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A Study of the Classification of Pink Pigment Producing Organisms

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A STUDY OF THE CLASSIFICATION OF PINK PIGMENT PRODUCING ORGANISMS

by

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LIFE

Marion Walter Zielinski was born in Chicago, Illinois, February 18, 1938.

He was graduated from St. Ignatius High School, Chicago, Illinois, June 1956, and attended Loyola University, Chicago, Illinois, from September 1956 to June 1960 when he graduated with the degree of Bachelor of Science.

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TABLE OF CONTENTS

LIST OF FIGURES

HISTORY AND REVIEW OF LITERATURE

Pink pigment bacteria were isolated and studied as early as 1926 by Snow and Fred. They can be found in soil, water (fresh, distilled, and sea), and in air. Pink Corynebacteria and pink cocci are often found in the soil and air; pink gram negative rods are found in water. The latter are the subjects of this thesis.

Recently, Leifson (1962) studied bacteria found in distilled water and also marine bacteria. Research (e.g., Leifson 1962 and Flannery 1956) has been done on both fresh and salt water bacteria (primarily the latter); but very little attention has been paid to classification of these organisms, especially the pink bacteria.

Formerly, gram negative pink bacteria had been placed into several genera, for example, Pseudomonas, Vibrio, Proteminobacter, and Cellulomonas. Some of the pink rods studied by the author were species of the above genera. In respect to these bacteria, the object of the thesis was to determine whether they should remain as classified or whether they should be reclassified.

The Genus Pseudomonas (Bergey, 1948a) is composed of gram negative rods that are polar monotrichous, lophotrichous or non motile. Some produce a water soluble pigment which turns green upon being exposed to oxygen. Glucose is oxidized; nitrates are reduced to nitrites and sometimes to nitrogen gas. The chromogenic species are salt tolerant. According to Leifson (1963), the Genus Pseudomonas should be limited to gram negative rods, motile by a polar monotrichous flagellum and capable of only oxidising carbohydrates.

Cellulomonas (Bergey, 1948b), as originally proposed, was based on a single physiological property, the digestion of cellulose. It included such diverse types of bacteria as polar flagellate species; gram variable, non motile rods; and peritrichous non spore forming gram negative rods.

The Genus Vibrio (Bergey, 1948c) is composed of gram negative rods capable of fermenting several carbohydrates and possessing a polar monotrichous flagellum. Protaminobacter (Bergey, 1948d) is composed of gram negative, polar monotrichous rods which oxidize glucose.

MATERIALS AND METHODS

CULTURES

The distilled water bacteria were acquired from Leifson who isolated them from samples withdrawn from distilled water storage tanks. The storage tank samples were sent to him from various points on the globe. The marine micro-organisms were also acquired from Leifson; these samples were collected by him along the Atlantic Coast near Connecticut.

Cultures # 334, # 301, # 306, # 44, # 48, and # 215 are from distilled water. Cultures F, R, # 50, and # 75 are of marine origin.

When in use, all stock cultures were transferred every two to three months. Cultures not in use were transferred every six months. When tests were being run, the cultures were transferred every one to two weeks. Cultures not in immediate demand were incubated at 20° C. and then stored at 4° C.. Cultures in constant use were kept at 20° C.. In helping to establish the identity and purity of the individual cultures, carbohydrate tests were run three times over a period of a year and a half.

Serratia marcescens and Pseudomonas aeruginosa (A.T.C.C. 14149) were used as control organisms. Pseudomonas rubescens, Cellulomonas rossica, Protaminobacter rubrum (A.T.C.C. 8457), and Vibrio rubicundus (acquired

from Dr. Rudolph Hugh, George Washington University, Washington D.C.) were tested to see if they should be reclassified or not. Rhodospirillum rubrum was tested to see if any new facts could be added to our present knowledge of this spiral shaped organism.

BASE MEDIA

Since marine organisms and distilled water organisms were the two types used, two types of base media were employed. Phosphate salts, peptone, and yeast extract were the basic constituents of the medium for the distilled water organisms; the latter two plus artificial sea water comprised the sea water medium.

Distilled Water Medium (1X)

KH_2PO_4.....0.015%

K_2HPO_4.....0.035%

Bacto - Casitone.....0.2%

Yeast Extract.....0.1%

pH.....7.0±.1

Sea Water Medium (1X)

Bacto - Casitone.....0.2%

Yeast Extract.....0.1%

Artificial Sea Water Components

NaCl.....2.3%	$CaCl_2$.....0.11%
Na_2SO_4.....0.37%	H_3BO_3.....0.0026%

Table continued on Page Five.

KCl.....0.066%	$SrCl_2$.....0.0024%
KBr.....0.0098%	NaF.....0.0003%
$MgCl_2$.....0.498%	$FeSO_4.7H_2O$...0.001%

Tris buffer.....0.05%

pH.....7.5±.1

The incubation temperature, unless otherwise stated, was twenty degrees Centigrade (20° C.).

Hydrogen ion concentrations in media were standardized by the application of the Gillespie Method, using brom thymol blue for distilled water media (pH 7.0±.1) and phenol red for sea media (pH 7.5±.1).

TEMPERATURE TOLERANCE

These tests were performed in broth at four different temperature levels, 4°C., 20°C., 25°C. to 30°C., and 37°C..

OSMOTIC TOLERANCE

Both distilled water and salt water bacteria were grown in distilled water medium plus varying sodium chloride concentrations. All tubes were macroscopically and microscopically checked for growth, motility, and filament formation.

OXYGEN TENSION

A base broth was used as the medium. It was heated to drive off the oxygen. Once the broth cooled to 30°C., the tubes were immediately inoculated; this was followed by a petrolatum seal and a rubber stopper, thus reducing the oxygen tension.

Anaerobic conditions were produced by incubating streaked plates in a carbon dioxide (CO_2) atmosphere. The incubation chamber was evacuated to a negative pressure (- 15 lbs.) and then filled with carbon dioxide to a positive pressure (10 lbs.). This cycle was completed three times. The plates were incubated at room temperature for five days.

STAINS

The gram stain (Hucker's), flagella stain (Leifson), and capsule stain (India ink and methylene blue) were performed with cultures grown in broth at 20°C.. Cells, to be measured, were 24 to 48 hours old and stained with methylene blue.

MOTILITY

Motility was determined in the sodium chloride solutions plus at all four temperatures of the base broth medium. A loopful of the culture would be placed on a slide and checked under high power at room temperature.

PIGMENTATION

Pigment production was checked at the four temperature levels. Broth and agar bases were used.

CARBOHYDRATE TESTS

The base media for the carbohydrate tests were the two types first mentioned. They are similar to the Hugh - Leifson oxidative - fermentative medium. Different concentrations of indicator and agar were used.

The constituents of the base media for carbohydrate tests are:

Distilled Water Base (1X)	Sea Water Base (1X)
Bacto - Casitone.....0.2%	.....0.2%
Yeast Extract.....0.1%	.....0.1%
KH_2PO_4.....0.015%	.....
K_2HPO_4.....0.035%	.....
Tris buffer.....	.....0.05%
Brom thymol blue....0.0065%	.....
Phenol red.....	.0.00050% to 0.00075%
Agar agar.....0.4%	.....0.4%

Artificial sea water for marine organisms.

Carbohydrates were prepared by dissolving in distilled water and sterilizing by Seitz filtration. Sterile solutions were kept at refrigerator temperatures (4°C.) until needed.

Carbohydrate stock solutions were prepared as 10X and 5X resulting in final concentrations of the carbohydrate in the test media of 1% and .5%. Carbohydrates, that were sparingly soluble in cold water, were heated and then distributed in the soft agar solution (base media). Once the solution cooled, the agar helped to distribute the sparingly soluble sugar.

Each test was observed for oxidation, fermentation, and growth. All organisms were capable of growth in the base medium itself without the production of acid.

OTHER MEDIA

Production of indol, reduction of nitrate, gelatin liquefaction, urease production, citrate utilization, hemolysis of blood and production of catalase were also tested. The various media were prepared using standard concentrations, omitting salts for distilled water organisms and adding artificial sea water for the marine bacteria. Except for the urea and citrate tests, no commercially prepared powders were used.

Indol production was tested for by the addition of 3% Bacto - tryptone to the base medium. After a period of 48 hours incubation, a reagent was added directly to the culture. If the test were positive for indol, a red color would develop at the surface of the broth.

Reagent :

Paradimethylaminobenzaldehyde.....2 grams

Ethyl alcohol (95%).....190 ml.

Hydrochloric acid (concentrated)....40 ml.

Gelatin liquefaction was tested for in a 12% gelatin medium containing .1% yeast extract and 0.2% Bacto Casitone. After a period of 8 to 10 days incubation, the tubes were checked for the presence or absence of liquefaction.

Hemolysis of blood (human) was detected using the basic methods as outlined by Schaub (1958). The base media utilized was the major difference.

Nitrate reduction was tested for in base broth containing 0.1% potassium nitrate (KNO_3). Following a period of 48 hours incubation, nitrites were tested for by the addition of four drops each of the following reagents.

A. Sulphanilic acid.....4 grams

5N Acetic acid.....500 ml.

B. Alpha - napthylamine acetate...2.5 grams

5N Acetic acid.....500 ml.

The formation of a red color indicated that nitrites were present. Negative nitrite (NO_2) tests were proven to be negative by the addition of zinc (Zn) dust which reacts by reducing any nitrates (NO_3) present to nitrites resulting in a red color and thus assuring that the nitrate was not utilized. If a red color does not develop, then the nitrate has been reduced to nitrogen gas.

Urease production and citrate utilization were tested for in commercially prepared preparations. Bacto urea broth concentrate and Simmons citrate agar were used. Proteus vulgaris (urease positive) and Escherichia coli (urease negative) were used as control organisms for the urease test; Aerobacter aerogenes (citrate positive) and Escherichia coli (citrate negative) for the citrate test.

A positive urease test would turn the medium from a neutral pH (orange) to a basic pH (red) in 24 to 48 hours; a positive citrate test would turn the blue green medium (neutral pH) to blue (alkaline pH).

Bacto Urea Broth

Yeast extract...0.01%

KH_2PO_4.....0.91%

K_2HPO_4.....0.95%

Urea.....2%

Phenol red.....0.001%

Simmons Citrate Agar

$MgSO_4$.....0.02%

NH_4PO_4.....0.1%

K_2HPO_4.....0.1%

Sodium citrate.....0.2%

NaCl.....0.5%

Agar.....1.5%

Brom thymol blue...0.008%

The catalase test was performed in the base broth medium. Four drops of 30% hydrogen peroxide (H_2O_2) were added to approximately four milliliters of broth culture. Bubble formation indicated a positive test.

MINIMAL MEDIUM

The minimal salts medium (1X) consisted of

$CaCl_2$.....0.005%

Na_2SO_4.....0.02%

$MgCl_2.6H_2O$.....0.02%

K_2HPO_4.....0.02%

NH_4Cl.....0.1%

It was supplemented with the following

Sodium acetate.....0.1%

Glucose.....1%

Acid hydrolyzed Casamino acids....0.2%

A straight wire was used to inoculate the initial 2 ml. of medium. If macroscopic growth appeared, then again, by means of a straight wire, a 5 ml. tube was inoculated from the 2 ml. culture. If this 5 ml. culture was positive, another 5 ml. culture would be inoculated following the above mentioned procedure.

The marine bacteria were tested using the artificial sea water broth base instead of the minimal salt medium mentioned above. The supplements added remained the same as for the distilled water bacteria.

SURVIVAL TESTS

All organisms under study were placed into distilled water, distilled water plus minimal medium salts, demineralized water, well water (Yorkville, Illinois), and artificial sea water. A loopful of organisms was inoculated into each screw cap tube which contained 5 ml. of the liquid. At various time intervals, the cultures would be checked for growth on their respective base media. The survival tubes were stored at room temperature.

MEASUREMENTS

Cells were measured by means of a micrometer eyepiece calibrated by use of a calibrated stage micrometer. The cells were grown at 20°C. for 24 to 48 hours depending on the culture. They were stained with methylene blue and measured under oil immersion. For each culture, measurements were taken of ten different rods, the average value being used to calculate the final value in microns.

The wavelength and amplitude of a flagellum were measured using the above procedure. Flagella stain (Basic fuchsin - Leifson) was used to stain the flagella.

SEROLOGY

A limited serological study was conducted using several cultures as antigens. The antiserum was produced in rabbits. The basis for the choice of organisms as antigens was carbohydrate utilization. Number forty-four (44) did not produce acid from any sugars; number forty-eight (48) produced acid from a few carbohydrates; Pseudomonas aeruginosa produced acid from a moderate number of carbohydrates.

Antigens were prepared from cells grown on the base medium for distilled water organisms (the marine organisms were not included). Prescription type bottles (about 500 ml. volume), containing approximately 60 ml. of base medium plus one milliliter of inoculum, were incubated for one week for the somatic antigen and three days for the flagellar antigen.

The cells were collected in .85% sodium chloride solution. They were washed three times by centrifugation and then placed at refrigerator temperatures (4°C.) in 5 ml. amounts of the .85% sodium chloride solution.

Two types of antigens were produced, the somatic (O) antigen and the flagellar (H) antigen. Somatic antigens were prepared by boiling the washed cells for two hours; the flagellar antigen was prepared by addition of five tenths per cent (0.5%) formaldehyde to washed cells.

After standing at refrigerator temperature (4°C.) for 24 hours, the antigens were centrifuged. Two and one half ml. (2.5 ml.) of .85% NaCl was again added to the tubes.

Twenty-five hundredths of a milliliter (.25 ml.) of the two and one half ml. (2.5 ml.) suspension above was then diluted with .85% NaCl to a volume of 5 ml.. Using a Klett Summerson Colorimeter, readings were taken and compared to a previously prepared standard curve. The antigen concentrations were one hundred million to one billion cells per milliliter.

Serum controls were run in order to determine whether the rabbits to be immunized already possessed antibodies against the antigens to be injected. Blood was withdrawn via the marginal ear vein; new needles (disposable 25 guage) were used each time the vein would be tapped for blood or injected with antigen.

The ear was swabbed with alcohol (70%) and shaved with a razor. Xylol was then placed on the vein to produce a cooling sensation and dilate the vessel. Tube dilution tests for antibodies were negative.

Six rabbits weighing about 2.5 kilograms apiece were numbered one to six and injected in the following manner. Rabbits one and two were injected with antigens 44H (organism 44, flagellar antigen) and 44 O (organism 44, somatic antigen) respectively; rabbits three and four, 48H and 48 O; rabbits five and six, Pseudomonas aeruginosa H and Pseudomonas aeruginosa O.

The animals were immunized by injection of the antigen into the marginal ear vein on alternate days. Twenty-five hundredths milliliter of antigen was injected as doses one and two; five tenths milliliter as doses three and four; one milliliter as doses five and six.

One tenth milliliter serum diluted in .85% NaCl (2 ml.) was tested for antibody using serial dilutions of 1:20, 1:40, 1:80 thru 1:10,240. One tenth milliliter of the respective antigen was added to each tube. All agglutinations were incubated in a water bath at 52°C. for two hours followed by overnight incubation at 37°C. for O agglutination. The tubes were grossly examined for agglutination.

RESULTS

TABLE I

CARBOHYDRATES

Including the control organism, the bacteria tested did oxidize carbohydrates, that is, they utilized carbohydrates in the presence of oxygen. The fact, that they are not fermenters, means they cannot use organic hydrogen acceptors; and, because they depend on oxygen as the final hydrogen acceptor, they are oxidizers.

Protaminobacter rubrum, # 44, # 334, and Vibrio rubicundus did not oxidize any of the sugars tested. Dulcitol, inulin, melezitose, and sorbose were not oxidized by any of the micro-organisms tested. Adonitol, erythrite, and inositol were utilized by Serratia marcescens only. The above facts are mentioned here because they are not recorded on the table.

TABLE II

TEMPERATURE TOLERANCE

All the organisms grew at 20°C. and at room temperature (25°C. to 30°C.). Of those that grew at 37°C., some produced a much heavier growth at this temperature than they did at 20°C. or room temperature. No growth was visible at 4°C..

STAINS

All the bacteria were gram negative. Rhodospirillum rubrum was the only organism which was not rod shaped. Most of the organisms were polar monotrichous. Although all possessed capsules, the capsules were not as large as those around Klebsiella pneumonia.

Organisms # 334, # 301, # 44, and # 48 appeared quite frequently in aggregates. The other organisms were observed primarily as single cells.

TABLE III

OSMOTIC TOLERANCE

These tests were run at 20°C.. At the first sign of growth, the organisms were observed under the microscope with the high power objective lens. In general, an increase in osmotic pressure tended to decrease motility and increase chain formation. Most filamentous forms were observed at concentrations preceding the salt concentration at which no growth was visible. Increasing the osmotic pressure also prolonged the incubation time necessary to produce visible growth. Pigment production decreased with increasing osmotic pressure. At times, macroscopic growth was not visible; but, detection with the microscope was possible.

In general, the distilled water organisms did not grow at concentrations above one per cent sodium chloride. The marine organisms that grew above four per cent sodium chloride needed more time to produce visible growth.

TABLE IV

OXYGEN TENSION

A large number of the organisms are aerobic and are incapable of existence at reduced oxygen tensions. Nine organisms were capable of growth under reduced oxygen tension, four of the nine were capable of growth under anaerobic conditions. Pseudomonas aeruginosa, Serratia marcescens, Cellulomonas rossica, and # 334 (distilled water type) can be considered facultative. The other organisms are aerobes, some capable of growing under reduced oxygen tension.

PIGMENTATION

Petri dish cultures gave the best conditions for determination of pigment color. At twenty degrees Centigrade and room temperature, the pink pigment was produced.

The pink pigment would not appear under the anaerobic conditions mentioned above; but, upon exposing the cultures to aerobic conditions, pigmentation began to appear. Number 334 turned orange as did Serratia marcescens; Cellulomonas rossica remained white. Pseudomonas aeruginosa's green water soluble pigment also began to appear. In each case when the pigment was to appear, it did so in 24 to 48 hours after exposure to oxygen. Continuation of growth under aerobic conditions was not determined.

From the osmotic tolerance tests, one observes that pigmentation begins to gradually decrease as the sodium chloride concentration increases. Whether this decrease is due to the NaCl increase or whether it is due to the decrease in growth is not known.

Minimal medium tests showed that the pink organisms needed more time to produce pigment when supplemented with glucose and sodium acetate than when supplemented with acid hydrolyzed Casamino acids. Pseudomonas aeruginosa's pigment, on the other hand, could be observed only in the Casamino acid supplement.

OTHER REACTIONS

Proteus vulgaris was urease positive; Escherichia coli urease negative. Aerobacter aerogenes was citrate positive; Escherichia coli citrate negative. All the other organisms studied were urease negative; except for Cellulomonas rossica, all other organisms were citrate negative. None of the pink pigment bacteria produced indol.

TABLE V

MINIMAL MEDIA

All except three of the organisms were capable of utilising Casamino acids (acid hydrolyzed) as the sole source of energy and body building protein. Quite a few utilized sodium acetate or glucose as the sole sources of energy and somatic protein. Micro-organisms that utilized the Casamino acids produced much heavier growth in less time than when they grew in the presence of the other supplements.

SURVIVAL TESTS

The results reported here are those recorded the last time previous to the writing of this thesis. This means, that if a tube shows growth, the bacteria have survived for approximately four months. The cultures needed

longer incubation periods before growth appeared. Some showed two different pink colonies, a fast growing light pink one and a slow growing dark pink colony. Many that did survive had their numbers greatly diminished.

TABLE VI

SIZE

The rods averaged two microns in length; one-half micron (0.5) in width. Flagella averaged wavelengths of one and a half (1.5) microns and amplitudes of .44 to .62 microns.

TABLE VII

SEROLOGY

All the antigens produced antibody (low titer). Protaminobacter flagellar antigen cross reacted with the flagellar antibody from Pseudomonas aeruginosa, # 44, and # 48. Protaminobacter somatic antigen cross reacted with somatic antibody from # 44 and # 48. Number 215 flagellar antigen slightly cross reacted with flagellar antibody from Pseudomonas aeruginosa; # 334 somatic antigen cross reacted with somatic antibody from # 44 and # 48.

PIGMENT

The pigment cannot be found in the medium. If the cells are grown in a broth medium, upon centrifugation, the cells plus their pigment can be found on the bottom with a clear supernatant above. The pigments exact location in the cell was not determined.

Attempts were made to extract the pigment with chloroform, petroleum ether, and acetone; the latter was the only successful solvent. Using petroleum ether and acetone (ratio 1 : 1) as a solvent, the pigment was chromatographed (Lederer). Chromatography results were inconclusive.

TABLE I

CARBOHYDRATES

	Arabinose	Cellobiose	Dextrin	Dextrose	Esculin	Galactose	Levulose	Lactose	Maltose	Mannitol	Mannose	Melibiose	Raffinose	Rhamnose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose
# 301	+	+	+	+	-	+	+	+	+	-	+	+	+	-	-	-	+	-	+
# 306	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Serratia marcescens	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+
Rhodospirillum rubrum	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas rubescens	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
# 48	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellulomonas rossica	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
# 215	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Pseudomonas aeruginosa	+	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	+
Marine (F)	+	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+
Marine (R)	+	+	-	+	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+
Marine (50)	+	-	-	+	-	+	+	-	+	+	-	-	-	+	-	+	+	-	+
Marine (75)	+	-	-	+	-	+	+	-	+	+	-	-	-	+	-	+	+	-	+

(+) acid production (-) no acid production (0) no test

TABLE II

	Shape		Gram		Growth				Motility				Flagella			
	Rod	Coccus	Positive	Negative	4°C.	20°C.	25°C.-30°C.	37°C.	4°C.	20°C.	25°C.-30°C.	37°C.	Mono.	Peri.	Lopho.	Capsules
# 334	+	-	-	+	+	++	+++	++	-	-	-	+	-	-	-	+
# 301	+	-	-	+	+	++	+++	++	-	-	-	-	-	-	-	+
# 306	+	-	-	+	+	++	+++	++	-	+	+	+	+	-	-	+
Serratia marcescens	+	-	-	+	+	++	+++	+++	+	+	+	+	-	+	-	+
Rhodospirillum rubrum	-	-	-	+	+	++	+++	++	-	+	+	+	-	-	+	+
Protaminobacter rubrum	+	-	-	+	+	+++	++++	+++	-	+	+	-	-	-	-	+
Pseudomonas rubescens	+	-	-	+	+	+++	++++	++++	+	+	+	+	+	-	-	+
# 44	+	-	-	+	+	++	+++	-	-	+	-	-	+	-	-	+
# 48	+	-	-	+	+	++	+++	++	-	+	+	-	+	-	-	+
Cellulomonas rossica	+	-	-	+	+	++	+++	++++	-	+	+	+	+	-	-	+
# 215	+	-	-	+	+	++	+++	++	-	+	-	-	+	-	-	+
Vibrio rubicundus	+	-	-	+	+	++	+++	-	-	+	-	-	+	-	-	+
Pseudomonas aeruginosa	+	-	-	+	+	+++	++++	++++	-	+	+	+	+	-	-	+
Marine (F) and (R)	+	-	-	+	+	++	++++	+++	-	-	-	-	-	-	-	+
Marine (50) and (75)	+	-	-	+	+	++	++++	+++	-	-	-	-	-	-	-	+

TABLE III

OSMOTIC TOLERANCE (% NaCl)

	Growth										Motility									
	0.25%	0.50%	0.75%	1.00%	2.00%	3.00%	4.00%	5.00%	6.00%	7.00%	0.25%	0.50%	0.75%	1.00%	2.00%	3.00%	4.00%	5.00%	6.00%	7.00%
# 334	+	+	+	+	+	+	+	+	-	0	-	-	-	-	-	-	-	-	0	0
# 301	+	+	+	+	-	0	0	0	0	0	-	-	-	-	0	0	0	0	0	0
# 306	+	+	+	+	-	0	0	0	0	0	+	+	+	-	0	0	0	0	0	0
Serratia marcescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Rhodospirillum rubrum	+	+	+	+	-	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0
Protaminobacter rubrum	+	+	+	+	-	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0
Pseudomonas rubescens	+	+	+	+	+	+	+	+	-	0	+	+	+	+	+	+	+	+	0	0
# 44	+	+	+	+	-	0	0	0	0	0	-	-	-	-	0	0	0	0	0	0
# 48	+	+	+	+	-	0	0	0	0	0	+	+	+	-	0	0	0	0	0	0
Cellulomonas rossica	+	+	+	+	+	+	+	-	0	0	+	+	+	+	+	+	-	0	0	0
# 215	+	+	+	+	-	0	0	0	0	0	+	+	+	-	0	0	0	0	0	0
Vibrio rubicundus	+	+	+	+	-	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	-	0	+	+	+	+	+	+	-	-	0	0
Marine (F) and (R)	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Marine (50) and (75)	-	-	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0

(+) growth - motility (-) no growth - no motility (0) no test

TABLE IV

	Miscellaneous Tests					Oxygen Tension			Pigmentation (Pink Pigment)			
	Citrate	Urease	Gelatin	Nitrite	Catalase	Aerobic	Reduced Oxygen Tension	Anaerobic	4°C.	20°C.	25°C.-30°C.	37°C.
# 334	-	-	-	-	+	+	+	+	0	+	+	+
# 301	-	-	+	-	-	+	+	-	0	+	+	+
# 306	-	-	-	+	+	+	-	-	0	+	+	+
Serratia marcescens	+	-	+	+	+	+	+	+	0	+	+	+
Rhodospirillum rubrum	-	-	-	+	-	+	-	-	0	+	+	+
Protaminobacter rubrum	-	-	-	+	+	+	-	-	0	+	+	+
Pseudomonas rubescens	-	-	+	+	-	+	+	-	0	-	-	-
# 44	-	-	-	-	-	+	-	-	0	+	+	0
# 48	-	-	-	+	-	+	-	-	0	+	+	+
Cellulomonas rossica	+	-	-	+	+	+	+	+	0	-	-	-
# 215	-	-	-	+	-	+	+	-	0	+	+	+
Vibrio rubicundus	-	-	-	-	+	+	-	-	0	+	+	0
Pseudomonas aeruginosa	-	-	+	+	+	+	+	+	0	-	-	-
Marine (F) and (R)	+	-	-	+	-	+	-	-	0	+	+	+
Marine (50) and (75)	-	-	-	+	-	+	+	-	0	+	+	+

(0) no visible growth

TABLE V

	# 334	# 301	# 306	Serratia marcescens	Rhodospirillum rubrum	Protaminobacter rubrum	Pseudomonas rubescens	# 44	# 48	Cellulomonas rossica	# 215	Vibrio rubicundus	Pseudomonas aeruginosa	Marine (F)	Marine (R)	Marine (50)	Marine (75)
MINIMAL MEDIUM PLUS SUPPLEMENTS																	
Salts plus glucose	-	-	+	-	-	-	-	+	+	+	-	-	+	-	-	-	-
Salts plus sodium acetate	-	-	-	+	-	+	+	-	-	+	-	+	+	-	-	+	+
Salts plus Casamino acids	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
(+) visible growth (-) no growth																	
SURVIVAL TESTS																	
Distilled water salts	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-	-	-
Artificial sea water	+	-	-	+	+	-	+	+	-	+	-	-	+	+	+	+	+
Well water	+	+	+	+	-	-	+	-	+	+	+	+	+	-	-	-	+
Distilled water	-	+	+	-	-	-	-	+	-	+	-	+	+	-	-	-	-
Demineralized water	+	-	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
(+) survival (-) death																	

TABLE VI

	# 334	# 301	# 306	Protaminobacter rubrum	Pseudomonas rubescens	# 44	# 48	Cellulomonas rosea	# 215	Vibrio rubicundus	Pseudomonas aeruginosa	Marine (F)	Marine (R)	Marine (50)	Marine (75)
Length (microns)	1.76	1.76	1.92	2.20	2.82	2.29	2.46	2.50	1.41	1.50	0.97	1.41	1.76	1.32	2.11
Width (microns)	0.53	0.62	0.62	0.88	0.44	0.53	0.70	0.35	0.53	0.70	0.35	0.53	0.35	0.79	0.88
Flagellar wavelength (microns)	0	0	1.76	0	1.50	1.50	1.76	1.76	1.94	1.23	1.58	0	0	0	0
Amplitude (microns)	0	0	0.44	0	0.62	0.53	0.44	0.44	0.53	0.62	0.44	0	0	0	0
Aggregates	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-
Single cells	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Strains of Rubriomonas	-	-	+	-	-	+	+	-	+	+	-	-	-	-	-

(0) flagella stains did not take

TABLE VII

'H' and 'O' AGGLUTINATION TITERS WITH SELECTED ANTISERA

Antigen	Antisera					
	# 44 H	# 44 O	# 48 H	# 48 O	P. aeruginosa H	P. aeruginosa O (rabbit died)
# 44 (Control) 'H'	1:80	0	-	0	-	0
# 44 (Control) 'O'	0	1:40	0	-	0	0
# 48 (Control) 'H'	-	0	1:80	0	-	0
# 48 (Control) 'O'	0	-	0	1:40	0	0
Pseudomonas aeruginosa (Control) 'H'	-	0	-	0	1:80	0
Pseudomonas aeruginosa (Control) 'O'	0	-	0	-	0	0
# 215 'H'	-	0	-	0	1:20	0
# 215 'O'	0	-	0	-	0	0
# 334 'H'	-	0	-	0	-	0
# 334 'O'	0	1:40	0	1:40	0	0
Protaminobacter rubrum 'H'	1:40	0	1:40	0	1:40	0
Protaminobacter rubrum 'O'	0	1:40	0	1:40	0	0

(-) no antigen antibody reaction

(0) no serological testing

DISCUSSION

Gram negative rods can be divided into three main groups based on how they do or do not utilize carbohydrates. There are those which ferment, oxidize or do not attack carbohydrates.

The first group can be divided into polar and non polar (peritrichous) flagellates. Aeromonas and Vibrio comprise the polar flagellated genera; the Family Enterobacteriaceae comprises the non polar flagellates.

The second group (oxidize carbohydrates) is divided into two main groups, the polar and non polar flagellates. The polar flagellates can be subdivided on the basis of pigment production. Chromobacterium (purple) and Xanthomonas (yellow) producing a water insoluble pigment; Pseudomonas and Acetomonas producing a water soluble pigment or none at all; Photobacterium producing luminescent (light producing) pigments.

The non polar flagellates of the second group are also subdivided on the basis of pigment solubility. Chromobacterium (purple) and Flavobacterium (yellow) produce water insoluble pigments; Agrobacterium and Achromobacter produce a water soluble pigment or none at all.

Finally there is the non carbohydrate utilization group which is divided like the second group in respect to flagellation and pigmentation (no luminescent pigments). Organisms with polar flagella and water insoluble

pigments have not been classified. Here is where the pink bacteria may be able to be classified. Some bacteria produce no pigments and are polar flagellated. In the non polar, water insoluble pigment category, no organisms are classified; but, non pigment producers are found in the Genus Alkaligenes.

Carbohydrate metabolism has always played an important role in the classification of micro-organisms.

Fermentation is the utilization of a carbohydrate under anaerobic conditions; oxidation requires oxygen as the final hydrogen acceptor. The means of determining acid production resulting from carbohydrate breakdown is by being able to observe the color change of an indicator. The color change is initiated by the acid produced. What about the organisms that do not produce excessive amounts of acid? They must go unnoticed unless they are tested in minimal medium and carbohydrate supplements. According to Bisset (1952a), many Pseudomonads complete the oxidation of carbohydrates and do not produce organic acids.

For the present, we must say that fermenters and oxidizers, in their own respective environments, must produce acid in sufficient quantities to overcome the phosphate buffer in the process of breaking down the carbohydrate. If they do not, they will not be considered as oxidizers or fermenters.

On the basis of carbohydrate utilization, the pink organisms discussed fall into the second and third groups, the oxidizers and non oxidizers

respectively. Some produced so little acid that at times the observer is led to believe that the pigment is somehow the orange yellow discoloration (acid ph of brom thymol blue) which would appear in the upper most strata of the tube and along the path of inoculation.

A few of the organisms (# 306, # 44, and # 48) did not show positive results in the carbohydrate base medium plus glucose but were capable of glucose utilization for growth (minimal medium - Table V). Since the Hugh Leifson method depends on acid production, slow acid production, or insufficient acid production, or acid utilization by the micro-organism would give negative results.

The marine bacteria, under optimal conditions, were capable of producing acid from a wider variety of sugars. The tris buffer at the specified pH is a weak buffer. This factor may have contributed toward the results.

In comparison to the Pseudomonads, both fresh and salt water bacteria required 24 to 48 hours more incubation time for positive results. All the tests were run at least in duplicate, some in triplicate; results were consistent.

Flagellation also plays a major role in the classification of the gram negative rods. Motile and non motile forms can be found in water. All those whose flagella stain worked, that were motile, were found to be monotrichous; the spirillum, lophotrichous.

Using flagellation as a norm, one would tend to say that the pink monotrichous organisms should be kept in the Family Pseudomonadaceae but placed into a new Genus (Rubriomonas). This Genus could then represent gram negative rods, motile by polar monotrichous flagellation, producing a pink to red pigment and incapable or capable of producing small amounts of acid from oxidation of carbohydrates.

Sodium chloride tolerance is of limited taxanomic value in the classification of an organism (Leifson, 1963). It is the belief among many taxanomists (Leifson, 1963) that a bacterium should not be placed into a different species merely because it grows or does not grow in sea water. In other words, if an organism fits a certain Genus (e.g. Pseudomonas), merely because it grows in sea water (4% salt concentration), does not mean that it should be classified differently.

Leifson (1963) considered a halophobe to be any organism that does not grow in a 1% NaCl medium.

Flannery (1956) divides NaCl responses into two groups.

I. Halophobes

A. Salt-sensitive organisms that grow only in media containing less than 2% NaCl.

B. Salt-tolerant micro-organisms which grow best in media containing less than 2% NaCl, but will grow in media containing more than 2%.

II. Halophiles

A. Facultative halophiles will grow in media containing less than 2% NaCl but will grow best in media containing more than 2%.

B. Obligate halophiles grow only in a medium containing more than 2% NaCl.

Except for # 334, all the distilled water bacteria (cultures identified by numbers) tested, Proteminobacter rubrum, plus Vibrio rubicundus did not grow in base broth with sodium chloride concentrations above 1% and can thus be considered halophobes.

The two classified Pseudomonads grew in 5% but not 6%; Cellulomonas rossica in 4%; two marine organisms plus Serratia marcescens in 7%; two marine organisms did not grow at all. Because two marine micro-organisms did not grow at all, it appears, that the test medium used base broth plus NaCl) did not possess the proper environment found in the artificial sea water. Thus the marine micro-organisms might be dependent on other factors besides osmotic pressure (4%) from the ocean.

Classification of these organisms was definitely not aided in any way by this study; but the test, as insignificant as it is in classification, has helped to group the organisms into two minor groups: those which do not grow in 2% NaCl and those which do grow in 2% NaCl.

The role which the other reactions play in classifying micro-organisms is primarily that of differentiating genera of a family (if all species in a particular Genus have the same definitive trait or of differentiating species of a specific Genus. All the organisms were urease negative; most did not liquefy gelatin.

In general, the organisms of the proposed Genus Rubriomonas are citrate and urease negative; catalase and gelatin positive and negative; indol negative; and non-hemolytic.

The survival tests showed that the organisms tested were capable of existing in different water environments. Water environments containing small amounts of mineral supported life the longest. Distilled water and demineralized water supported the least amount of life. If these organisms do land in the various water environments, we can see that they are capable of surviving. Another thing one must remember is that the sources of nutrient are replenished and of much greater magnitude in the natural state than they are in the test tube.

The organisms are aerobic, some are capable of growth at reduced oxygen tensions. The optimal temperature is in the range of 25° C. to 30° C..

Serology was useful in that the antigenic relationships were established. Other than this, the results are of little use.

Just how much importance should be placed on pigmentation. At present, some genera can be said to have been formed because of a pigment produced, namely, Xanthomonas, Flavobacterium, and Chromobacterium.

Very little work has been done with bacterial pigments. The determination of the solubility and the observation of a color change of a pigment in a solvent have been the only techniques applied. The following is an outline of the classification of pigments (Buchanan).

CLASSIFICATION OF PIGMENTS (BUCHANAN AND FULLMER)

I. No color change in medium; the pigment is diffuse in protoplasm, incorporated in granular globules, found in cell wall, infiltrates capsulor sheaths, and can be found intracellularly or cellularly.

A. Soluble in chloroform

1) Insoluble in alcohol

2) Soluble in alcohol - (Carotinoids)

a) Giving lipocyan reaction

(1) Soluble in chloroform

(2) Soluble in alcohol

b) Not giving lipocyan reaction

(1) Change color with acids or bases

(2) Do not change color with acid or base

(a) Fluorescent

(b) Non-fluorescent

B. Insoluble in chloroform

1) Water soluble (Anthocyanins)

2) Water insoluble

a) Soluble in alcohol

b) Insoluble in alcohol

(1) Soluble in bases

(2) Insoluble in bases

II. Pigments producing color change in medium (extracellular).

A. Soluble in water

1) Pigment soluble in chloroform

2) Pigment insoluble in chloroform

a) Pigment changed by acid or base

b) Pigment not changed by acid or base

B. Insoluble in water

1) Pigment soluble in some one or more of the usual solvents, exclusive of alkalies.

2) Pigments not soluble in some one or more of the usual solvents, exclusive of alkalies.

The pink pigment has been produced steadily for the two years that the organisms have been studied. It appears that the organisms do not mutate much in this respect.

Rhodospirillum rubrum is a spiral organism possessing bipolar lophotrichous flagellation, oxidizing few sugars, fermenting none. It is capable of withstanding 1% NaCl, being motile at the same time. With the

increase in osmotic pressure, the spirals become very long. Except for the fact that the organism is not motile at 4° C., it is capable of growth and motility at all temperatures tested. This aerobic organism is pink; the pink pigment turns light green when boiled in water as was shown when the organism was boiled to produce somatic antigen.

Pseudomonas rubescens does not produce a pink pigment; the organism appearing to be colorless (no pigment) or white. Only once did it appear that a pigment was produced. This was on an aged agar slant; even then, it was hard to distinguish. Otherwise, the organism appeared to be a member of the Genus Pseudomonas (Gessard) and should not be reclassified.

Cellulomonas rossica, contrary to Kellerman and McBeth (1933), is incapable of utilizing cellulose. This monotrichous organism is capable of oxidizing only glucose, growing at reduced oxygen tensions, and not capable of producing pigment. It should be placed in the Genus Pseudomonas.

Vibrio rubicundus is a pink rod, polar monotrichous, and incapable of oxidizing sugars or growing at reduced oxygen tensions. Other than the fact that most Vibrios lose their curvature upon being cultured on artificial media (Bisset, 1952b), contrary to Grotton (1942), the organism still shows more tendencies toward the new Genus proposed rather than the Genus Vibrio.

Protaminobacter rubrum may also be placed into the new Genus. This pink rod, contrary to Den Dooren de Jong (1926), is incapable of oxidizing carbohydrates.

The marine organisms were pink non motile rods capable of oxidizing carbohydrates. They should be placed into a Genus other than Pseudomonas.

The bacteria studied are gram negative rods which produce a pink pigment. These polar monotrichous organisms are aerobic and do or do not oxidize some sugars. The basis for establishing the new Genus is that the proposed Genus differs from the Genus Pseudomonas in the type of pigment produced. Pseudomonas produces a blue-green water soluble pigment; Rubriomonas produces a pink pigment attached to the soma of the cell.

SUMMARY

It is the hope of the author that some knowledge has been added to the field taxanomy. Rhodospirillum rubrum, Cellulomonas rossica, Pseudomonas aeruginosa, Vibrio rubicundus, Protaminobacter rubrum, and other previously unclassified pink pigment producing organisms were studied.

The author has concluded that certain pink pigment producing organisms (five strains) warrant the formation of a new Genus, Rubricomonas in the Family Pseudomonadaceae.

CHARACTERISTICS OF THE GENUS RUBRIMONAS

MORPHOLOGY

Rod-shaped, 1.14 microns to 2.46 microns (average 1.92 microns) by 0.53 microns to 0.70 microns (average 0.62 microns). Flagellation polar monotrichous. Flagella have 2 to 3 curves with an average wavelength of 1.64 microns and an average amplitude of 0.51 microns. Five strains are to be included in the new genus; # 306, # 44, # 48, # 215, and Vibrio rubicundus; also, possibly Protaminobacter rubrum (A.T.C.C. 8457).

CULTURAL CHARACTERISTICS

Colonies on agar are smooth, edges entire, not translucent. Growth on agar slants is moderate, smooth, pink to red, and not translucent. Growth in agar stab is confined primarily to the surface. Growth in broth is moderate with pellicle formation. No growth in broth under anaerobic conditions. Temperature relations, mesophilic; pH relations, neutrophilic; oxygen relations, aerobic; pigmentation, pink to red.

PHYSIOLOGICAL CHARACTERISTICS

Few carbohydrates are oxidized. The following tests are uniformily negative: citrate, urease, indole, gelatin, blood hemolysis. Nitrate and catalase variable.

HABITAT

Found in water.

PHOTOMICROGRAPHS

Figure 1. Photomicrograph of distilled water organism #44.

Leifson flagella stain. X 1000.

Figure 2. Photomicrograph of distilled water organism #48.

Leifson flagella stain. X 1000.

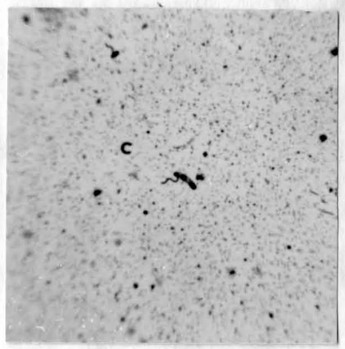

Figure 1

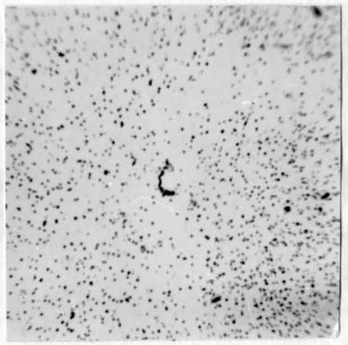

Figure 2

Figure 3. Photomicrograph of distilled water organism # 306.

Leifson flagella stain. X 1500.

Figure 4. Photomicrograph of distilled water organism # 215.

Leifson flagella stain. X 1700.

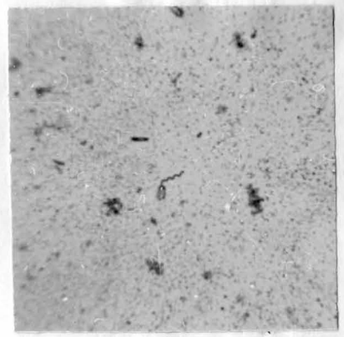

Figure 3

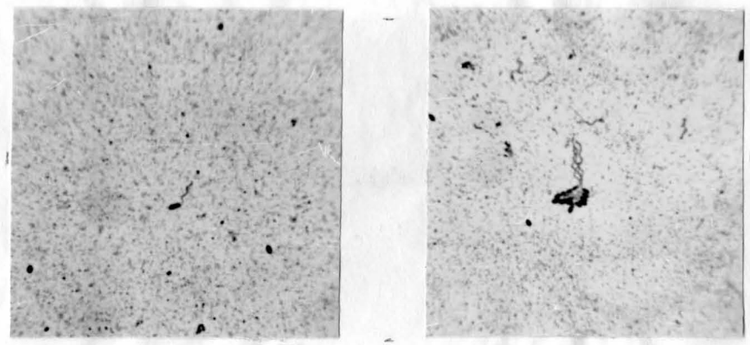

Figure 4

Figure 5. Photomicrograph of Vibrio rubicundus. Leifson Flagella stain. X 1000.

Figure 6. Photomicrograph of Cellulomonas rossica. Leifson flagella stain. X 1000.

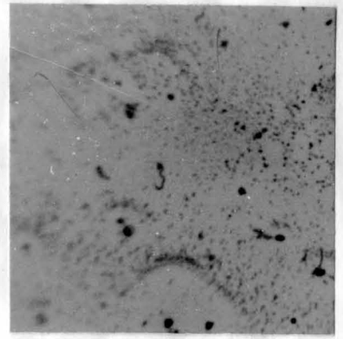

Figure 5

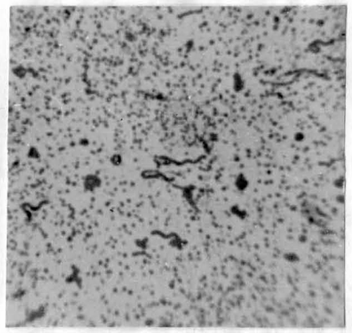

Figure 6

Figure 7. Photomicrograph of Pseudomonas rubescens. Leifson flagella stain. X 1000.

Figure 8. Photomicrograph of Pseudomonas aeruginosa. Leifson flagella stain. X 2000.

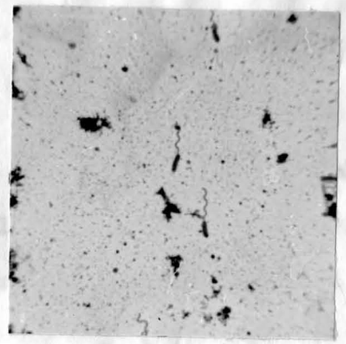

Figure 7

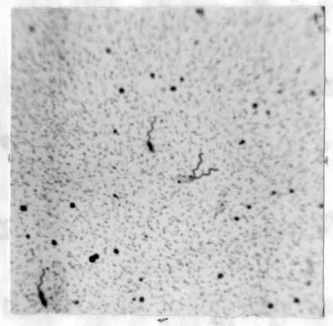

Figure 8

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APPROVAL SHEET

The thesis submitted by Marion Walter Zielinski has been read and approved by two members of the Department of Microbiology and one member of the Department of Biochemistry.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

January 15, 1964
Date

R. F. Baumann
Signature of Adviser