PHYSIOLOGY AND NATURAL DISTRIBUTION OF THE BACTERIUM *CARYOPHANON LATUM* IN THE FRESH WATERS OF MISSOURI

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A. Introduction and Objectives

The non-human fecal pollution by small feed-lots and the stream-watering of cattle and other ruminants is a major problem in pollution loading in small streams. Industrial and municipal water pollution is more easily shown, in most instances, primarily because the pollutants and their source of entry to the stream are known and methods for demonstrating the pollution are available.

The majority of our smaller streams and rivers, however, are lined with small farms where construction of adequate feed-lots and watering systems is economically unfeasible. In most areas of the country, attempts to force stream improvement by restrictions on their use have become political and social misadventures. This failure is due primarily to our inability to clearly demonstrate to the individual farmer, voter, and the courts that a particular individual or farm is directly responsible for polluting an area of the stream.

Our concern has been focused primarily on fecal indicators and most obviously those of human origin. And rightly so, for they are of prime importance. But what of the fecal pollution caused by farm stock and especially by ruminants?

Any attempt to develop a group-specific bio-indicator technique is dependent upon the availability of an organism found only in the polluting material, in this case ruminant feces, and in the characteristics of the organism which will allow it to be identified easily and, hopefully, in as short a time as possible.

One particular genus of bacteria, Caryophanon, provides all of the necessary characteristics. Caryophanon latum is found exclusively in the feces of ruminants and, under natural conditions, is there in abundance. As members of that group of organisms known as the 'higher bacteria' they have a size and general morphology which makes them easily distinguished from the other forms, (Pringsheim, E.G., 1957) and its large defined nucleus (Robinow, C.F., 1956; Pringsheim, E.G. and C.F. Robinow, 1947) allows for easy detection after preliminary isolation.

The fact that this organism is an aerobic heterotroph and occurs in trichomes of large size (3 x 30 u) are factors favoring the development of specific techniques (Bergey's Manual of Determinative Bacteriology, 1957). Primarily because of its unusual morphology, especially its large nucleus, the few studies that have been made on this organism were concerned with molecular biology and cytology. Until now little interest has been shown for the elucidation of their physiology and ecology. Thus the literature citations concerning this organism are few and irrelevent to our work.

The objective of this research program was to study the physiology of Caryophanon latum and to develop a selective medium for growing the organism to the exclusion of most, if not all, other organisms found in stream water. After development of this selective medium, the procedure was to be evaluated on sample stream waters with and without inoculation with the organism.

The primary goal of this project is the development of a technique for the detection and possible enumeration of organisms of the genus Carophanon in natural waters. Demonstration of the presence of these organisms would prove fecal pollution by ruminants and enumeration methods would make it possible to locate the point of pollution.

B. Statement of the Problem

One of the major technical problems in maintaining present water quality is in the field determination of non-human fecal pollution, especially that resulting from bovine and other ruminant farm stock. Many forms of life thrive in our small rivers. The presence of a bio-indicator microorganism unique to the feces of these animals, but ubiquitous in terms of its presence there would aid us materially in making proper pollution determinations in the future.

The problem which confronts us is the selection of a type organism and development of a selective growth medium which can be used in the membrane filter technique for water analysis. The organism selected was Caryophanon latum. Its selection was based on its presence in and restriction to ruminant fecal material. The problem which now confronts us is the complete study of the physiology of this organism, especially its growth requirements, and the construction of a medium and conditions for growth which will inhibit all other forms of life and accelerate the growth of this organism. A final improvement would be the use of this medium in conjunction with the membrane filter technique.

C. Method of Investigation

The objective of this program was to develop a differential medium for the specific, quantitative culture of the ruminant fecal organism C. latum. Since this organism has been inadequately studied in the past, it was necessary to characterize the organism biochemically and physiologically before an attempt at developing a differential medium could be made. Thus the investigation can be divided into three different areas: 1) physiology and growth requirements of the organism, 2) development of a selective medium usable with the membrane filter technique, and 3) demonstration that the technique can be used to analyze water containing the organism.

In order to accomplish the characterization of the organism, standard microbiological methods were employed. It was necessary to know and incorporate all the essential nutrients in the medium, in order to get as rapid a culture growth as possible. It was also necessary to find inhibitors to which this organism is resistant, in order to provide a selective environment. Thus, a nitrogen source, a carbon source, a source of essential nutrients (vitamins) and minerals, and some form of antibiotic to which C. latum is resistant, were required. After the appropriate nutrient combinations had been determined, its applicability for use with the Millipore membrane filter technique had to be ascertained. First, in pure culture and second, with stream samples enriched with C. latum.

The formulas for the media employed in this investigation are shown in Appendix A.

The laboratory strain of the test organism Caryophanon latum was purchased from the American Type Culture Collection (ATCC). This was the type organism for which the test was being developed. The organism was carried in stock culture by frequent transfer on Green-Top Agor. Growth on this semi-solid medium was slow and limited to the top one-eighth inch of the agar; but the cell concentration was quite high and provided a very good source of inoculum for the test media. Because of the need for a solid growth

medium, a characteristic of this organism, quantitative methods could not be employed during the initial phase of the investigation.

This requirement for a solid growth surface is not unique for C. latum. It is, however, not a common requirement with bacterial cultures. A high priority was given to the investigation of the need for the solid medium and to the development of a means of growing the culture in a liquid medium whereby quantitative evaluations of cell concentration could be obtained.

The organism, a chemoheterotroph, requires both organic carbon and a nitrogen source for growth. Since growth was obtained on both Green Top Agor and the Yeast Extract-Peptone Agar (YEP) recommended in the literature, these media were employed as basal solid media and as nitrogen sources. Various carbohydrates were examined by either adding them to the basal medium with a pH indicator or by adding the previously grown cells to KEY carbohydrate fermentation tablets.

Various standard laboratory media were examined for their ability to provide the necessary nutrients including nitrogen sources, vitamins, ions, and essential substrates. Standard nitrogen sources such as peptone, trypticase, tryptone, etc., were also added to find their potential in increasing the growth rate.

In order to restrict the growth of other organisms that would be expected to appear in natural waters, various antibiotics and inhibitors were examined in order to find out which ones had no apparent effect on the test organism. By incorporating the inhibiting materials to which C. latum was resistant, it will be possible to prevent the contaminating species from overgrowing the type colonies and would provide a selective medium.

Various physical and chemical parameters were investigated. These included the optimum pH, optimum temperature, and the presence of extractable substances in agar which would allow growth in liquid media. The latter was considered important since it would

change the organism's need for a solid surface to a simple nutritional requirement which could be satisfied in the preparation of the media.

Growth on a medium alone does not in itself demonstrate that the organism will grow in the standard membrane filter assay when that medium is incorporated in the procedure. This has been shown in the past when other standard media were employed (ex. EMB Agar). Following the development of a selective medium it was necessary to test it by examining sterile water samples that had been artificially polluted with C. latum. This test served three basic purposes. 1) It showed that no inhibitory chemicals were present in the membrane filters. 2) It demonstrated that the nutrients and the inhibitors could diffuse through the membrane and provide sufficient growth for detection. 3) It provided a means of quantitatively analyzing the solutions for cells.

An extension of the above evaluation was made by adding C. latum cells to creek water which contained naturally occurring bacteria. In this way the effect of inhibitors on this background growth could be seen and further improvements on the selective properties of the medium could be made.

Following completion and success of the above investigations, field studies of the water analysis assay procedure could be attempted and a complete evaluation of the usefulness of the technique could be ascertained.

D. Results

1. Carbohydrate Sources.

Cultures of C. latum were unable to utilize most of the carbohydrates examined (Table I). Of the carbohydrates examined, only xylose and dextrin showed any response. One interesting aspect of this study was the growth that occurred on the gels formed by the Key tablets containing dextrin and xylose. Apparently all the nutrients necessary for growth are present in these tablets and further studies on this aspect of the problem are indicated. Starch agar (Difco) yielded no growth.

2. Nitrogen Sources.

The organism was examined for the production of the enzymes urease and gelatinase and neither enzyme was found under the cultural conditions employed. In an attempt to increase the growth rate obtained on Green Top and YEP agar media various substitutes were examined.

Trypticase-yeast extract-acetate agar (TEA), Stock Culture agar (Difco), and Brain Heart Infusion agar (BBL) did not improve the growth rate; however, trypticase did replace the peptone in YEP with equal growth. The addition of Casamino Acids did not improve the growth rate. The substitution of malt extract for peptone in YEP agar enhanced the growth somewhat but the rate was still considered poor.

Other standard media were examined with negative results. These included nutrient broth (Difco) containing glucose, lactose, or sucrose; trypticase broth (BBL), litmus milk, and blood agar.

3. Effect of lipids.

Since lipids could be required as either a source of carbon or as an essential nutrient they were examined for their

growth promoting ability. Trilutyrin, incorporated in YEP agar, showed that a lipase enzyme was produced by the culture and growth seemed to be enhanced somewhat.

4. Antibiotics and inhibitors.

The effects of various antibiotics and inhibitors is shown in Table 2. All of the sulfa drugs examined showed no inhibitory effects on the growth of the organism. Streptomycin was the only other antibiotic examined which was not inhibitory. Table 3 shows the effects of increasing concentrations of streptomycin on the growth on YEP agar. All of the dyes incorporated into the medium were inhibitory (Table 2).

5. Optimum temperature and pH.

Cultures grown on YEP agar were incubated at 25 C, 30 C and 37 C for 16 hours. No apparent difference in the quantity of growth with respect to the incubation temperature. When incubated at 44 C, no growth occurred. pH 7.5 was found to be the optimum.

6. Effect of agar source on growth.

The effects of the source and purity of the agar was also examined. As seen in Table 4 only Technical agar (Difco) resulted in better growth, but the enhancement was so great that excellent growth was obtained in 24 hours. The color of the colonies when grown with technical agar was yellow as opposed to the normal clear to opaque, white colonies obtained on the more pure types of agar. The pigment was soluble in methanol, but not in acetone, ethanol, ether, or ethyl acetate.

7. Growth in liquid medium.

Water and methanol extracts of technical agar made at room temperature were added to YEP broth and incubated at 30 C with shaking. Good growth throughout the medium was obtained in 24 hours with the water extract, but not with the methanol extract.

TABLE I

CARBOHYDRATES EXAMINED

Glucose	sod. succinate	Cellobiose
Galactose	sod. citrate	Fructose
Sucrose	sod. acetate	Starch
Xylose	Dextrin	Inulin
Trehalose	Adonital	Arabinose
Raffinose	Inositol	Rhamnose
Sorbitol	Dulcitol	Melibiose

TABLE 2

SENSITIVITY TO VARIOUS INHIBITORS

Inhibitor	Result
Aureomycin	Sensitive
Chloromycetin	Sensitive
Penicillin	Sensitive
Polymixin B	Sensitive
Streptomycin	Resistant
Tetracycline	Sensitive
Triple Sulfa	Resistant
Thiosulfil	Resistant
Sulfamethoxypyridazone	Resistant
Elkosin	Resistant
Gantrisin	Resistant
Sulfadiazine, Merazine, Thiazole	Resistant
Crystal Violet, Malachite Green, Safanin, Methylene Blue, Basic Fuchsin	Sensitive

TABLE 3

EFFECT OF STREPTOMYCIN CONCENTRATION

Conc. of Streptomycin	Growth in 24 Hours	Morphology
None	++	Normal
50 ug/ml	++	Normal
100	++	Normal
150	++	Normal
200	+	Short, wide cells
250	+	Short, wide cells
500	+	Irregular, yeast-like
1000	-	-----
1500	-	-----

TABLE 4

EFFECT OF AGAR SOURCE

Source	Growth in 24 Hours	Pigmentation of Colony
Purified Agar (Difco)	-	-
Lab. Grade Agar (Difco)	+	None
Technical Grade Agar (Difco)	+++	Yellow
Shredded Agar (NBC)	-	-

E. Conclusions

The results obtained in the physiological and biochemical studies provided a basic assay medium which meets the criteria outlined previously. This medium will probably have to be altered as more information is obtained and further field testing is performed. The medium selected and used for trials was essentially the YEP medium, originally used as a basal maintenance medium, supplemented with 100 ug/ml streptomycin. One exception to its formulation is the use of technical grade agar in place of the usual laboratory grade agar commonly employed in media.

Apparently some necessary water soluble nutrient or trace metabolite is removed during the manufacturing process which is necessary for the vigorous growth of C. latum. Investigations are now being made as to the nature and, hopefully, the identification of the substance. Further testing in the field at this point would be useless until the required substance can be included in the medium and the best growth of the organism obtained.

Initial laboratory trials with pure cultures of C. latum demonstrated that the membrane filter technique does in fact provide a quantitative estimation of the cell concentrations when the above medium is used.

When perfected this assay procedure will find a place in the testing procedures for water quality. Several requests have already been made for incorporating this procedure in research projects throughout the state, but its actual use will have to wait until the above question is answered and extensive field trials are performed.

F. Presentations and Reports

"Development of a selective membrane filter technique for assaying for Caryophanon latum". Paper presented to the Missouri Water Resources Research Center.

G. Training Accomplished

Two technicians were trained in various phases of microbiological water quality techniques under the auspices of this grant.

Mr. Ralph Carroll, undergraduate technician, was responsible for the initial investigations on the physiology of Carophanon latum.

Mrs. Joyce Hill, B.S. in Microbiology, was responsible for the later investigations involving the development of the assay medium and for preliminary laboratory and field testing.

H. Bibliography

Pringsheim, E.G., 1957. Bacteriol. Rev. 21, 69.

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APPENDIX A

Green Top Agar (ATCC #32)

Yeast Extract	2.0 g
Tryptone	1.0 g
Sodium Acetate	1.0 g
Soil Extract	50.0 ml
Agar	2.0 g

Water to 1.0 liter. Adjust pH to 7.5.

Yeast Extract-Peptone Agar (YEP)

Yeast Extract	5.0 g
Peptone	5.0 g
Sodium Acetate	0.1 g ? 0 g
Agar	1.0 - 2.0 g

Adjust to pH 7.5. Water to 1.0 liter

KEY Carbohydrate fermentation tablets purchased from:

Key Scientific Products Company
Los Angeles, California

Trypticase - Yeast Extract - Acetate Agar (TEA)

Trypticase	5.0 g
Yeast Extract	5.0 g
Sod. Acetate	0.1 g
Agar	2.0 g

Wager to 1.0 liter. pH to 7.5