1954

Some taxonomic considerations of the genus Pseudomonas

Herbert Halpenny James
The University of Montana

SOME TAXONOMIC CONSIDERATIONS OF THE GENUS PSEUDOMONAS

by

HERBERT HALPENNY JAMES JR.
B.S. Montana State College, 1951

Presented in partial fulfillment
of the requirements for the degree of
Master of Arts

MONTANA STATE UNIVERSITY
1954

Approved by:

E. E. Jeffers
Chairman, Board of Examiners

Gordon B. Castle
Dean, Graduate School

May 17 1954
Date

ACKNOWLEDGMENTS

The author wishes to express his heartfelt thanks to Mr. E. L. Jeffers for his patience, suggestions and guidance throughout the course of this investigation. Appreciation is also due Dr. D. M. Hetler for his assistance and opinions. I am equally indebted to Mr. V. J. Vilk for his time saving pointers and assistance with the experimental animals, to Mr. J. L. Rogers for his drawings, and to Mrs. Pauline Niemeyer for the preparation of the necessary media for this investigation.

H. H. J., Jr.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

The present study was undertaken to examine possible antigenic relationships among certain strains of Pseudomonas, myxobacteria and Pasteurella tularensis.

The usual methods employing morphology and biochemistry for classification often have proven quite unsatisfactory for grouping members of the genus Pseudomonas (1a). The generic title was first proposed for polar-flagellated organisms by Migula in his 1894 outline (14). Winslow, et. al. (27) classified the group on morphology and the basis of production of certain pigments which are water soluble. These pigments are usually green, blue, and yellowish green.

Tobie used a combination of pigment production and biochemistry for classification (25). Serology was applied by Seleen and Stark (20). The results indicated little correlation among the strains of this group. They considered flagellar and somatic antigens, production of pigments (pyocyanin, fluorescin, or neither) and biochemical reactions. Pigment production was shown to be affected by media constituents. Christie (2) observed differentiation of 138 strains of Pseudomonas pyocyaneus by their hemolysis of

sheep red blood cells, and somatic and flagellar and somatic antigen reactions. Munoz, Scherago and Weaver, (15) in a study of representative groups of Pseudomonas, concluded that serological reactions, in addition to morphological and biochemical features, were essential in the identification and classification of species in this genus.

Meader, in 1924, (12); and Meader, Robinson, and Leonard in 1925 (13) found twenty-one monotrichous strains of the genus Pseudomonas to be serologically homogeneous. Elrod and Brown (5) and others showed the genus Pseudomonas (in some cases strains of the same species) to be antigenically heterogeneous. The methods employed were essentially the same in these two studies. The organisms were grown on plated media, suspended in absolute alcohol, centrifuged and resuspended in physiological saline. They were checked by the macroscopic slide agglutination method. There is a possibility that this apparent contradiction of results may have been due to the presence or absence of flagella.

Salvin and Lewis (19) found fifty-six strains of Pseudomonas pathogenic for man and animals. Hodgkiss showed members of this group to be pathogenic for fish (7). He claimed to have found Pseudomonas ichthyodermis to cause a large necrotic lesion in a plaice. In the particular specimen discussed, cavities were formed by destruction of

the muscle through infiltration. There was no damage to the skin surface. Other members of this genus have been implicated in diseases of plants.

Jeffers (unpublished) has indicated that members of this group are commonly found in infected fish in the presence of certain myxobacteria. Kopper (11) demonstrated enzymes to be metabolic products of the Pseudomonas. Vilk (unpublished) found that quiescent myxobacteria were induced to grow upon addition of either dead or living pseudomonas to the media. It could be the metabolic products of the Pseudomonas are responsible for the increased activity of the myxobacteria in their presence.

With reference to the myxobacteria, they were first named as another group of the soil bacteria in 1892 by Thaxter (24). It was noted by Ordal (16) that typical myxobacterial fruiting bodies were produced in the laboratory on fish media. The color of these fruiting bodies can be varied by altering the media constituents (1b). Sniesko, McAllister, and Hitchner (21), showed that certain of the myxobacteria behave as parasites toward associated bacteria. Johnson (8) published an account of certain members which attack chitin. The author has noted that fingerling aquarium trout showed marked softening of the skull upon autopsy, when water was innoculated with cultures of acquatic myxobacteria from hatchery waters.

One of the Myxobacteriales pathogenic for fish was studied by Garnjobst (6a) in organisms isolated from a bullhead. The isolation of these organisms from the cutaneous and connective tissues was accomplished. The genus Cytophaga, with which we are concerned in this investigation, does not produce the characteristic fruiting bodies. Stanier used morphology to classify the members of this group (22).

Pasteurella tularensis was first recorded as a plague-like disease of rodents in 1912 by McCoy and Chapin (11). They also showed it to occur as a parasite in more than thirty forms of animal life in nature. The first reported human case was in the conjunctiva of the eye of a meat butcher. This was diagnosed by Wherry and Lamb (26) in 1913 from McCoy and Chapin's description. Parker, Steinhaus, Kohls, and Jellison showed that nearly all of the natural waters in the northwestern United States were contaminated from time to time with this organism (18).

PROBLEM TO BE INVESTIGATED

Organisms, when commonly found together at the same source, or in the same hosts, may possibly be related. Somatic antigens in many of the isolations may display similarity. Because Pseudomonas and the myxobacteria are commonly found together it was thought that they may be somehow related. Serological methods were employed to

examine this possibility. Similar tests were run with the *P. tularensis* organisms and homologous anti-sera.

CHAPTER I

PROCEDURE

MATERIALS USED

A. Media used included the following:

1. A modification of the Tryptone Agar as suggested by Ordal and Rucker (17). The medium was modified by the addition of 0.05% K_2HPO_4 and 0.1% yeast extract. This medium was used for the cultivation and maintenance of the myxobacteria and for the cultivation but not the maintenance of the Pseudomonas strains.

2. Nutrient Agar as described in the Difco Manual. This medium was employed in the maintenance of the Pseudomonas (4).

3. Cystine-Glucose-blood Agar as described by Parker, Steinhaus, Kohls, and Jellison (18). This media, along with Cystine-Glucose-Heart Agar is used at the Rocky Mountain Laboratories of the United States Public Health Service at Hamilton, Montana, for the cultivation and maintenance of P. tularensis.

ORGANISMS USED

The Pseudomonas strains used in this study were taken

from the stock cultures collected by Raymond (unpublished) in the waters of western Montana. Organism #1 was taken from the gills of a 2-inch cutthroat trout in the Arlee Hatchery. Organism #3 was taken from the kidney of a 3-inch eastern brook trout from the hatchery waters at Post Creek. Organism #4 was taken from the interior of a 3-inch eastern brook trout also, taken from the waters of the Post Creek hatchery. Organism #5 was taken directly from the waters of Post Creek.

The myxobacteria used in this work were taken from stock cultures collected by Jeffers (unpublished) from various fish in Montana hatcheries. Organism A was isolated from a lesion on the gills of a fingerling rainbow trout from the Anaconda hatchery. Organism C was taken from the gills of a fingerling rainbow trout picked up at the Arlee hatchery. Organism #2 was believed to be an unusual member of the myxobacteria due to its color, morphology, odor, etc. as described by Garnjobst (6b). It was isolated from a spring beside the small falls at McDonald Lake in the Mission Range in Montana.

The strains of P. tularensis used in this investigation were isolated from waters of certain western Montana streams. Only the prepared antisera were used by the author. The antisera used was pooled positive antisera which had been taken from infected guinea pigs twenty-one days after

innoculation.

ANTIGEN PREPARATION

Ordal's modified medium was prepared as agar slants. These were innoculated and incubated at room temperature for 48 hours. It was noted that the strains of Pseudomonas grew much more rapidly than the myxobacteria. To have uniformly-aged colonies with the maximum growth, it was decided to incubate all cultures for 48 hours.

One ml. of sterile physiological saline was used to wash the organisms from the medium. Blake bottles were innoculated with the material washed from the agar slant to provide a larger growth area. The bottles were inverted and incubated for the 48 hour period.

The organisms were removed from the blake bottle with 10 ml. of physiological saline and transferred to sterile 60 ml. vaccine bottles containing 8-10 sterile glass beads to break up the clumps of organisms. Five per cent phenol was employed to kill the organisms. The suspension was passed through filter paper to remove any clumps which had not been broken up. After sufficient time had lapsed for the phenol to kill the bacteria, the suspension was diluted to 40 ml. and kept in storage at 4°C.

It was found that this mixture, after storage for a period of six weeks to two months, formed a heavy mucoid mass in the bottom of the sterile vaccine bottles. This

occurred only in the bottles containing the strains of Pseudomonas. When a freshly phenolated mixture was heated in a 56°C waterbath the clot was found to form within an hour. It is believed that this was a chemical reaction between the phenol and certain products of the Pseudomonas, inasmuch as the slime, when observed under the microscope, showed few if any organisms. The slime was milk white in color, and could not be removed by filter-paper filtration. It was found that by using small 1 ml. pipettes the mass could be sucked up and removed in this manner. Care was taken not to innoculate any of this product into the animals being immunized, nor to get it into any of the tubes when performing the agglutination tests, as far as was possible.

The P. tularensis antigen was prepared at the Rocky Mountain Laboratories. Guinea pigs were innoculated with 10 ml. of suspected creek water. Water was collected in sterile 200 ml. flasks. Occasionally mud was used. In this event, 500 ml. bottles were employed, 3 tablespoonfuls of top mud were mixed with 300 ml. of water, and allowed to settle. The animals were innoculated intraperitonealy. The guinea pigs were observed and 21 day survivors were sacrificed. Blood was extracted for antisera. The tissues were cultured on the media or in some cases ground up and reinjected into another series of test animals.

Plates were streaked periodically with the suspensions from the vaccine bottles to determine the death of the organisms and to see if contamination had taken place.

ANTIGEN STANDARDIZATION

At the outset the Pseudomonas organisms were prepared as a vaccine and a turbidimetric method was used for standardization (9).

The myxobacterial vaccines were standardized by Wright's method (23). This method was considered to be more accurate than the nephelometer tubes and successive Pseudomonas antigens were standardized in this manner.

ANTISERA PREPARATION

For control purposes 20 ml. of blood was removed from the heart of each experimental rabbit prior to innoculation. Each serum was checked at this time for pre-existing antibodies which might act as agglutinins against the antigen to be innoculated. The normal sera of the rabbits selected for immunization did not show agglutinins against the strain to be innoculated.

After a series of innoculations, the blood was removed from the heart of the animals. The sera were preserved by addition of equal volumes of glycerol and stored in the refrigerator at 4°C until needed.

The titres were determined by running antigen-antibody

reactions using the macroscopic agglutination test (3).

In all cases, the agglutination tests were run twice.

Innoculations and bleeding of the rabbits were carried out as outlined by Zinsser and Bayne-Jones (28).

ANTIBODY

ANTIGEN	1	2	3	4	5
1	1/800	—	—	1/10	—
2	—	1/800	—	—	1/10
3	—	—	1/1600	1/10	—
4	—	1/10	—	1/3200	—
5	—	—	1/10	—	1/1600

TABLE I

PSEUDOMONAS TITRES and CROSS REACTIONS

CHAPTER II

RESULTS

Table I shows the cross reactions and titres obtained in tests run on the *Pseudomonas* strains. It is clear that there was little or no common antigenicity among the members of this group. It has been found that flagellar antigens of this group produce higher agglutinins. With the comparatively high titres demonstrated, had there been any real common antigens of the somatic type, they should have demonstrated themselves in the higher dilutions.

Table II shows the cross reactions and titres obtained in tests run on the myxobacteria and the strains of *P. tularensis*. The titres of the myxobacteria were much lower on a comparative basis than those of the *Pseudomonas*. This was due probably to the fact that the animals used were merely given two booster shots rather than the whole series because they had been innoculated six months previously with the same organisms. A common antigenicity is indicated among those members of the myxobacteria used. The complement-fixation technique was employed in the case of the *P. tularensis* antigen by the workers at the Rocky Mountain Laboratories.

ANTIBODY

ANTIGEN	A	C	TULARENSE
1	—	—	—
2	1/50	1/100	—
3	—	—	—
4	—	—	—
5	—	—	—
A	1/200	1/100	—
C	1/200	1/100	—
TULARENSE	—	—	1/160

TABLE II

MYXOBACTERIA and TULAREMIA TITRES

MYXOBACTERIA, TULAREMIA, PSEUDOMONAS CROSS REACTIONS

Dilutions in all cases were run as high as 1:3200 in the event that the reaction should carry that far. It should be noted that organism #4 is the only one whose titre ran this high.

Following the supposition earlier established concerning the possibility of organism #2 being a member of the genus Cytophaga, the only organism carrying myxobacterial agglutinins was organism #2.

There was insufficient evidence to warrant any supposition that there are any antigenic relationships among these three genera of the water bacteria.

CHAPTER III

DISCUSSION

The members of the genus Pseudomonas show some relationship within the group. However, these relationships are not strong or sharply defined. Confusion exists within the general area of the group. It may be the somatic antigens which are probably responsible for the reactions indicated.

There is a definite relationship among the members of the myxobacteria employed. The author's supposition that organism #2 was a member of this group was readily supported when agglutinins were found present for the known myxobacteria but not for the Pseudomonas.

The antibody built up by the myxobacteria soon loses its capacity to react. This was demonstrated by the animals used for the experiment. Less than a year before, these same rabbits had been immunized against the same species of Cytophaga used in the innoculations but showed no agglutinins present for any of the organisms to be innoculated when checked by the macroscopic agglutination test prior to innoculation.

At the Rocky Mountain Laboratories four strains of

P. tularensis antigen were checked agains the antisera of the strains *Pseudomonas* and myxobacteria using the pooled positive antisera as a control.

Dr. Lackman of the Rocky Mountain Laboratories staff communicated to the author, that on previous occasions, water and soil organisms have been found that have shown a titre as high as 1:80 with the *P. tularensis* sera using the complement-fixation technique. This would indicate that there are some water and soil organisms that possess antigens in their make up common to those of the tularemia causing bacteria.

SUMMARY AND CONCLUSIONS

1. Two strains of the genus Cytophaga of the Order Myxobacteriales, four strains of the genus Pseudomonas, and one suspected species of the Cytophaga were innoculated into experimental laboratory rabbits.

2. The titres ran high but lack of cross reactions indicated no common antigens among the Pseudomonas strains and the myxobacteria.

3. Pooled antisera of Pasterurella tularensis was obtained and agglutination tests were performed using the strains of Pseudomonas and Cytophaga as antigens. No common antigens were found among these three groups of bacteria.

4. Slime was found to be formed by the members of the Pseudomonas in the sterile vaccine bottles. It was believed to be a chemical reaction between the phenol used to kill the organisms and some of their metabolic products.

BIBLIOGRAPHY

(1) Breed, R. S., Murray, E. G. D., and Hitchens, A. P. Bergey's Manual of Determinative Bacteriology, 6th Ed., Williams and Wilkins Co., Baltimore, 1948, (a) p. 7, 32, and 218. (b) pp. 1005-1050.

(2) Christie, R. Observations on the Biochemical and Serological Characteristics of Pseudomonas Pyocyanea. Australian Jour. Exptl. Biol. and Med. Sci. Vol. 26, 1948, pp. 425-437.

(3) Committee On Bacteriological Techniques, Manual of Methods Of Pure Culture Study of Bacteria, Leaflets I to IX, Soc. of Am. Bact., Geneva, N. Y.

(4) Difco Manual of Dehydrated Culture Media, 8th Ed. Difco Laboratories, Detroit, 1948.

(5) Elrod and Brown, A. C. Pseudomonas aeruginosa, Its Role as a Plant Pathogen. Jour. of Bact., Vol. 44, 1942, pp. 633-644.

(6) Garnjobst, Laura. Cytophaga Columnaris (Davis) in Pure Culture: A Myxobacterium Pathogenic to Fish, Jour. of Bact., Vol. 491949, (a) p. 113, (b) p. 124.

(7) Hodgkiss, W. and Shewan, J. M. Pseudomonas Infection in A Plaice. Jour. Path. and Bact. Vol. 62, 1950, pp. 655-657.

(8) Johnston, D. E. Some Observations on Chitin Destroying Bacteria. Jour. of Bact. Vol. 24, 1932, pp. 335-340.

(9) Kolmer, Approved Laboratory Technique. 3rd Ed., 1941, D. Appleton-Century Co., N. Y., pp. 545-547.

(10) Kopper, Paul H. A Note on the Component Enzymes of Pseudomonas "Creatinase". Arch Bio-Chem. Vol. 19, 1948, pp. 171-172.

(11) McCoy, R. and Chapin, D. Bacterium Tularense, a Plague-Like Disease of Rodents. Jour. Inf. Dis. Vol. 10, 1912, p. 61.

(12) Meader, P. D. Serological and Cultural Reactions of Bacillus Pyocyaneus. Amer. Jour. Hyg. Vol. 4, 1924, p. 403.

(13) _____, Robinson, G. H., and Leonard, V. Pyorubrin, A Red Water Soluble Pigment Characteristic of B. Pyocyaneus. Jour. of Bact., Vol. 17, 1925, pp. 633-634.

(14) Migula, An Outline for Bacteria. Arb Bact. Inst. Karlsruhe Vol. 1, 1894, p. 237.

(15) Munoz, J., Scherago, M. and Weaver, R. H. A Serological Study of Members of The Pseudomonas Genus. Jour. of Bact., Vol. 57, 1949, pp. 269-278.

(16) Ordal, Erling J. Studies on Myxobacteria, Jour. of Bact., Vol. 51, 1946, p. 579.

(17) _____, and Rucker, R. R. Pathogenic Myxobacteria. Proceedings of the Society for Experimental Biology and Medicine, Vol. 56, 1944, p. 15.

(18) Parker, R. R., Steinhaus, Ed. A., Kohls, Glen M., and Jellison, Wm. L., Contamination of Natural Waters and Mud With Pasteurella Tularensis and Tularemia in Beavers and Muskrats in The North Western United States. National Institute of Health, Bul. 193, 1951.

(19) Salvin, S. B., and Lewis, K. L. External Otitis, With Additional Studies on the Genus Pseudomonas, Jour. of Bact., Vol. 51, 1946, pp. 495-506.

(20) Seleen, L. A., and Stark, C. N. Some Characteristics of Green-Fluorescent Pigment Producing Bacteria. Jour. of Bact., Vol. 46, 1943, pp. 491-500.

(21) Sniesko, S. F., McAllister, Joan, and Hitchner, E. R. Further Studies on the Biology of Certain Myxobacteria. Jour. of Bact., Vol. 43, 1943, pp. 28-29.

(22) Stanier, R. Y. Studies on Non-Fruiting Myxobacteria I. Cytophaga Johnsonas, N. Sp., A Chitin Decomposing Myxobacteria. Jour. of Bact., Vol. 53, 1947, pp. 297-315.

(23) Stitt, E. R., and Clough, Paul W., and Branham, Sara E., Practical Bacteriology, Hematology, and Parasitology, 10th Ed., Blankiston Co., 1948, p. 304.

(24) Thaxter, R. On the Myxobacteriaceae, A New Order of Schizomycetes, Bot. Gas., Vol. 17, 1892, p. 389.

(25) Tobie, R. A. Proposed Biochemical Basis for the Genus Pseudomonas. Jour. of Bact., Vol. 49, 1945, p. 459.

(26) Wherry, Wm. B., and Lamb, B. H. Infection of Man With Bacterium Tularense. Jour. of Inf. Dis., Vol. 15, 1914, pp. 331-340.

(27) Winslow, et. al. The Families and Genera of The Bacteria, Report of Committee. Jour. of Bact., Vol. 2, 1917, p. 555.

(28) Zinsser, Hans, and Bayne-Jones, Stanhope. A Textbook of Bacteriology. D. Appleton Century Co., 1939.