1974), includes the nonfruiting myxobacteria under the order *Cytophagales*. The most prominent family in this order is *Cytophagaceae*, and the most prominent genera are *Cytophaga* and *Flexibacter*. All of the isolates obtained in this study were from these two genera. The distinguishing characteristic between these two genera is the ability of the *Cytophaga* members to degrade agar, cellulose, or chitin.

As is evident from the results, several of the isolates were able to degrade chitin and/or carboxymethylcellulose. These isolates were thus considered to be members of the genus *Cytophaga*. Further characterization was difficult and attempts to speciate the isolates were unsuccessful.

In order to facilitate speciation of both the *Cytophagas* and *Flexibacters*, the isolates were tested for their ability to utilize a wide variety of carbon sources. Representatives from each class of isolates were investigated and a wide ranging carbon utilization pattern was developed.

On the basis of their ability to degrade chitin or CMC, the *Cytophaga* isolates could be divided into three different categories. The categories, the tentative groups within the categories, and the ability of the isolates to degrade chitin and CMC are listed in Table 8. The isolates in Category 1 were able to degrade only chitin. The isolates in Category 2 were able to degrade both chitin and CMC. The isolates in Category 3 were able to degrade only CMC. An examination of Table 7 indicates that Categories 1 and 2 are similar to one another, but quite different from category 3. The isolates in Category 3 generally used carbon sources not used by the rest of the isolates and were not as capable of using carbon sources readily utilized by the remaining isolates.

Table 9 lists the recognized species within the genus *Cytophaga* and their primary identifying characteristics.

Table 8

Cytophaga Categories

Categories	Groups Within Each Category	Degradation of Chitin/CMC	
1	8, 9, 10, 11	+	-
2	12	+	+
3	13, 14	-	+

It also lists the applicable results for the *Cytophaga* isolates obtained in the present study. An examination of this table reveals that all three categories closely resemble *C. johnsonae*. However, there are two major differences and one potential difference. *C. johnsonae* uses arabinose and does not produce catalase. The isolates in the first two categories are able to use arabinose, whereas the isolates in the third category are unable to use this carbohydrate. Also, all the isolates in each of the categories produce catalase. The isolates in the second and third categories are able to utilize CMC while *C. johnsonae* does not use cellulose. These potential differences between categories 2 and 3 and *C. johnsonae* should be investigated further by examining a strain of *C. johnsonae* along with the present isolates.

These results suggest the three categories represent new species. A tentative description of these categories is presented in Table 10.

Table 9

Characteristics of the Species of Genus *Cytophaga* and Applicable Characteristics of the *Cytophaga* Isolates

	Attack on									
	Agar	Cellulose	Chitin	Starch	Glucose	Galactose	Xylose	Arabinose	Catalase	NaCl Required
1. *C. hutchinsonii*	-H	C	-H		+	-	-	-	+	-
2. *C. rubra*	-H	C	-H		C	-	C	-	+	-
3. *C. johnsonae*	-H	-	C	C	C	C	C	C	-	-
4. *C. krezmieniewskae*	L	D	-	D	+	+	+	-	+	+
5. *C. diffluens*	L	dD	-	d	d	d			-	+
6. *C. lytica*	H	-	-	H	+	+			+	+
7. *C. salmonicolor*	dH		-	+		+		+	+	+
8. *C. fermentans* subsp. *fermentans*	H;-C	-C	-C	+	+	-	+		+	+
9. *C. fermentans* subsp. *agarotorans*	H	-	-H	+	+	+	+			+
Cytophaga Categories										
1 and 2	-H		+H	+	+	+	+	+	+	-
3	-H		+H	+	+	+	+	-	+	-

For the carbon compounds listed, + or - refers to utilization, or not, of a given substrate. Where the data permit, more explicit information is conveyed by: D = decomposed; l = liquified; C = serve as (-C will not serve as) source of carbon and energy; H = hydrolyzed; -H not hydrolyzed; d = some strains positive, some strains negative.

Table 10

Percentage of Isolates Per Category Positive for Each of the Characteristics

Characteristics	Categories 1	2	3
H_2S Production	63	0	50
Nitrate Reduction	90	0	0
Glucose Oxidation	100	100	100
Starch Hydrolysis	100	100	100
Gelatin Liquefaction	100	100	100
Catalase	100	100	100
Glucose Fermentation	0	0	0
Tyrosine Degradation	18	0	50
Growth 37	0	0	0
24	100	100	100
10	100	100	100
4	100	100	100
Chitin Degradation	100	100	100
CMC Degradation	0	100	100
Bacterial Cell Lysis			
Dead: *P. fluorescens*	100	100	100
E. coli	100	100	100
S. aureus	90	100	100
B. subtilis	100	100	100
Live: *P. fluorescens*	100	100	100
E. coli	90	100	50
S. aureus	81	100	100
B. subtilis	90	100	100
Penicillin Resistance	9	0	0
Casein Hydrolysis	100	100	100

Table 11 presents the recognized species of the genus *Flexibacter* and their primary identifying characteristics. It also lists the applicable results for the *Flexibacter* isolates. An examination of this table and the results obtained in the present investigation reveal a wide difference between the *Flexibacter* organisms isolated in this study and known species of *Flexibacter*.

The *Flexibacter* isolates used in this investigation all produce catalase, whereas *F. succinicans* hydrolyzes starch and utilizes glucose, sucrose, and galactose, as do the isolates. However, there is no mention of the ability of *F. succinicans* to utilize acetate, hydrolyze gelatin, or produce hydrogen sulfide. *F. succinicans* is also able to grow anaerobically. The isolates studied in the present investigation, however, are strict aerobes.

The isolates resemble several of the other genera. However, none of these genera possess catalase and two fail to hydrolyze starch.

Based on the above comparisons, it is reasonable to assume that the *Flexibacter* isolates represent new species. A tentative description of these species is presented in Table 12.

As is evident, the critera which are currently available for the characterization and identification of *Cytophaga* and *Flexibacter* are far from adequate. *Cytophaga* and *Flexibacter* isolates may undergo many changes during

Table 11

Differential Characteristics of the Species of Genus *Flexibacter* and Applicable Characteristics of the *Flexibacter* Isolates

	Starch hydrolysis	Carboxymethyl cellulose depolymerization	Carbon Compounds Utilized						H_2S production	Gelatin hydrolysis	Catalase
			Glucose	Galactose	Sucrose	Acetate	Lactate	Glycerol			
1. *F. flexilis*	-	-	d	d	+	d	-	-	+	+	-
2. *F. aggregans*	-	d	+	+	+	d	d	d	-	-	-
3. *F. giganteus*	+	-	+	d	-	-	-	-	d	+	-
4. *F. tracuosus*	d	-	+	d	d	d	-	d	d	+	-
5. *F. succinicans*	+		+	+	d		-	-			+
6. *F. columnaris*	-		+							+	
Flexibacter Isolates	+	d	+	+	d	+			d	+	+

+ = 90% or more strains positive

- = 90% or more strains negative

d = some strains positive, some strains negative

Table 12

Percentage of *Flexibacter* Isolates Positive for Each Characteristic

Characteristics	*Flexibacter* Isolates
H_2S Production	69
Nitrate Reduction	91
Glucose Oxidation	100
Starch Hydrolysis	100
Gelatin Liquefaction	100
Catalase	100
Glucose Fermentation	0
Tyrosine Degradation	16
Growth 37	0
24	100
10	100
4	100
Chitin Degradation	0
CMC Degradation	0
Bacterial Cell Lysis	
Dead: *P. fluorescens*	92
E. coli	92
S. aureus	94
B. subtilis	85
Live: *P. fluorescens*	98
E. coli	92
S. aureus	98
B. subtilis	98
Penicillin Resistance	14
Casein Hydrolysis	100

subcultures and, in addition, investigators often use different characterization procedures. This could account for the discrepancies in the results of various investigations of these organisms. Much of what is known about the nonfruiting myxobacteria, and *Cytophaga* in particular, is drawn from older work and is in need of corroboration. The works of Lewin and Lounsbery (1969) and Christensen (1977) have contributed greatly to the knowledge of these organisms. More work is needed, however. One solution might be a study of the carbon sources able to be utilized by *Cytophaga* and *Flexibacter*. The investigation of aerobic pseudomonad organisms made by Stanier, Palleroni, and Doudoroff (1966) would serve as an excellent reference for such a study.

A second beneficial area of study might involve an investigation into the varieties of cartenoid pigments present in nonfruiting myxobacteria. The pigments which were extracted in the present study are presumed to be carotenoids based on their yellowish-orange color. Lewin and Lounsbery (1969) have performed a preliminary study on carontenoid pigments extracted from several *Flexibacter* species. Seven different carotenoid pigments were identified based on their absorption spectra and their migration patterns on different qualities of chromatography paper (Eastman Kodak Company, Rochester, New York), using various solvent system. These pigments aided in the

speciation of the *Flexibacter* isolates studied by Lewin and Lounsbery (1969). Undoubtedly further investigation would prove quite beneficial.

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LITERATURE CITED

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