Iowa State University
Digital Repository

Retrospective Theses and Dissertations

Iowa State University Capstones, Theses and Dissertations

1940

The fluorescent bacteria in dairy products

Earl Raymond Garrison
Iowa State College

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Agriculture Commons, Food Microbiology Commons, and the Microbiology Commons

Recommended Citation

Garrison, Earl Raymond, "The fluorescent bacteria in dairy products " (1940). *Retrospective Theses and Dissertations*. 13675.
https://lib.dr.iastate.edu/rtd/13675

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

المنارة للاستشارات

www.manaraa.com

NOTE TO USERS

This reproduction is the best copy available.

UMI®

THE FLUORESCENT BACTERIA IN DAIRY PRODUCTS

by

Earl R. Garrison

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject Dairy Bacteriology

Approved:

Signature was redacted for privacy.

In charge of Major work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College
1940

UMI Number: DP12709

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI®

UMI Microform DP12709

Copyright 2005 by ProQuest Information and Learning Company.

All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

QR121
G1939

TABLE OF CONTENTS

INTRODUCTION........ 7

STATEMENT OF PROBLEM........ 10

DEFINITION OF FLUORESCENT BACTERIA........ 11

HISTORICAL........ 12

Fluorescence of Bacterial Cells........ 12

Factors Influencing the Production of Fluorescent Pigments by Bacteria........ 14

Variation Among Pure Cultures of Fluorescent Bacteria........ 35

Protective Action of Fluorescent Pigments on Bacteria........ 39

Toxicity of Fluorescent Bacteria Towards Other Microorganisms........ 41

Pathogenicity of Fluorescent Bacteria........ 42

Enzymes Formed by Fluorescent Bacteria........ 48

Fluorescent Bacteria Isolated from Sources Other than Dairy Products........ 58

Action of Fluorescent Bacteria on Dairy Products... 63

METHODS........ 79

Detection of Fluorescent Bacteria on Plates........ 79

Staining Procedures........ 79

Incubation Temperature........ 79

Absorption Spectra of Broth Cultures and Solutions of Certain Fluorescent Susbstances........ 80

T16713

General Characteristics.................................... 80

Action on litmus milk.................................... 80

pH and titratable acidity.................................... 80

Fermentation of carbohydrates.................................... 81

Proteolysis.................................... 81

Lipolysis.................................... 81

Hemolysis.................................... 82

Nitrate reduction.................................... 82

Formation of hydrogen sulphide.................................... 82

Production of indol.................................... 83

Utilization of urea.................................... 83

Formation of catalase.................................... 84

Production of ammonia.................................... 84

Growth in Uschinsky's medium.................................... 84

Diastatic action.................................... 84

Production of acetylmethylcarbinol.................................... 85

Formation of chlororaphine.................................... 85

Lactose Determination.................................... 85

Protein Breakdown in Skimmilk.................................... 85

Fat Acidities.................................... 86

EXPERIMENTAL.................................... 87

Isolation of Fluorescent Bacteria from Dairy Products and Other Materials.................................... 87

Distribution of the Fluorescent Bacteria.................................... 89

Milk.................................... 90

Unpasteurized sweet cream.................................... 92

Unpasteurized sour cream........................ 92

Ice cream...................................... 92

Freshly made sweet cream butter................ 93

Butter not freshly made from sweet cream....... 93

Water.. 94

Miscellaneous materials........................ 94

Sources of the Cultures Studied in Detail............ 94

Influence of Various Factors on Fluorescence........ 96

Composition of the medium...................... 96

pH of the medium............................... 97

Oxygen supply.................................. 98

Light.. 99

Incubation temperature......................... 100

Heating at 48.9°C.............................. 101

Age of the culture............................. 101

Absorption Spectra of Broth Cultures and Solutions of Certain Fluorescent Substances.................. 102

General Characteristics of the Fluorescent Bacteria. 109

Morphology..................................... 109

Growth on beef infusion agar................... 109

Growth in beef extract-peptone broth........... 110

Growth temperatures............................ 110

Growth of selected cultures in skimmilk at 5° and 21°C.................................... 111

Action on litmus milk.......................... 113

Fermentation of carbohydrates.................. 114

Acid development and utilization of lactose in skimmilk and lactose broth...................... 115
Proteolysis.................................... 119
Lipolysis...................................... 120
Hemolysis...................................... 120
Nitrate reduction.............................. 121
Formation of hydrogen sulphide................. 121
Production of indol............................ 121
Utilization of urea, formation of catalase, production of ammonia and growth in Uschinsky's medium... 122
Diastatic action, production of acetylmethylcarbinol and formation of chlororaphine......... 122
General Resistance of Fluorescent Bacteria.......... 123
Viability in culture media..................... 123
Resistance to heat............................. 124
Resistance to chlorine......................... 125
Tolerance of sodium chloride................... 126
Ability to grow in alkaline and acid media...... 129
Protein Breakdown in Skimmilk....................... 133
Action on Butter of Selected Cultures of Fluorescent Bacteria................................ 135
Keeping Qualities of Fresh Sweet Cream Butter Containing Fluorescent Bacteria.......................... 140
Growth of Fluorescent Bacteria in Water on Cottage Cheese.. 144

Species of Fluorescent Bacteria.................. 147

Key to the Identification of Fluorescent Bacteria.. 153

SUMMARY.. 155

ACKNOWLEDGMENT................................. 160

LITERATURE CITED............................... 161

INTRODUCTION

Bacteria that produce a water soluble, fluorescent pigment under suitable conditions are widely distributed in nature and are frequently encountered in dairy products. The marked proteolytic and lipolytic properties of many of these organisms and their ability to grow at relatively low temperatures make them potential agents of deterioration of milk and its products during holding. Defects in such products due to the action of this group of bacteria are rather common under commercial conditions and have been reported by several investigators.

The inhibitory effect of acid on the fluorescent organisms restricts their action to products with a pH not greatly reduced below that of normal milk. Accordingly, they are not important in the development of off-flavors in raw milk and cream or other products containing acid-forming organisms, except when held at temperatures too low to permit a rapid growth of the acid producers. Since the fluorescent bacteria are easily killed by heat, they should be destroyed by the usual pasteurization exposures, but recontamination from various plant sources can occur readily. Because of their psychrophylic nature, any of these organisms thus introduced after pasteurization might grow extensively during prolonged holding of dairy products at the usual holding temperature

and thus produce deterioration.

The addition of butter culture to cream before churning would be expected to restrict the growth of the fluorescent bacteria in butter, while even moderate salting of butter would be another important factor in their control. Unsalted and unripened cream butter, however, should be a suitable medium for the growth of these bacteria and when this occurs the flavor and odor might be adversely affected. The high acidity of most cheeses should largely prevent reproduction of the fluorescent bacteria, but they might grow in certain soft varieties, such as cottage, that have a reduced acidity due to washing the curd and thereby limit the length of the storage period.

The fluorescent bacteria isolated from both normal and defective milk products by various investigators have not always been studied sufficiently to be identified accurately. Consequently, it is not definitely known what species of these organisms are commonly present in dairy products or what species are the most important in producing defects in such materials. Information on the general distribution of fluorescent bacteria in milk and its products is also somewhat limited. This is due partly to the fact that the method commonly followed in making routine bacterial analyses of milk and cream does not permit detection of these organisms, both the medium and incubation temperature being unfavorable for both their growth and pigment production. Comprehensive information on the dis-

tribution of the fluorescent bacteria in milk and its products can only be obtained by the use of a procedure which permits fairly accurate detection of these organisms. Cultures isolated in such an investigation can be studied and information obtained on their classification.

STATEMENT OF PROBLEM

The purposes of this investigation were:

1. To determine the prevalence of fluorescent bacteria in certain dairy products.

2. To isolate and study cultures of fluorescent bacteria from dairy products.

3. To study the resistance of the fluorescent organisms to heat and chlorine.

4. To ascertain the effect of acidity and salt on the growth of the fluorescent organisms.

5. To determine the action of pure cultures of fluorescent bacteria on various dairy products.

6. To classify the fluorescent bacteria found in dairy products.

DEFINITION OF FLUORESCENT BACTERIA

Substances that have the power to absorb certain wave lengths and re-emit the energy in longer wave lengths instead of converting it into heat are said to be fluorescent. Such substances are visible under ultra-violet light because they emit visible light rays during the irradiation. Examination under ultra-violet light is therefore a convenient method of detecting fluorescent substances since they will be visible under this kind of light, while substances that are non-fluorescent will be invisible.

The dried cells of several species of bacteria show a characteristic fluorescent color when viewed under ultra-violet light, as was first observed by Arloing, Policard and Langeron (1925). Many species of bacteria, however, particularly certain species in the genera Pseudomonas and Phytomonas (Bergey, Breed, Murray and Hitchens, 1939) secrete a water soluble pigment under suitable conditions that is greenish colored in natural light and bluish-green to greenish-yellow under ultra-violet light. The bacteria included in this study produced a water soluble pigment and fluorescent colonies on beef infusion agar, pH 7.0 to 7.2, when incubated at a temperature of 20° to 25°C. for 72 hours and examined under ultra-violet light.

HISTORICAL

Fluorescence of Bacterial Cells

Arloing, Policard and Langeron (1925) made a brief study of the fluorescent color of dried bacterial cells under ultra-violet light and proposed a rough method of differentiation between organisms on this basis. Gassul and Žolkevič (1927) also found that each species of bacteria produced a characteristic fluorescent color and could be differentiated by examination under ultra-violet light. They stated that cells for such an examination should be obtained from cultures of the same age and grown on the same medium at a uniform temperature. Danielsen (1929), however, found no regularity in the shade of fluorescent color produced by various species of bacteria or by different strains of the same species. He disagreed with Gassul and Žolkevič (1927) in their belief that each species of bacteria has a definite characteristic fluorescence in ultra-violet light.

Lasseur, Dupaix and Lecaille (1931) grew various bacteria and yeasts on peptone agar at a pH of 7.0; the cells were washed three times in physiological salt solution or distilled water, then centrifuged and examined under ultra-violet light. All cells examined produced a fluorescent color to a greater or less extent, but these investigators concluded that fluorescence depends too much on the personal factor to be used

as a basis for differentiation between bacterial species. The fluorescent colors of certain species examined were:

Bacillus caryocyaneus	-	bluish-white
Bacillus chlororaphis	-	yellowish-white
Bacillus fluorescens albus	-	white
Bacillus fluorescens aureus	-	white
Bacillus fluorescens putidus	-	white
Bacillus pyocyaneus	-	green, yellowish-green or violet-orange

Pulvertaft (1934) examined the cells of several species under ultra-violet light and concluded that fluorescent color was useful in distinguishing between bacterial cultures. He stated that cultures used for this purpose should be grown aerobically on a non-fluorescent meat medium containing only the initial breakdown products of pancreatic extracts. The fluorescent substances in the cells were quite soluble in absolute alcohol or acetone but were only slightly soluble in ether or chloroform.

Ropetti (1938) found that under ultra-violet light many microorganisms in the living state gave an orange to red or yellow fluorescence which showed several well defined bands on spectral analysis. Species that produced bacteriofluorescein could also produce a porphyrine. Red yeasts produced protoporphyrine while *Actinomyces* species produced caproporphyrine.

Factors Influencing the Production of Fluorescent Pigments By Bacteria

The blue pigment of Pseudomonas aeruginosa, pyocyanine, was apparently first studied and named by Fordos (1860). He extracted this pigment from blue-green pus with chloroform and observed that acid turned it red while alkali turned it blue. Later (1863), some old crystals of pyocyanine that had turned yellow with age were dissolved in ether and when the ether was evaporated yellow crystals, called pyoxanthase, were obtained. The organism causing the formation of blue pus in wounds was first isolated by Schroeter (1872) and named Bacterium aeruginosum. Gessard (1882) also made several isolations of the organism responsible for the blue-green coloration of the cloth dressing on wounds and found that it produced the blue pigment, pyocyanine, of Fordos. He therefore proposed the name Bacillus pyocyaneus for the organism.

Hueppe (1884) studied a blue-green pus organism that produced a greenish fluorescence in gelatin and an organism, isolated from water, that first developed a greenish fluorescence and later a violet to blue-black color in gelatin. He concluded that the color formed by these organisms varied with the chemical compounds in the medium.

Ledderhose (1888) made a brief study of factors affecting the formation of pigment by Bacillus pyocyaneus. He observed that this organism produced a blue color in cultur-

al media, while Bacillus fluorescens formed a yellow-green color and did not develop the characteristic odor of blue pus.

Gessard (1892) was the first to make a careful study of the relationship between the composition of the medium and the production of the fluorescent pigment by Bacillus pyocyaneus. Fluorescence was produced in a water extract of lean beef, while a blue tint but no fluorescence was developed in a 2 per cent solution of market peptone. A synthetic medium composed of ammonium succinate 10 gm., potassium phosphate 5 gm., magnesium sulphate 2.5 gm., and water 1000 ml. supported growth and the production of fluorescence; the use of 0.0625 gm. potassium phosphate per liter resulted in the production of pyocyanine but no fluorescence; 0.125 gm. or slightly more potassium phosphate per liter supported the production of both pyocyanine and fluorescence; while when 1.3 gm. or more potassium phosphate was used fluorescence alone was produced. The base united with the phosphate was not important in the production of fluorescence. A correct proportion between the amounts of nitrogen and phosphate in the medium was believed to be necessary for the simultaneous production of pyocyanine and the fluorescent pigment. An excess of nitrogen resulted in the formation of pyocyanine alone while an excess of phosphate gave only the production of fluorescence. Lecithin in place of potassium phosphate in the medium was very effective in

supporting the production of fluorescence. Phosphoglycerate also was believed to support the development of fluorescence.

Charrin and Dissard (1893) used a synthetic medium, composed of monopotassium phosphate, disodium phosphate, calcium chloride, magnesium sulphate, potassium bicarbonate and water, to which different substances were added to determine their effect on the production of chromogenic substances by _Bacillus pyocyaneus_. Peptone added to this medium supported good growth but only little color production; asparagine gave good growth and abundant pigmentation; dextrose and glycogen gave slight growth and weak chromogenesis; lactic acid gave a weak growth; while no growth occurred with urea.

Thumm (1895) reported that the presence of magnesium sulphate and potassium phosphate in the medium was of the greatest importance in the formation of pigment by _Pseudomonas aeruginosa_ and _Pseudomonas syncyanea_. Pigment production occurred only in the presence of atmospheric oxygen. When these bacteria were grown with an acid-forming organism, a yellow pigment developed, but there was no fluorescence. The green fluorescent pigment of _Pseudomonas pyocyaneus_ was found to be non-crystalline and soluble only in water and dilute alcohol. In concentrated solution it was dark orange to red-brown and exhibited a pale blue fluorescence in reflected light. Alkali changed the color to a fluorescent green.

Lepierre (1895) studied the formation of pigment by a pathogenic fluorescent organism in peptone broth made from various protein materials. He stated that the development of fluorescence depended on the presence in the medium of meat extracts, such as xanthine and creatine, and soluble albuminoids. Heating under pressure was found to destroy the fluorescigenic power of the medium.

Jordan (1897-1899) studied the production of fluorescence by six species of fluorescent bacteria, including a culture of Bacillus fluorescens liquefaciens isolated from Lake Michigan water. He found that (a) although both phosphorus and sulphur were essential for the production of the fluorescent pigment, only traces were needed and enough was sometimes supplied as impurities in other chemicals, while the nature of the base associated with the phosphate and sulphate was not important; (b) the presence of the methyl or methylene group was coincident with superior nutritive value and fluorescigenic power; (c) pigment was not produced in an acid medium and when pigment was present in an acid medium the acid concealed its presence; (d) diffuse daylight was unfavorable to pigment production; (e) chemical substances favorable to growth and pigment production in certain concentrations checked pigment formation when present in excess, although growth was more abundant; and (f) the production of fluorescent pigment was not of vital importance to the cell. Jordan (1897) also studied six cultures of Bacillus pyocyaneus

and found that both phosphate and sulphate were essential for the formation of the fluorescent pigment but were not necessary for the production of pyocyanine. Asparagine, ammonium citrate, ammonium lactate and ammonium succinate were all suitable sources of nitrogen for the production of both pigments. The ability to produce pyocyanine under conditions of artificial cultivation was lost sooner than the fluorescigenic power. The fluorescent pigment was slowly oxidized by the action of light, air and certain chemical reagents to a yellow pigment, while pyocyanine was similarly oxidized to a black pigment. Jordan concluded that the fluorescent pigment of this organism was similar to the pigments produced by other fluorescent bacteria. Cultures at first showed a delicate robin's egg blue which changed to green as the solution became more alkaline, due to bacterial growth, and in strongly alkaline solution were deep green without fluorescence. The color was destroyed by the addition of acid but was restored by the addition of alkali. When the fluorescent pigment was oxidized, as in old cultures, it was yellow or yellow-brown. The pigment was soluble in water but was insoluble in chloroform.

Gessard (1901) reported that Bacillus pyocyaneus, when growing in suitable media, first gives rise to a red pigment, then later to a black pigment. The black pigment was believed to be due to oxidation of tyrosine and was only formed in media containing this amino acid. No blackening occurred

in gelatin, but milk was rendered black after which other colors were formed, the sequence depending on the amount of air present. The name, Bacillus pyocyaneus melanogène, was given to this variety of the organism. McCombie and Scarborough (1923), however, found that the black pigment produced by oxidation is not due to an interaction with tyrosine. They prepared several salts of pyocyanine and, after a careful study of them, concluded that the probable formula was $C_{26}H_{28}O_3N_4$, but that it may lose one or two molecules of water, and the salts may be derived from any one of the three forms.

Jirou (1901) studied the biochemical properties, growth characteristics and pigment production of 14 species of fluorescent bacteria, including Bacillus pyocyaneus, Bacillus chlororaphis, Bacillus fluorescens mesentericus, the organism of blue milk and other fluorescent organisms isolated from water, a case of empyema and the exudate from angina. To produce fluorescence these organisms required a simple source of inorganic nitrogen, a hydrocarbon or glycerol and a phosphate mineral. Phosphate was indispensable for the production of fluorescence, but the amount required varied with the growth conditions.

Sullivan (1905), using a synthetic culture medium for studying the formation of bacterial pigments, found that pyocyanine production is independent of the presence of either a phosphate or sulphate, while the production of the

fluorescent pigment requires both. The formation of pyocyanine and the production of fluorescent pigment were believed to be closely related functions since Bacillus pyocyaneus produced either one or both of these pigments, depending on the composition of the medium. Production of the fluorescent pigment was favored by a high phosphate content and a slight alkalinity, while the production of pyocyanine was favored by a low phosphate content and a slightly acid reaction. Ammonium malate, ammonium tartrate and ammonium oxalate in concentrations under 0.4 per cent supported growth but not pigment formation, while ammonium lactate, ammonium citrate and ammonium succinate supported pigment production. The formation of pigment was also dependent on the reaction of the medium, growth temperature and the presence of oxygen.

Lasseur (1909) reported that several factors affected the formation of chlororaphine by Bacillus chlororaphis, but the composition of the medium was of most importance; among the sources of nitrogen utilized by this organism only peptone permitted the production of the green crystals. The most favorable temperature for crystal formation was near 25°C. The pigment was insoluble in water, ether and benzene and precipitated from cultural media as long, flexuous needles in the form of bundles or rosettes. It was slightly soluble in methyl, ethyl and amyl alcohols but was very soluble in acetone. The crystals were yellow in an oxidizing medium and green in a reducing medium. The green crystals

were unstable and oxidized rapidly, while the yellow crystals were stable. Later, Lasseur (1911) employed a medium composed of water 100 gm., asparagine 0.7 gm., glycerol 2.5 gm., dipotassium phosphate 0.1 gm., magnesium sulphate 0.5 gm., calcium chloride 0.04 gm. and ferrous sulphate 0.01 gm. for the growth of Bacillus chlororaphis and found that it supported abundant crystal formation and the development of a noticeable fluorescence. Dipotassium phosphate, glycerol, magnesium sulphate and ferrous sulphate were indispensable for the production of chlororaphine, while calcium chloride favored its formation. A temperature of 24° to 30°C. was most suitable for crystal production, while none were formed at 37°C. The optimum oxygen content of the air for crystal formation was around 35 to 65 per cent. The presence of magnesium, sulphate and phosphate in the medium was essential for the formation of fluorescence, while the presence of calcium chloride and iron was considered favorable. The nature of the nitrogen and carbon compounds and the proportion of these compounds to the mineral substances in the medium also affected the development of fluorescence. The optimum temperature for the production of fluorescence was 22° to 24° C., while a temperature of 37°C. inhibited its appearance except with a few strains. Xanthoraphine, a yellow water soluble material, was believed to be the mother substance from which chlororaphine was formed. When xanthoraphine was reduced in an acid medium, a green color appeared but, when reduced in a

neutral medium, green crystals of chlororaphine were precipitated; the green crystals were transformed by oxidation into oxychlororaphine, a yellow, slightly water soluble substance. The formula for oxychlororaphine was given as $C_{14}H_{10}N_3O$. Kögel and Postowsky (1930) showed that xanthoraphine was the same product as phenazine-alpha-carbonamide, while chlororaphine was a dimolecular mixture of phenazine-alpha-carbonamide and its derived dihydro or dihydrophenazine. Oxychlororaphine was found to have the formula $C_{13}H_9N_3O$. Elema (1933) studied the oxidation-reduction potential of chlororaphine and found that a reversible reduction of phenazine-alpha-carbonamide in aqueous solution occurred at pH 0 to 3, resulting in a color change from yellow to green to orange by the successive addition of hydrogen.

Lasseur (1911a) found that iron was specifically required for the production of chlororaphine by Bacillus chlororaphis in a synthetic medium and could not be replaced by manganese, nickel, cobalt, zinc, chromium or boron. Ferrous sulphate gave the best results, but ferrous citrate and ferrous chloride also gave satisfactory crystal formation. The use of 0.3 mg. of ferrous sulphate per 1000 ml. of medium favored the appearance of a green fluorescence only, while the addition of 1.0 mg. gave uniform production of chlororaphine. Lasseur, Thiry and Dupaix (1930) showed that the development of a violet tint and the blackening of the surface membrane during the growth

of <u>Bacillus caryocyaneus</u> in the synthetic medium of Lasseur (1911) was dependent on the presence of iron in the substrate. Lasseur, Marchal, Dupaix and Renaux (1932) determined the role of iron in the production of pigment by <u>Bacillus cyaneofluorescens</u> in this synthetic medium. Iron was found necessary for the formation of the blue-violet, brown or black pigment characteristic of this organism, but the amount needed varied with the culture, only traces being required in some cases. They found it necessary to work with carefully purified reagents and with the progeny of single cells in order to show the variations in iron requirements for the production of pigment by different cultures of the organism.

Nogier, Dufourt and Dujol (1913) observed a culture of <u>Bacillus pyocyaneus</u> that produced a red pigment, in addition to the green fluorescent and brown pigments. This pigment was formed only on suitable media and was not believed to be a degradation product of the brown pigment. Pigment production occurred at 15°, 37° and 46°C. but only slowly at the highest temperature. The red material was soluble in alcohol or acetic acid but not in chloroform or ether.

Gessard (1917) reported that a certain culture of <u>Bacillus pyocyaneus</u> first produced a yellow color in 2 per cent peptone solution and then, after a time which varied with the aeration of the medium, a red zone appeared on the surface instead of the blue-green color that occurs with the ordinary organism; the red color finally extended throughout

the mass of the liquid. This organism was designated Bacillus pyocyaneus erythrogène. Factors affecting the production of fluorescence and the red color by this organism were studied by Mammelle (1918). The red pigment was very evident in 24 to 48 hours in gelatin-peptone-glycerol medium but was not noticeable in peptone broth until after 5 or 6 days; the color was partly lost after the cultures had aged for several months.

Blanchetière (1917) found that a medium composed of sodium chloride 5 gm., disodium phosphate 1 gm., dipotassium phosphate 1 gm., asparagine 3 gm. and distilled water 900 ml. supported growth and pigment production by Bacterium fluorescens liquefaciens. The maximum yellowish-green fluorescent color was produced at 25°C.; no fluorescence was formed at 37°C., but after a long time a yellowish color sometimes appeared.

A spectographic analysis of the pigments produced by Bacillus pyocyaneus was made by Cluzet, Rochaix and Kofman (1921). Filtered broth cultures of the organism were examined under ultra-violet light and the absorption bands noted. The combined pigments (pyocyanine and the green fluorescent pigment) produced by this organism gave total absorption of ultra-violet rays starting from 4,500Å. The absorption band for pyocyanine extended from 3,800 to 3,550Å, while the green fluorescent pigment gave total absorption from 4,250 to 3,580Å.

The absorption band of the melanogène pigment varied from 4,300 to 3,000Å, depending on the concentration in the medium. The pigment erythrogene gave total absorption of ultraviolet light starting at 4,700Å.

Gessard (1919) classified the _Bacillus pyocyaneus_ organisms into four varieties on the basis of the pigments produced in 2 per cent peptone water. These are:

Bacillus pyocyaneus var. _pyocyanogène_-produced the blue pigment pyocyanine.

Bacillus pyocyaneus var. _melanogène_ --produced a black pigment.

Bacillus pyocyaneus var. _erythrogène_ -produced a red pigment (yellow at first).

Bacillus pyocyaneus var. _achromogène_ - produced no pigment.

Later, (1920) he divided these four varieties into 16 subvarieties on the basis of pigment production in peptone water, bouillon and glycerol-peptone-gelatin medium.

Meader, Robinson and Leonard (1925) stated that _Pseudomonas pyocyaneus_ produced three water soluble pigments (a) fluorescent, yellow by transmitted light and green by reflected light; (b) pyocyanine, bright blue; and (c) pyorubin, bright red. The fluorescent pigment was found to be non-specific, pyocyanine was specific and pyorubin was characteristic of _Pseudomonas pyocyaneus_. Each of these pigments were derived by oxidation of a specific leuco-base that was formed anaerobically. The fluorescent pigment and pyocyanine

acted as pH indicators, but the color of pyorubin was not changed by addition of acid or alkali.

Gubitz (1928) stated that <u>Bacterium fluorescens</u> and other fluorescent organisms failed to produce fluorescence at 0°C. but developed a brown color after aging at that temperature. Types of <u>Bacterium fluorescens</u> with a maximum growth temperature around 35°C. formed a yellow color but were not fluorescent after several transfers at 30°C.; when again grown at 18°C., however, abundant fluorescent pigment was produced.

Lasseur, Thiry, Dupaix and Oliver (1930) reported that <u>Bacillus caryocyaneus</u> failed to grow in the synthetic medium of Lasseur when phosphate was excluded; 50 mg. of dipotassium phosphate per 100 ml. of medium was necessary to give good growth and fluorescence. An amount of phosphorus that supported normal growth of the organism also resulted in the appearance of fluorescence, which denoted that phosphorus only indirectly affected the fluorescent function of the organism. Growth occurred but no fluorescence was produced in the absence of magnesium sulphate; when 300 to 600 mg. of magnesium sulphate per 100 ml. of medium was added to an 8 day old uncolored culture, fluorescence appeared in 24 hours. Since magnesium chloride supported the production of fluorescence, it was concluded that magnesium, and not sulphate, was the important substance in the production of fluorescence. Cultures grown in the synthetic medium devoid of magnesium sul-

phate, but containing 1 to 2 mg. of magnesium chloride per 100 ml., developed a yellow color which was transformed into a green fluorescence when magnesium sulphate, additional magnesium chloride, manganese chloride or zinc sulphate was added; this green coloration was produced in the presence of oxygen and also when the air was replaced with nitrogen.

Dupaix (1930) studied an organism, originally isolated by Beijerinck from rotten willow wood, which closely resembled <u>Pseudomonas pyocyaneus</u> but varied enough to constitute a different species and was named <u>Bacillus caryocyaneus</u>. It produced a blue pigment, caryocyanine, that closely resembled pyocyanine but was very unstable and easily transformed into a dark brown pigment. In the synthetic medium of Lasseur (1911), the organism produced a thin white voile and a green fluorescent pigment at the top of the medium in 24 hours; after 48 hours the coloration extended down in the tube, and a blue pigment appeared which later masked the green fluorescence; after 15 days the liquid was colored an intense blue by reflected light and violet-red by transmitted light. A pH of 7.2 was the most favorable for the production of fluorescence, but a pH of 6.0 was the most favorable for the appearance of the blue-violet pigment. Some cultures grew at 39°C. but produced no pigment at that temperature.

Elema (1931) determined the oxidation-reduction potential of pyocyanine in the pH range 1.3 to 11.5. In the more physiological region (pH 6.0 to 9.0) pyocyanine was found to occupy

a place on the oxidation-reduction scale between methylene blue and indigo trisulphonate. In the pH region below 6.0, the pigment showed the remarkable color change from red to green to colorless during the reduction process. The relation between Eh and percentage reduction indicated that reduction occurs in two steps, each involving one electron.

Lasseur, Oliver, Dupaix and Maguitot (1930) found that a pH of 7.0 was the most favorable for production of the green fluorescent pigment by <u>Bacillus caryocyaneus</u> in the synthetic medium of Lasseur (1911), although a slightly acid medium was more favorable for growth. Both the green fluorescent and the blue-violet pigments were formed in this medium with an initial pH as low as 5.4, but since the medium became progressively more alkaline with the growth of the organism it was impossible to establish a close relationship between the development of fluorescence and the reaction of the medium. Lasseur, Dompray and Dupaix (1931) obtained growth of <u>Bacillus chlororaphis</u> in a synthetic media at initial pH values that varied from 5.4 to 7.64; after incubation the pigment, chlororaphine, occurred in inoculated tubes at pH values ranging from 6.58 to 7.47, depending on the original pH of the medium.

With <u>Bacillus chlororaphis</u>, <u>Bacillus caryocyaneus</u> and <u>Bacillus cyaneo-fluorescens</u>, Lasseur, Fribourg and Grojean (1932) showed that the pH varied with the depth of the medium in the tube, being highest at the top where growth was abun-

dant and lowest in the depth of the tube where growth was scant; fluorescence appeared at a pH near 7.0 and did not develop at the initial pH of 5.4. Lasseur and Dupaix-Lasseur (1936) also reported that Bacillus chlororaphis tends to convert the pH of the medium in which it is growing towards a reaction most favorable for its development.

Lasseur, Dupaix-Lasseur and Marchal (1936) measured the amount of fluorescence in bacterial cultures by means of a spectral photometer and found that Bacillus chlororaphis produced the maximum fluorescence when the pH of the medium was near 6.4. About 100 mg. of magnesium sulphate per 1000 ml. of medium was sufficient to induce development of maximum fluorescence.

Georgia and Poe (1931) made a comprehensive study of the inorganic constituents necessary for the production of fluorescent pigments by bacteria. Magnesium, phosphate and sulphate in the medium were found to be essential for pigment production. Even highly purified chemicals sometimes contained enough of these substances as impurities for the development of fluorescence. Media sterilized in soft glass test tubes sometimes dissolved enough magnesium to permit production of fluorescence. A synthetic medium composed of magnesium sulphate 0.5 gm., dipotassium phosphate 0.5 gm., asparagine 3.0 gms. and distilled water 1000 ml. proved to be very satisfactory for the production of bacterial fluorescence.

Georgia and Poe (1932) found considerable variation in the ability of _Pseudomonas fluorescens_ and closely related organisms to produce fluorescence in broths prepared from various commercial brands of peptone, even when the reaction was adjusted to a pH of 6.9 to 7.1. Some peptones failed to support the production of fluorescence with any of the organisms studied, while certain peptones favored the formation of fluorescence with some organisms but not with others. The addition of phosphorus, magnesium, and/or sulphate to the media that did not support pigment production resulted in the development of fluorescence; the addition of purines, meat bases or asparagine to the peptones that were deficient in fluorescigenic power did not stimulate pigment production. Ordinary sterilization exposures did not appreciably affect the fluorescence producing ability of a medium but a prolonged exposure at higher pressures reduced the amount of pigment produced. A 3 per cent peptone broth gave good growth but less pigment production than a broth containing 0.5 per cent peptone. Fluorescence was produced in media ranging in pH from 6.1 to 8.1 but was most abundant in the pH range from 6.8 to 7.3; the most pigment appeared in the cultures after 3 to 4 days incubation.

Lacey (1932) observed that certain plant pathogens, which closely resembled _Bacterium fluorescens liquefaciens_ and _Bacterium fluorescens non-liquefaciens_ in cultural characteristics, produced a brown coloration of the agar after a

green fluorescence had first developed. The fluorescent plant pathogens produced a green fluorescence in broth at pH 5.2 to 5.8 in 6 days, while *Bacterium fluorescens liquefaciens* and *Bacterium fluorescens non-liquefaciens* failed to produce fluorescence in broth at a pH as low as 6.9. The plant pathogens produced fluorescence in broth at 20°C. in 6 days but the other two organisms did not produce fluorescence at a temperature below 22°C.

Robinson (1932) concluded from his investigation that suitable forms of nitrogen, organic carbon, magnesium and phosphorus dissolved in proper concentration in distilled water and adjusted to a pH of 6.0 to 9.0 comprised a satisfactory medium for the aerobic growth of *Bacillus pyocyaneus*; anaerobic growth also required the addition of nitrate. The formation of the two pigments of the organism required only those substances necessary for growth, but the pigmentary function was sensitive to two other factors which were of minor relevance to the function of aerobic growth; these factors were the degree of atmospheric oxidation and the concentration of medium constituents. A high phosphate concentration inhibited pigment formation. The occurrence of traces of pigment in anaerobic tubes was found to be an index of inefficient deoxygenation. A medium consisting of sodium citrate ($5\frac{1}{2}$ H_2O) 1.0 per cent, calcium sulphate 0.025 per cent, magnesium sulphate ($7H_2O$) 0.05 per cent, ammonium chloride 0.1 per cent, monopotassium phosphate 0.2 per cent and ethyl

alcohol 0.25 per cent in distilled water and dispensed in tubes with a surface / volume of 0.25 was quite suitable for pigment production.

Labrousse (1934), working with certain phytopathogenic bacteria, found that they produced a definite fluorescence on beef extract medium but only if magnesium and phosphate were present.

Clara (1934) investigated the ability of several plant pathogens to produce the green fluorescent pigment in different media at a temperature of 27°C. About half the organisms studied developed a green fluorescence in beef extract-peptone broth and beef extract-peptone agar at pH 6.95 in 10 days; the addition of magnesium sulphate or of magnesium sulphate and dipotassium phosphate to beef extract agar had no appreciable effect on pigment production. Only a few organisms produced pigmentation on potato-dextrose agar at pH 6.7 in 9 days or in Cohn's solution in 7 days. Only part of the organisms studied formed a green fluorescence after 7 to 15 days growth in Uschinsky's solution when made as originally recommended in 1893, but when the pH was adjusted to 6.95 all but one of the organisms produced a pronounced green fluorescence in 3 days. Green fluorescence was also reported as being produced on starch agar by some organisms. A medium composed of magnesium sulphate (anhydrous) 0.5 gm., dipotassium phosphate (anhydrous) 0.5 gm., asparagine 5.0 gm. and distilled water 1000 ml. was very satisfactory for the production of fluores-

cence by the plant pathogens. All the organisms studied always produced the green fluorescent pigment when repeatedly cultured in this solution, regardless of the time of isolation which extended over two years.

Turfitt (1936), working with 100 strains of fluorescent bacteria isolated from water, feces, soil and other sources, found that peptone and glycerol in the medium favored pyocyanine production, while gelatin supported the development of considerable fluorescence. When the amount of peptone in Gessard's medium was increased from 2.0 to 2.5 per cent, fluorescence was not detectable. Bacto-peptone gave the best results of any peptone used. If magnesium or phosphate was omitted from the medium, growth occurred but no color was formed; if a trace of heavy metals was added, or tap water used for making the medium, growth resulted but pigment formation was completely inhibited. Strains of Bacillus pyocyaneus could not be induced to produce fluorescence and strains of Bacillus fluorescens could not be induced to produce pyocyanine by long cultivation on different media suitable for such production.

Turfitt (1937) extracted and purified by electrodialysis the green fluorescent pigment produced by the pyocyaneus-fluorescens group of organisms. He obtained an amorphous, greenish powder with an ash content of 0.4 per cent that was readily soluble in water, phenol or acetic acid, slightly soluble in dilute aqueous alcohol but insoluble in all other organic

solvents tried, including chloroform, ether and alcohol. Dilute aqueous alkaline solutions showed a green fluorescence but became colorless and non-fluorescent upon acidification; more concentrated, alkaline solutions had a red color and an intense green fluorescence. The red color that occurs in aged agar slant cultures of the organisms was attributed to an increased concentration of the green fluorescent pigment, although it was pointed out that some modified form of the pigment might also be produced by oxidation. Turfitt did not agree with Meader, Robinson and Leonard (1925) that the red color which they designated pyrorubin was a separate pigment from the green fluorescent pigment. When ammonium hydroxide was added to the colorless material from below the surface of a 4 day old culture, a green fluorescence was produced; the addition of hydrogen peroxide to this colorless material did not produce any color. The formula for the purified substance was found to be C_4H_7NO. A spectrographic analysis in an alkaline solution showed a well defined wave band with maximum absorption at 4,100Å, while in an acid solution the maximum absorption was at 3,700Å. The pigment obtained from different organisms was the same by analytical and spectrographic analyses. The substance gave a positive Millons test.

Orla-Jensen, Otte and Snog-Kjaer (1936) observed that the fluorescent bacteria and Bacterium pyocyaneum grew better and produced more pigment in untreated milk than in milk to which bios and lactoflavin had been added. They believed that bios

prevented these bacteria from producing pigment in milk.

Variation Among Pure Cultures of Fluorescent Bacteria

Charrin and Phisalix (1892) noted that *Bacillus pyocyaneus* grew abundantly at 43°C. but produced no color characteristic odor at that temperature. If a culture grown at 43°C. was transferred to fresh medium and incubated at a lower temperature, color was again produced, but if the culture was carried through several transfers at 43°C. before being held at a favorable temperature, the chromogenic function was not recovered. The colorless cultures were fatally pathogenic to guinea pigs and rabbits, and when recovered after death of the animals the organisms still failed to produce pigment in broth at a favorable temperature.

Jordan (1897) found that *Bacillus pyocyaneus* lost its ability to produce pigment under conditions of artificial cultivation, but the ability to produce pyocyanine was lost sooner than the fluorescigenic power.

Gessard (1919) isolated from the exudate of a wound a culture of *Bacillus pyocyaneus* that produced pyocyanine in peptone solution and both pyocyanine and a green fluorescence in bouillon but later failed to produce pyocyanine after several alternate passages in these media. The name *Bacillus pyocyaneus achromogène* was given to this variety of the organism.

Gessard (1920) gave the name *Bacillus pyocyanoides* to cul-

tures of Bacillus pyocyaneus that had become so altered by growth on laboratory media that they no longer closely resembled the original or typical culture and failed to produce pyocyanine in any medium. The "pyocyanoides" were divided into two races--Race F produced a green fluorescence in bouillon, while Race S was achromogenic. Later, Gessard (1925) reported that a culture of Bacillus pyocyaneus melanogène lost the ability to form pyocyanine and liquefy gelatin after a series of transfers on peptone-glycerol-gelatin medium.

Blanc (1923) observed that a culture of Bacillus pyocyaneus grown in bouillon 24 hours at 37°C. and then 10 to 12 days at room temperature showed both normal and modified colonies (small, clear, wrinkled) when inoculated on gelatin slopes. A modified colony was inoculated into bouillon and incubated as before after which the culture was filtered through a Chamberland filter and the filtrate then mixed with another modified colony and again spread over a gelatin slope. After each treatment in this manner the culture produced less pyocyanine and fluorescent pigment than previously and after passage through the third filtrate became permanently achromogenic and lost the ability to liquefy gelatin. Recently isolated cultures were less resistant to this change than stock cultures grown on an artificial medium. The addition of a few drops of glycerol to the medium augmented the change in colony type. The alteration of the organism was attribut-

ed to the action of the decomposition products in the filtrate and to the lysogenic action of the glycerol.

Lagrange (1927) added one drop of bacteriophage from cultures of certain organisms in the genera Salmonella, Shigella, Escherichia and Staphylococcus to a broth culture of Pseudomonas fluorescens and 24 to 72 hours later plated the culture on gelatin, nutrient agar and nutrient agar plus sucrose. Various modifications were noted in the colonies. that developed. A bacteriophage never gave the same result with all 12 organisms used. Some bacteriophages produced a division of the cultures into two distinct varieties; one variety developed a pale colony on agar while the other formed a deep colored colony. Gelatin liquefying cultures permanently lost the ability to liquefy gelatin. On one occasion the filtrate from contaminated soil water induced a gummy variant with a stock culture, but the variation was not permanent with all sub-cultures isolated. A filtrate from the excreta of hens, however, imparted a permanent ability to certain cultures to form a gummy substance. Later, Lagrange (1928) found that Pseudomonas fluorescens developed a gummy and vitreous type of colony along with the normal type on sucrose agar. The variant cultures evolved into a homogeneous type of organism.

Lacey (1931) reported that Bacterium trifolarium, which closely resembled Bacterium fluorescens non-liquefaciens except that it was pathogenic to Vicia faba, exhibited various

types of growth on bouillon agar, from translucent, smooth, round, slightly convex colonies with entire or slightly crenated margins to a transparent, flat growth spreading in a thin film over the surface. Each type of colony could give rise to the other types on replating, and all were identical in virulence and biochemical reactions. Lacey (1932) also noted a varability among different strains of certain green fluorescent organisms in coagulating and peptonizing milk, reducing nitrates, liquefying gelatin and fermenting sucrose or dextrose. The ability to liquefy gelatin gradually became weaker as the organisms were carried on agar.

Lasseur, Marchal and Dupaix (1930) found that some cultures of Bacillus caryocyaneus grew in a synthetic medium (Lasseur, 1911) at 39°C. but developed no color at that temperature; after 20 passages at 39°C., these cultures failed to produce any fluorescent pigment when again incubated at 25°C. The achromogenic character was permanent.

Lasseur and Dupaix-Lasseur (1934) reported that Bacillus chlororaphis produced both rough and smooth colonies on agar plates with variations among each type. Colonies designated as Ra were elevated, transparent, wrinkled and granulated with lacerate edges, while the Rb colonies were flat and wrinkled with lobate edges; colonies with smooth surfaces were also formed. Lasseur and Marion (1936) noted that it was possible to assist in the transformation of one type of colony into another by altering the surface tension and pH of

the medium or by holding the culture for different periods in sealed tubes. These workers also (1937) observed that lowering the surface tension of solid and liquid media with taurocholate and glycoholate produced modifications in the growth of both S and R dissociation types of Bacillus caryocyaneus.

Lasseur, Dupaix-Lasseur and Marchal (1937) found that Bacillus pyocyaneus produced both a green and a red type of growth on potato-glycerol medium. The Bacillus pyocyaneus erythrogène variety of this organism also formed three different colony types on agar plates; these were attributed to dissociation.

Lasseur, Dupaix-Lasseur and Palgren (1937) noted that Bacillus caryocyaneus produced both rough and smooth types of colonies on agar plates; the S type cultures formed essentially the green fluorescent pigment while the Rb cultures produced mainly the blue pigment.

Protective Action of Fluorescent Pigments on Bacteria

Oker-Blom (1913) concluded from his investigation that the germicidal action of ultra-violet light was due to the direct action of these rays on the bacterial protoplasm and not to the oxidizing action of ozone or hydrogen-peroxide produced in the medium, as was formerly believed.

Burge and Neill (1915) demonstrated that broth cultures of fluorescent bacteria were more resistant to ultra-violet

light than similar cultures of non-fluorescent bacteria. They believed that the fluorescent bacteria protected themselves from the coagulative effect of the ultra-violet rays by converting the short waves into longer waves and thus disposing of the energy in the absorbed short waves; the non-fluorescent bacteria were more easily destroyed than the fluorescent bacteria because they were unable to dispose of the energy in the absorbed short waves.

Dewey and Poe (1938) exposed diluted asparagine broth cultures, 12 to 48 hours of age, of several fluorescent organisms and of Aerobacter aerogenes and Escherichia coli to ultra-violet light for intervals of 1 to 40 minutes and then determined the numbers of surviving cells by plating the irradiated cultures. Asparagine broth was more satisfactory than lactose or plain broth for this study because it did not protect the organisms from the ultra-violet rays. Cultures of Pseudomonas organisms were more resistant to the ultra-violet light than cultures of Aerobacter aerogenes and Escherichia coli. Pseudomonas cultures that had lost the ability to produce pigment were less resistant than pigmented cultures. The resistance of undiluted cultures increased with the amount of pigment present; dilution decreased resistance to ultra-violet light since it reduced pigment concentration.

Toxicity of Fluorescent Bacteria Towards Other Microorganisms

Olitsky (1891) showed that substances were produced during the growth of Pseudomonas fluorescens liquefaciens that were markedly inhibitory to Eberthella typhosa, Bacillus anthracis, Vibrio cholerae, Serratia marcescens and Staphylococcus aureus.

Frost (1904) found that old broth cultures of Pseudomonas fluorescens and Pseudomonas putida contained a thermostable and filterable substance that was bactericidal for Eberthella typhosa.

Rahn (1906) reported that Bacillus fluorescens produced an iso-inhibiting substance that was thermolabile and distinct from the hetero-inhibiting substances described by other investigators.

Lewis (1929) demonstrated, by the seeded plate method, that Pseudomonas fluorescens produced a thermostable, filterable, dialyazable, alcohol soluble bacterio-toxin that was both inhibitory and bactericidal for other organisms. The toxin was not specific but was more active against certain species of bacteria than against others; spore-forming soil bacteria and micrococci were very sensitive, while the colon bacteria and Serratia marcescens were quite resistant. The amount of toxin produced by a culture of the organism depended on the composition of the medium and the amount of oxygen available. The toxin was not weakened by desiccation over long periods.

Gubitz (1928) observed that the fluorescent organisms included in his study were very unfavorable to the growth of *Oidium casei* on the surface of cheese.

Pathogenicity of Fluorescent Bacteria

Lesage (1888) studied a rod-shaped, spore forming organism that caused diarrhea and green colored stools in infants. Growth occurred at 20°C. but was most rapid at 25° to 35°C. A green water soluble pigment was produced on peptone-gelatin and gelatin was liquefied.

Lartigau (1898) reported an outbreak of dysentary attributed to *Bacillus pyocyaneus* in two families in which there was a total of 15 cases and 4 deaths. The source of infection in each outbreak was a contaminated well from which the family used water. The patients' stools turned green upon exposure to the air. The isolated cultures produced indol, liquefied gelatin and acidified litmus milk with precipitation of the casein.

Ducamp and Planchon (1894) isolated a green fluorescent, gelatin-liquefying, motile, rod-shaped organism from water that was pathogenic for laboratory animals. It closely resembled *Bacillus fluorescens liquefaciens* in cultural characteristics, except that it produced a pellicle but no odor in bouillon. The pigment was insoluble in ether, chloroform, alkali or alcohol; pyocyanine was not produced. Milk was first coagulated, then digested to a yellow-green solution.

Pigmentation was greatest at room temperature but 37°C. was optimum for growth; weak growth occurred at 40°C. Gelatin colonies appeared as small, yellow droplets with notched edges.

Bonjean (1899) reported that water can be the vehicle for <u>Bacillus pyocyaneus</u> infections and that water containing this organism should be rejected for human use.

Rocha, Lepierre and Fonseca (1900) described a green fluorescent producing organism that caused illness in a soldier, the infection evidently being contracted from water. They stated that the saprophytic fluorescent bacteria in water may sometimes be pathogenic for humans.

Berger (1926) isolated from aquarium water an organism resembling <u>Pseudomonas fluorescens</u> that was pathogenic for fish. The organism grew well at 10° to 20°C. but did not grow at 37°C. The use of sewage polluted water was responsible for transmission of the infection to the fish.

Several investigators have found <u>Pseudomonas aeruginosa</u> associated with udder infections in dairy cows. Mundhenk (1922) isolated <u>Bacillus pyocyaneus</u> from milk samples obtained from cows with severe mastitis. He considered these organisms only as secondary invaders of the udder, however, since they were associated with colon bacteria in the milk.

Pickens, Welch and Poelma (1926) cited cases in which <u>Pseudomonas aeruginosa</u> caused severe udder infections in dairy

cows. The cultures isolated from these infections did not produce indol.

Cherrington and Gildow (1931) found that a persistent outbreak of mastitis in a dairy herd was caused by *Pseudomonas aeruginosa*. Contaminated water in which the cows waded was considered responsible for the outbreak, since further cases did not develop after the cows were excluded from the water supply. An agglutination titer of 1:100 or over for the blood serum and 1:50 or over for the milk serum was demonstrated for the infected animals.

Cone (1938) reported that *Pseudomonas aeruginosa* was associated with, and probably the cause of, a mastitis outbreak of extreme severity in 17 cows of one herd. The onset was sudden and accompanied by fever. With certain cows milk production practically ceased during the acute stage of the disease. Some of the cows continued shedding the organisms in small numbers in the milk long after the acute attack had subsided. Cone stated that cultures of *Pseudomonas aeruginosa* isolated from the infected cows (and also other strains studied) differed from the description of the species given by Bergey, Breed, Murray and Hitchens (1939) in their inability to produce indol and their ability to ferment the simpler carbohydrates. All cultures produced decided or slight acid reactions with arabinose, xylose, dextrose, levulose, galactose, glycerol or mannitol, while very alka-

line reactions were obtained with sucrose, maltose, lactose, raffinose, inulin, dextrin or dulcitol. Beef extract-peptone broth (pH 7.0 and containing brom thymol-blue) to which 1 per cent of the test material was added after sterilization and an incubation temperature of 37°C. were used for the fermentation studies. The cultures hydrolyzed fat, reduced nitrates to nitrogen and liquefied gelatin but were unable to hydrolyze starch.

Several investigators have observed a marked similarity between certain fluorescent phytopathogenic organisms in the genus *Phytomonas* and certain fluorescent organisms in the genus *Pseudomonas*. Burkholder (1930) reported that the green fluorescent bacterial plant pathogens of the genus *Phytomonas* of Bergey constitute a natural, closely related group with a distinct similarity to members of the genus *Pseudomonas*. As an example, it was stated that *Phytomonas marginale* can scarcely be distinguished from *Pseudomonas aeruginosa*. Eighteen species of green fluorescent organisms in the genus *Phytomonas* were listed and described. Some of these failed to produce fluorescence on beef extract agar, while certain species lost the ability to produce pigment after growing in pure culture for a time. Uschinsky's solution was suggested as a medium for determining the ability of an organism to produce fluorescence. Many of the non-pathogenic fluorescent organisms were found to be ubiquitous, occurring as common saprophytes in the soil, on seeds and on various parts of

plants. Certain members of the Phytomonas group that produced a yellow pigment also formed acid in litmus milk, even though they were non-lactose fermenters. This was thought to be an error and was attributed to the preparation of the milk and to hydrolysis of the lactose during sterilization.

Smith and Fawcett (1930) found a close agreement in cultural and biochemical characteristics between the fluorescent organisms Bacterium syringae, Bacterium cerasi and Bacterium citriputeale. All were motile rods, with 1 to 3 flagella, and all produced a small capsule; litmus milk was digested from the top down without coagulation or reduction of the litmus; acid but no gas was produced in sucrose, dextrose, galactose, levulose and glycerol broths; lactose and maltose were not fermented; and indol was not formed. Cultures grown on dextrose-potato agar for 1 year sometimes changed from the characteristic smooth to a wrinkled type of growth. The virulence and gelatin liquefying abilities of the rough form were less than of the smooth type.

Lacey (1931) isolated two strains of Bacterium trifolarium that caused a disease of Vicia faba and compared the organism with other species of green fluorescent bacteria. Isolated cultures of Bacterium fluorescens non-liquefaciens and Bacterium striate showed close cultural agreements to the organism but were not pathogenic for Vicia faba. The question was raised as to whether all plant pathogens of the green fluo-

rescent group are not merely parasitic strains of Bacterium fluorescens liquefaciens and Bacterium fluorescens non-liquefaciens. Lacey (1932) later found that certain pathogens causing diseases of potato tubers, lettuce and seeds of Medicago lupulina closely resembled Bacterium fluorescens liquefaciens in cultural characteristics.

Clara (1934) made a comparative study of the green fluorescent plant pathogens and three species of the Pseudomonas group (Pseudomonas aeruginosa, Pseudomonas fluorescens and Pseudomonas putrida). Pseudomonas fluorescens was found to be mildly pathogenic on the fruit of the Keiffer pear (Pyrus communis) and was considered as a border-line form, linking the closely related green fluorescent plant pathogens to the green fluorescent non-phytopathogenic forms.

Harris, Naghski, Farrell and Reid (1939) were able to produce R forms of Pseudomonas fluorescens by growing the organism in the presence of its homologous antiserum. When the R forms were grown in the presence of its homologous serum and killed cells of Phytomonas tobacci, S forms were obtained which proved to be culturally and serologically identical with strains of Phytomonas tobacci and produced identical lesions on the tobacco plant. The same relationship was also found to exist between Pseudomonas fluorescens and other members of the genus Phytomonas, including Phytomonas angulata, Phytomonas cerasi, Phytomonas prinulae and Phytomonas vignae. The investigators belieived that specificity and virulence are

associated with the nature of the soluble specific substance in this group of organisms.

Dowson (1939) concluded as a result of his work that the plant pathogens could be arranged in three distinct groups, one like *Bacterium coli*, one like *Pseudomonas fluorescens* and one unlike either. He proposed to eliminate the genera *Erwinia* and *Phytomonas* of Bergey and reclassify the plant pathogens in the genera *Bacterium*, *Pseudomonas* and *Xanthomonas* n.g. Twenty-seven species of plant pathogens similar to *Pseudomonas fluorescens* were listed as belonging in the *Pseudomonas* group. These organisms produce fluorescin on beef infusion and on starch agar and are non-producers of acid in lactose, maltose and salicin. Dowson found that *Bacterium fluorescens* and *Bacterium pyocyanea* both liquefied gelatin, produced acid in xylose, dextrose, mannose, sucrose, or glycerol, but not in lactose or sucrose, hydrolyzed starch and produced hydrogen sulphide and ammonia; *Bacterium pyocyanea* reduced nitrates but *Bacterium fluorescens* did not.

Enzymes Formed by Fluorescent Bacteria

Wood (1889) subjected cells of *Pseudomonas pyocyanea* to phenol and found that the ability to coagulate milk was destroyed before the gelatin liquefying ability was lost. Breymann (1902) killed the cells of *Bacillus pyocyaneus* with chloroform and then dried and pulverized them. One-tenth gram of

this preparation coagulated 10 ml. of milk containing 5 per cent phenol in 4 hours at 37°C.; after 2 days the milk was almost completely peptonized.

Von Sommaruga (1894) was one of the earliest workers to demonstrate the lipolytic properties of microorganisms by means of a planned technique. He determined the increase in acid values of 2 per cent emulsions of olive oil or beef fat in nutrient gelatin or nutrient agar after inoculating with different organisms and holding about 30 days at body temperature. The results denoted that many cultures of the *Bacillus pyocyaneus* group were able to split as much as 21 to 27 per cent of the olive oil and 8 to 12 per cent of the beef fat.

Fermi and Montesano (1895) reported that cells of *Pseudomonas fluorescens* contain the enzymes, amylase and invertase.

Růžička (1898) compared the morphological, cultural and biochemical characteristics of three cultures of *Bacillus fluorescens liquefaciens* and three cultures of *Bacillus pyocyaneus*. The organisms could not be differentiated morphologically and produced similar colonies on agar plates. Both liquefied gelatin, digested milk and formed indol. *Bacillus pyocyaneus* grew best at 37°C. while room temperature was most favorable for the growth of *Bacillus fluorescens liquefaciens*.

Eijkman (1901) reported that bacteria which were able to digest casein were also able to liquefy gelatin and that this relationship held without exception for all organisms studied. *Bacillus fluorescens* was not amyloclastic but *Bacillus pyo-*

cyaneus was slightly so. Both of these organisms were able to decompose tallow under a solid medium and were classified as rapid fat hydrolyzers. Some cultures produced a diffusible lipolytic enzyme, while others produced an enzyme which diffused less readily and was more sensitive to the pH of the medium.

Emmerling and Reiser (1902) studied the proteolytic action of Bacillus fluorescens liquefaciens on gelatin at 37°C. Gelatin was liquified first on the surface, and after several months a brown, green fluorescent solution with a strong odor of ammonia resulted; methylamine, trimethylamine, choline and betaine were also produced but indol, skatol and hydrogen sulphide were not. The work indicated that the organism peptonizes proteins, then slowly decomposes them to simpler amines and finally to ammonia. Fibrin was also decomposed by the organism, arginine, leucin and aspartic acid being identified in the decomposition products; urea was decomposed to ammonium carbonate; amygdalin was not attached; and starch and trehalose were slowly hydrolyzed. A slimy condition was produced in meat bouillon. The enzyme of this organism closely resembled the plant enzyme, papain.

Jorden (1903) questioned the validity of the assumption made by other workers that "pyocyanolysis" was readily a bacterial hemolysin. Although filtrates of Bacillus pyocyaneus cultures hemolyzed the red blood cells of several animals, the

the hemolytic ability was lost when carbon dioxide was passed through a filtrate until it was acid in reaction. On heating the carbon dioxide filtrate, the gas was expelled and the filtrate at once regained its alkalinity and hemolytic power. Also, since the hemolytic property of the filtrate was not destroyed by heating to 125°C. for 1 hour, Jordan concluded that the hemolytic power was due to the alkalinity of the filtrate. Abbott and Gildersleeve (1903) likewise demonstrated that filtrates of cultures of *Bacillus fluorescens* and *Bacillus pyocyaneus* were strongly hemolytic when alkaline but only slightly hemolytic when neutralized by addition of acid. Filtrates of *Bacillus pyocyaneus* could be heated to 100°C. for 15 to 30 minutes without losing the power to liquefy carbolized gelatin. Abbott and Gildersleeve believed that the so-called hemolysins of these and other bacteria are probably proteolytic enzymes.

Bierema (1909) showed that asparagine is utilized by *Pseudomonas fluorescens liquefaciens* and that urea and acetamide are utilized by *Pseudomonas fluorescens*.

Söhngen (1910) observed that many species of bacteria, including certain fluorescent types, were able to grow well on a substrate containing fat as the only source of carbon and ammonium chloride as the sole source of nitrogen. He showed that the lipases of *Bacterium punctatum*, *Pseudomonas pyocyaneus* and *Bacillus albus* were more resistant to heat

than those of Bacterium lipolyticum, Bacillus sutzeri, Pseudomonas fluorescens and certain molds. He believed that his evidence indicated the existence of two types of bacterial lipases, which differed in their activity in the presence of acid.

Wells and Carper (1912) demonstrated the existence of lipase in several species of bacteria, including Pseudomonas pyocyaneus, that had been killed with toluene. Pseudomonas pyocyaneus hydrolyzed olive oil, triacetin and ethyl butyrate.

Söhngen (1913) demonstrated that Bacterium fluorescens liquefaciens was able to decompose paraffin in a synthetic medium in which this substance and calcium carbonate were the only sources of carbon.

Crabill and Reed (1915) reported that erepsin, amidase and tryptase were produced by Pseudomonas pyocyanea; erepsin was produced by Pseudomonas fluorescens, and since amygdalin supported good growth of this organism it was accepted as evidence of the production of emulsin.

Blanchetière (1917) isolated a culture of Bacterium fluorescens liquefaciens from water and studied its action on asparagine in a synthetic medium. Asparagine was found to be attacked in two states, the first step being a rapid hydrolysis of the amide group and liberation of nitrogen, after which a slow hydrolysis of the aspartic group took place. With sufficient time, 90 to 100 per cent of the nitrogen could be recovered as ammonia. Later, Blanchetière (1920) showed

that the organism can utilize certain amino acids as the only source of carbon in a synthetic medium. Histidine, alanine and asparagine were readily attacked; leucine, phenylalanine and tryosine were attacked more slowly; glutamic acid and tryptophane were attacked quite slowly; while glycine was utilized only after 1 month incubation.

Supniewski (1923) reported that Bacillus pyocyaneus converted acetaldehyde to formic acid, acetone to acetic acid, acetic acid to formaldehyde and formic acid, acetone to acetic acid and formic acid, ethyl alcohol to acetaldehyde and acetic acid, glycerol to carbon dioxide, glyceric acid and lactic acid, lactic acid to acetic acid and pyruvic acid and methyl alcohol to formic acid. In a later publication (1924) he showed that the organism utilized urea, glycine, asparagine and potassium cyanate.

Quastel (1924) noted that succinic and fumaric acids are decomposed by Bacterium pyocyaneum to the lower fatty acids, chiefly acetic.

Waksman and Tomanitz (1925) determined the action of a culture of Bacterium fluorescens isolated from soil on various amino acids and casein in a synthetic medium. Glycine was decomposed only to a limited extent in the presence of dextrose, and only small quantities of ammonia accumulated as long as dextrose was present in the medium; in the absence of dextrose the reaction became more alkaline, due to ammonia formation. Alanine, phenylalanine, glutamic acid and asparagine

were also decomposed. Casein was not attacked but the amino acids and probably the polypeptides from it were utilized. Bacillus cereus attacked casein vigorously but could not use the carbon or nitrogen in the amino acids glycine, alanine and phenylalanine. When both of these organisms were grown in the same flask of medium, Bacillus cereus decomposed the casein while Bacterium fluorescens decomposed a large part of the amino acids as soon as formed. The Bacterium fluorescens cells in the medium greatly out-numbered those of Bacillus cereus.

Callow (1926) obtained a positive oxidase reaction with guaiacum and a culture of Pseudomonas fluorescens, which was accepted as evidence of the production of peroxidase by this organism.

DeJong (1926) demonstrated that Pseudomonas fluorescens was able to oxidize methyl alcohol, acetic acid, butyric acid, iso-butyric acid and caproic acid. Alpha-propylene glycol, trimethylamine glycol, malonic acid, quinic acid, glyceric acid, aconitic acid, beta-hydroxy butyric acid, pyruvic acid, propionic acid and acetate were all dissimulated or utilized by this organism. In most cases ammonia served as the source of nitrogen in the media employed to determine the utilization of these compounds.

Sherwood, Johnson and Radotincky (1926) made a study of the biochemical properties of 22 strains of Bacillus pyocyaneus and observed that certain variations existed. Prac-

tically all strains were able to utilize dextrose, although alkaline products sometimes obscured this phenomenon. The cultures were all negative to mannitol, arabinose and dulcitol and positive to sucrose. All strains produced indol. Eleven strains blackened lead acetate and two reduced nitrates. Hydrocyanic acid was produced by all strains as well as by a culture of Bacterium fluorescens liquefaciens.

Sears and Gourley (1928) found that Pseudomonas aeruginosa produced an acid reaction in dextrose-peptone medium and dextrose-peptone-meat extract medium when the nitrogen content was low, but only a small amount of the dextrose was utilized. When the nitrogen content of the medium was high, however, as represented by 2 per cent or more of peptone, an acid reaction was not produced but large amounts of dextrose were utilized. Sugars other than dextrose did not support acid production even when the nitrogen content was low, but many of these sugars could be utilized by the organism.

Widmann (1929) showed that Bacterium fluorescens in mass culture rapidly and quantitatively converted methyl glyoxal to optically pure d (-) lactic acid.

Waksman (1932) reported that Bacterium fluorescens decomposes urea and that Bacterium fluorescens and Bacterium pyocyaneum decompose uric and hippuric acids. He also stated that Bacterium pyocyaneum readily attacks aliphatic and cyclic amino acids as sources of energy, but not benzene derivatives. During the decomposition of amino acids by this

organism the carboxyl group is first removed and ammonia is formed. Tyrosine is completely decomposed and tryptophane is broken down to ammonium carbonate and indol, but the indol is subsequently converted to anthranilic acid.

Vickery (1936) tested the lipolytic activity of a number of organisms, including several species of Pseudomonas. All cultures grew rather vigorously on fatty beef tissue held at -1°C., and most of them were lipolytic.

Gorback and Pirch (1936) found that peptidase remained within the bacterial cells of Bacillus cereus var. fluorescens and of Pseudomonas aeruginosa and gradually increased during 7 days cultivation. They later (1937) reported that the proteinase present in cultures of Bacillus fluorescens liquefaciens is an autolysis product of dead bacterial cells and is not excreted by the living cells, as contended by Virtanen and Suolahti (1937). This conclusion was based on the fact that a formol titration value of zero was obtained for a casein-enzyme solution in citrate-phosphate buffer, pH 7.0, after 24 hours incubation at 40°C. The enzyme solution used was obtained by filtering a broth culture of the organism grown at 20°C. for 7 days. When gelatin was used, the same treatment as employed for casein showed considerable proteolysis.

Sandiford (1937) investigated the ability of 50 strains of Pseudomonas pyocyanea from various sources to produce indol and ferment dextrose. All strains were indol negative

and all produced acid from dextrose. Sandiford found that an acid containing reagent, such as that of Böhme, should not be used in testing for indol production by this organism because of the likelihood of false positive reactions. He noted that this factor probably accounts for the positive indol reactions reported for this organism by certain workers. Both Pseudomonas pyocyanea and Pseudomonas fluorescens were isolated from human urine and feces.

Virtanen and Suolahti (1937) isolated a culture of Bacillus fluorescens liquefaciens from water and measured the proteinase secretion of the organism by the amount of casein hydrolyzed. They showed that proteinase is secreted by the living cell at 20° to 22°C., and that the secretion is quantitative in 10 hour old cultures. They also showed (1937a) by means of formol titration that the organism can break down almost all the casein or gelatin in the medium. Since there was a minimum of autolysis, it was believed that the proteolysis must be due to the action of the living cells.

Maschmann (1937) reported that the proteinases of Bacillus pyocyaneus, Bacillus fluorescens liquefaciens and Bacillus prodigiosus were probably identical but did not belong to the papians and probably not to the trypsins. The optimum pH for the activity of the enzyme on gelatin was 7.0. Lasseur, Dupaix and Marchal (1931), however, found that Bacillus caryocyaneus and Bacillus pyocyaneus liquified gelatin slower at pH 7.0 and 7.8 than at a more alkaline or

more acid reaction. They suggested the use of a medium at pH 5.0 for organisms that grow at a low pH and a medium at pH 8.0 or 9.0 for organisms requiring an alkaline medium.

Fluorescent Bacteria Isolated from Sources Other than Dairy Products

The organism now known as Pseudomonas fluorescens was described rather completely by Flügge (1886) and given the name Bacillus fluorescens liquefaciens. He stated that gelatin colonies showed appreciable variation and that several types of the organism resulted when small differences in growth characteristics were considered. He regarded this organism as identical with Bacterium butyri fluorescens (Lafar), Bacillus fluorescens nivalis (Schmelck), Bacillus viscosus (Frankland and Frankland), Bacillus fluorescens minutissimus (Unna and Tommasali) and Bacterium melochlous (Winkler and Schroeter). Bergey, Breed, Murray and Hitchens (1939), however, placed Bacillus viscosus in a separate species (Pseudomonas viscosa) from Pseudomonas fluorescens. Flügge also described another fluorescent organism that produced an odor of herring brine in gelatin without liquefaction; he named it Bacillus fluorescens putidus.

Since the publication of Flügge, various bacteriologists have described, named and classified a large number of species of fluorescent bacteria. Migula (1900) described 22 species

of gelatin liquefying and 29 species of gelatin non-liquefying, fluorescent bacteria in the genus Pseudomonas. Chester (1901) described 13 species of fluorescent bacteria, all of which were included in the genus Pseudomonas. Bergey's "Manual of Determinative Bacteriology" (1939) includes 31 species in the genus Pseudomonas, 28 (90 per cent) of which are probably fluorescent, while 38 per cent of the species listed in the genus Phytomonas are designated as fluorescent. Part B, Appendix I to the genus Phytomonas includes species which cannot be distinguished from species included in the genus Pseudomonas, except for plant pathogenicity. Appendix II to the genus Bacterium includes at least one organism, Pseudomonas pictorum, that is probably fluorescent since it produces greenish-yellow colonies on gelatin. One of the spore forming organisms, Bacillus cereus var. fluorescens, forms a yellowish-green fluorescence in gelatin, milk and other media. Zimmerman (1890), Migula, Engler and Prantl (1895) and Lehmann and Neumann (1896) also described several species of fluorescent bacteria.

Guignard and Sauvageau (1894) isolated a rod-shaped gram negative organism from white worms that produced a greenish tint with fluorescence in beef bouillon. In milk and other culture media, emerald green, needle shaped crystals were formed. Gelatin was liquefied. Milk was first coagulated, then became yellow, alkaline and viscous and developed an

aromatic odor similar to coumarin. The organism grew best at 25° to 30°C. and formed spores after 5 to 6 days.

Wright (1895) isolated a rod-shaped bacterium with polar flagella from water and named it Bacillus fluorescens mutabilis. It liquefied gelatin and produced a blue-green fluorescent color. Litmus milk was reduced, then coagulated with a firm clot, after which the curd was slowly digested; a bluish ring appeared on the wall of the tube at the surface of the coagulated milk. Indol was not produced. Neither Chester (1901) nor Bergey, Breed, Murray and Hitchens (1939) included this organism with the fluorescent bacteria that they described.

Ravenel (1896) studied a green pigment producing organism which he isolated from soil and named Bacillus fluorescens undulatus; it was not included in the lists of fluorescent bacteria by Chester (1901) or Bergey, Breed, Murray and Hitchens (1939). The organism was a slender rod, with polar flagella, that grew in chains and formed small oval spores. Gelatin colonies were circular, about 1 mm. in diameter, with finely striated border, convex, elevated, granular and gray in color; gelatin was not liquefied but a greenish coloration appeared on the surface. Growth on agar slants was thin and translucent, and a faint green color was imparted to the agar. Litmus milk was not coagulated but became deep blue after 3 weeks. Indol was not produced.

Fuller and Johnson (1899) isolated seven species of fluorescent bacteria (Bacillus fluorescens liquefaciens, Bacillus

fluorescens non-liquefaciens, *Bacillus fluorescens ovalis*, *Bacillus pyocyaneus*, *Bacillus viridis*, *Bacillus fluorescens incognitus* and *Bacillus proteus fluorescens*) from the Ohio river and studied their morphological, cultural and biochemical characteristics. Indol was produced only by *Bacillus viridis*. Dextrose was fermented by *Bacillus pyocyaneus*.

Schmidt-Nielsen (1902) studied several psychrophilic microorganisms capable of growing at 0°C., among which were the fluorescent organisms *Bacterium aquatile fluorescens non-liquefaciens*, *Bacterium tarde fluorescens* and *Bacterium proteus fluorescens*. The first two of these organisms were isolated from water.

Löhnis and Kuntze (1907-1908) isolated organisms that resembled *Bacterium fluorescens* from several samples of stable manure. They stated, however, that some of the cultures were non-fluorescent.

Pribram and Pulay (1915) concluded, from their work with 10 cultures of fluorescent bacteria, that although considerable variation existed among these organisms they could be placed in two main groups. One group was composed of those varieties that resembled *Bacterium fluorescens*, while the other group consisted of the varieties that were similar to *Bacterium putidum*. The first group liquefied gelatin and produced fluorescence at 37°C. The second group did not liquefy gelatin and failed to show fluorescence at 37°C.,

although it grew at that temperature. Both groups were identical morphologically.

Blanchetière (1917) stated that bacteria of the <u>Pseudomonas fluorescens liquefaciens</u> group of Flügge were the most widespread, gelatin-liquefying bacteria in the drinking water of Boulogne, France.

Tanner (1918) found fluorescent bacteria rather common in water from the Mississippi river and especially abundant in water from the Illinois river. One-hundred strains of fluorescent bacteria isolated from water fell into 27 groups. All strains were motile, gram-negative, long or short rods and usually grew in chains. Four strains were spore formers. About half the cultures liquefied gelatin, reduced nitrate, and produced hydrogen sulphide; about 40 per cent peptonized casein; 40 per cent fermented lactose; 30 per cent fermented sucrose; 15 per cent fermented glycerol; 100 per cent fermented dextrose; and none of the cultures produced indol or hydrolyzed starch. All strains were facultative anaerobes. Four types of fermentation occurred in plain milk; (a) coagulation in 2 days with a solid curd and often decomposition, leaving a golden colored solution; (b) clearing without coagulation and with green fluorescent color; (c) no change in 20 days; and (d) milk rendered slimy.

Marchal (1937) isolated an organism from marl water that produced rose colored fluorescent colonies on the synthetic medium of Lasseur (1911) but achromogenic colonies on ordinary

agar. The cells were rod-shaped, often in diplobacilli arrangement and negative to gram stain. The optimum growth temperature was 18° to 25°C., with no growth at 37°C. Milk was peptonized after 4 days without coagulation. Gelatin was liquefied. Dextrose, lactose, maltose and sucrose were fermented. Indol and hydrogen sulphide were produced and nitrates were reduced to gaseous nitrogen. Agar colonies varied from round types with smooth borders to spreading transparent types with irregular notched margins. The organism was considered a new species and was named Bacillus roseus fluorescens.

Action of Fluorescent Bacteria on Dairy Products

Hueppe (1884) studied the action on milk and other media of four greenish-blue, fluorescent bacteria obtained from water. One was the organism of blue-green pus; it coagulated milk, then peptonized the casein with the formation of ammonia, the result being a clear yellowish-green solution with a sediment at the bottom. Gelatin was liquefied and a dark green color was produced. The other organisms digested milk with the formation of ammonia, the result being a dirty-violet colored solution. Gelatin was liquefied and a violet to blue-black color was produced. All the organisms were rod-shaped.

Escherich (1886) isolated a motile, rod-shaped organism

from the intestinal tract of a child that had died from an intestinal disturbance and a non-motile, rod-shaped organism from air. Both produced a green, fluorescent, water soluble pigment on gelatin plates; the former organism liquefied the gelatin but the latter did not. After growing in milk for 14 days at 38°C., the gelatin liquefying organism reduced the fat content 56.7 per cent and the casein content 45.3 per cent, while the non-liquefying organism reduced the fat content about 8 per cent and the casein content nearly 35 per cent; the lactose in the milk was slightly decreased by both organisms.

Krueger (1890) investigated a sample of cheesy butter and isolated from it two types of yeast, Oidium lactis and three species of bacteria, one of which was stated to be Bacillus fluorescens non-liquefaciens although it was described as being a non-motile rod that produced terminal spores and grew both aerobically and anaerobically. On gelatin the organism produced leaf-like colonies; the gelatin was not liquefied but developed a dark green fluorescence at 16° to 18°C., the optimum temperature. A foul fermentation was quickly produced in sterile milk at 16° to 18°C.; the liquid developed a yellowish color, showed a green fluorescence and finally became slimy; the reaction gradually became acid and after 10 days a penetrating odor of trimethylamine was produced; ammonia and hydrogen sulphide were also

formed. Butyric and formic acids were produced from triglycerides. It was believed that the higher molecular weight fatty acids were decomposed to these two acids during fat hydrolysis.

Lafar (1891) isolated what he considered to be a new bacterial species and named it *Bacillus butyri fluorescens*. The organism produced rancidity in butter when inoculated into the cream before churning. Reinmann (1900), however, found this organism to be *Bacillus fluorescens liquefaciens*.

Reinmann (1900) inoculated *Bacterium fluorescens liquefaciens* into butter churned from sterile cream and noted the changes in flavor and odor that developed during several weeks holding at an unstated temperature. The butter had a fresh, desirable flavor when first prepared but developed a strong undesirable odor in a few days and after 2 or 3 weeks was completely inedible, although not rancid.

Schreiber (1901) observed that *Bacillus fluorescens liquefaciens* was the most frequent organism encountered on agar plates prepared from the surfaces of cylinders of butter that had been buried in the soil for various periods. Five cultures of this organism were isolated from soil and water and all decomposed cylinders of sterile butter when inoculated on the surfaces of cylinders held at room temperature. Cultures that were lipolytic when first isolated lost their ability to attack fat after being carried on gelatin for 1 year and 9 months. Schreiber stated that these organisms,

in the presence of nutritive material and oxygen, break down the fat and destroy the free fatty acids after they react with calcium carbonate.

Laxa (1901-1902) found that the total acid number of butterfat was 197.4 and the volatile fatty acid number was 0.94 after Bacillus fluorescens liquefaciens had grown in butter for one month; the control fat had an acid number of 12.2 and a volatile fatty acid number of 5.05. These data were considered to indicate that the organism essentially produces a splitting of the glycerides of the non-volatile fatty acids and not a proportionate splitting of all the glycerides. Laxa believed that the organism was unable to decompose the higher fatty acids to butyric and formic acids, as suggested by Krueger (1890).

Eicholz (1902) studied a non-spore-forming organism that grew at 3.5° to 7.0°C. and produced a strawberry aroma and an alkaline reaction in milk. It formed rosette and daisy-like patterns on gelatin but was non-fluorescent. The organism was named Bacterium fragi. Gruber (1902) isolated Pseudomonas fragariae from beets; it produced a strawberry like odor in both salted and unsalted butter in 14 days. The organism was a non-spore-forming rod, with 1 to 9 polar flagella, that produced fluorescence but did not liquefy gelatin. The colonies on gelatin plates were described as dew-drop-like, bluish, round, raised, shining and translucent. Later (1905), he isolated another organism, Pseudomonas fragariae II, from aged pasteur-

ized milk that had developed a strawberry like odor. This odor also developed in other media into which the organism was inoculated. The colonies on gelatin plates were large, round, arched, dirty-white and glistening. Gelatin was peptonized, and milk was coagulated by the production of acid. Growth was best at 18° to 22°C. and did not occur at temperatures above 34°C. The organism was rod-shaped and motile by means of a polar flagellum. Hussong, Long and Hammer (1937) studied an organism that developed three types of colonies, S(smooth), R(rough) and O(intermediate), on agar plates and produced a May apple or rancid odor in dairy products. The organism grew at 5° to 7°C. and was a frequent cause of defects in milk and butter. The ability of the organism to produce fluorescence was not stated. The R colony type was identified as _Bacterium fragi_, Eicholz, the C type as _Pseudomonas fragariae_, Gruber, and the S type as _Pseudomonas fragariae II_, Gruber.

Orla-Jensen (1902) investigated several microorganisms from the standpoint of their importance in the development of rancidity in butter under commercial conditions and in laboratory trials. _Bacillus fluorescens liquefaciens_ was always found in fresh butter, while _Bacillus fluorescens non-liquefaciens_ was only occasionally encountered; rancid butter often contained the former organism (along with many others) but not the latter. _Bacillus fluorescens liquefaciens_ was one of the two predominating liquefying bacteria in the surface layer of sweet cream butter held at room temperature for

3 days but was present only in small numbers in sour cream butter held under similar conditions. The organism did not grow in the interior of the butter. Butter made from sterilized cream, to which Bacillus fluorescens liquefaciens had been added before churning, contained 153,000, 55,000,000 and 0 organisms per gram in the surface layer after being held at room temperature 0 days, 1 week and 2 months, respectively; the total acid numbers of the surface butter were 1.2, 13.2, and 37.9, respectively, after the various holding periods, while the volatile acid number was 5.5 after 2 months holding. The butter had a bad taste and a butyric acid like odor after 1 week and after 2 months was completely inedible. The organism supposedly decomposed butterfat uniformly but utilized the non-volatile fatty acids in preference to the volatile fatty acids. Bacillus fluorescens liquefaciens died off when the volatile fatty acid number reached about 3.5. The presence of milk souring bacteria did not prevent the growth of Bacillus fluorescens liquefaciens in butter but, when sufficient acid had developed, hydrolysis of the fat was believed to be retarded. The addition of 2.9 per cent salt (21.6 per cent brine) to butter prevented growth and fat hydrolysis by the organism. Since Bacillus fluorescens liquefaciens is widespread in water, it was believed to be introduced into butter from this source. Pasteurization of cream at 85°C. destroyed all microorganisms injurious to the keeping quality of butter.

Müller (1903) found the generation time of *Pseudomonas fluorescens* at various temperatures to be 0.793 hours at 30°C., 1.052 hours at 25°C., 3.308 hours at 12°C., 9.456 hours at 6°C. and 23.74 hours at 0°C.

Kruyff (1907) isolated nine species of fat-splitting bacteria from soil, sewage, water, old butter and animal feces. All the species grew at 37°C. Only one of them was studied to any extent and it was identified as *Bacillus fluorescens liquefaciens*.

Wolff (1907-1908) isolated *Bacterium fluorescens* organisms from part of the milk samples examined but only after the samples had been held at a low temperature (5° to 7°C.) for several days (2 to 7). Under these conditions fluorescent bacteria comprised as much as 22 to 42 per cent of the total flora of certain samples. When the samples were held at 20°C., fluorescent colonies were only occasionally encountered on gelatin agar plates poured from the aged milk.

Barthel (1910) reported that *Pseudomonas fluorescens* can split fat to cause rancidity but is incapable of attacking glycerol.

Luxwalda (1911) grew *Streptococcus lactis* and *Bacterium fluorescens liquefaciens* together in milk and found that at 15°, 13° and 10°C. both species appeared to be benefitted by the association. After 6 days at 15°C., the milk was sour and coagulated and contained 1,700,000,000 *Streptococcus lactis* and 4,000,000 *Bacterium fluorescens liquefaciens* organ-

isms per ml. Since Bacterium fluorescens liquefaciens was able to live in a sour medium, it was believed that the milk souring bacteria produce something besides acid that hinders the growth of the fluorescent organisms in sour milk. Bacterium fluorescens liquefaciens produced a rennin coagulation and then peptonized the milk. At 3° to 5°C. the odor and taste of milk inoculated with this organism remained completely normal up to 19 days, even though it contained over 300,000,000 bacteria per ml., but after 20 days the milk was bitter and coagulated with alcohol.

Kendall, Day and Walker (1914) found that Bacillus pyocyaneus produced a transient, initial acidity in milk and later an alkaline reaction due to the production of ammonia. Since this organism does not ferment sugars they concluded (1914a) that this initial acid production preceding proteolysis was probably due, in part at least, to hydrolysis of fat. They later (1914b) demonstrated the lipolytic ability of the organism.

Kraaij and Wolff(1923) ascertained the ability of several organisms to split fat by inoculating them on nutrient agar to which had been added 0.5 per cent lecithin or a similar amount of fat; hydrolysis was evident by a clearing of the medium around the colony. Pseudomonas fluorescens was found to split both lecithin and fat.

Haag (1928) determined the lipolytic ability of a number of bacteria and concluded that Bacillus pyocyaneus was the

only organism in the group that was capable of splitting fat. While this organism could not cleave the triglycerides of palmitic and stearic acids, it was able to attack both of these acids in the free state. Bacterium fluorescens liquefaciens showed good growth on triolein and oleic acid.

Gubitz (1928) isolated several cultures of Bacterium fluorescens and related types of organisms, which included Bacterium punctatum and Bacterium putidum, from milk, butter, soil, water from various sources and plants and studied their growth at different temperatures. The optimum temperature of these organisms was under 30°C., and they were found to be one of the important types that grow at 0°C. The cultures of Bacterium fluorescens with a maximum growth temperature around 35°C. were designated as warm forms, while those with a maximum growth temperature near 27° to 30°C. were termed cold forms. The maximum and optimum temperatures of these organisms were reduced about 5°C. from the original by growing them in liquid medium for several generations at 0°C. The physiological properties, particularly the lipolytic and proteolytic characteristics, were not altered by holding at 0°C. for a considerable period. Growing the warm forms for 24 transfers at 30°C. raised the optimum and maximum growth temperatures about 5°C. Two types of colonies-(a) bluish, lobated, irregular shaped and spreading and (b) round, raised, discoid, smooth and glassy appearing-were produced by the fluorescent organisms. Growth on potato was very useful in

identifying the organisms. Bacterium fluorescens fermented dextrose and sometimes glactose; reduced nitrates to nitrites; produced a lusterous, blister-like, brownish growth on potato; developed an intense bitter taste in milk in 2 days at 18°C. and later peptonized the milk; hydrolyzed fat at 12° and 18°C.; failed to produce hydrogen sulphide; gave only weak growth in media containing 5 per cent sodium chloride; grew at a pH as low as 5.4 to 5.8; and was destroyed at a temperature of 63° to 64°C. for 30 minutes.

Shutt (1928-1929) found that contaminated water used in washing butter was responsible for an unclean and putrefactive flavor that developed on the surface of the butter during holding. The off-flavor occurred chiefly during the spring and summer months and was particularly common after periods of heavy rains. No defect of this nature was noted in butter from creameries that had good water supplies. The water supplies of creameries having difficulty with off-flavored butter contained large numbers of putrefactive bacteria, chief of which was Pseudomonas fluorescens. Sterile butter inoculated with this organism developed the typical surface flavor in 28 days at 25°C. Heating the water to 87.8°C. for 10 minutes was necessary to destroy the organisms. The trouble disappeared when the water was treated or when pure water was substituted for a contaminated supply. Neutralizing the cream to an acidity not less than 0.35 per cent was beneficial in avoiding the defect, since the organism grew

but feebly at pH 6.6. Surface-taint flavor occurred only in sweet cream or neutralized cream butter and never developed in sour cream butter.

Löhnis (1930) reported that Bacterium fluorescens and its closely related forms, which regularly occur in water, play an important role in the development of rancidity in butter stored with access to air. Since these organisms grow at low temperatures and decompose fat and protein, they were also found to be detrimental to the flavor of milk.

Newman (1930) examined three samples of milk with a bitter flavor and found that they contained mainly organisms of the Pseudomonas group. The bacteria grew well at 4°C. and produced a fluorescent blue-green, yellow or red pigment on agar plates. One type of colony was thin, flat and spreading, while the other type was moist, raised, glistening and circular or irregularly fringed. Colonies picked into sterile milk produced a strong quinine-like bitterness in the milk in 24 to 48 hours at room temperature. A different species of Pseudomonas was isolated from each sample of milk. One of the organisms corresponded to Pseudomonas ovalis.

Henneberg (1931) listed the fluorescent group as one of the most common types of organisms causing spoilage of milk, butter and cheese in Germany.

Orla-Jensen (1931) reported that Bacterium fluorescens liquefaciens frequently plays an important part in the development of rancidity in butter. Bacterium pyocyaneum,

however, grew so slowly at ordinary temperatures that it did not spoil butter under normal conditions. Bacterium fluorescens liquefaciens was listed as one of the most important fat hydrolyzing microorganisms. Since it is often added to butter by wash water or ice, he advised pasteurizing wash water or treating it with chlorine. He also stated that this organism produces a turnip, tallow and sometimes a soap taste in milk; turnip flavor was frequently noted in milk that had been held at a low temperature.

Rumment (1931) stated that numerous investigators have demonstrated that Bacterium fluorescens liquefaciens is a usual inhabitant of polluted water and causes rancidity in butter. The organism was used in his experimental work to determine the number of microorganisms that pass from the wash water into the butter and to determine the effect of these organisms on the keeping quality of butter. He found that sweet cream butter absorbed more organisms from the wash water than sour cream butter; that the firmer the consistency and the larger the butter granules the fewer were the bacteria that were traceable to the wash water; and that in sweet cream butter (pH 6.8) the organism increased rapidly and decomposed the fat intensely at higher temperatures but only slightly at lower temperatures, while in sour cream butter (pH 4.2 to 4.3) it did not increase and the fat remained unchanged in the cold but developed an unclean, tallow flavor and odor at higher temperatures. The fatty acids form-

ed in butter were found to have a germicidal effect on the organism.

Virtanen (1931) listed the most common defects produced in butter by bacteria as fermented, cheesy, putrid and "rank". The enzymes causing these defects were stated to be formed by proteolytic water bacteria of the Pseudomonas fluorescens and Pseudomonas punctatum groups. These bacteria were not easily destroyed by heat but were inhibited by the acidity of sour cream butter and by salt. It was reported that these bacteria usually do not cause defects when the water supply is not contaminated with them and when the milk is delivered daily; when the milk or cream is 2 or 3 days old, difficulty may arise even though the water supply is pure. The enzymes of these bacteria were not destroyed during pasteurization of the cream and sometimes caused defects in the butter in the absence of living bacteria. The catalase test for butter was recommended as a test for the presence of proteolytic bacteria, but a negative test was stated to be no assurance that this type of spoilage would not occur.

Henneberg (1931-1932) noted that protein decomposition without acid production is typical of the fluorescent group of bacteria, even though many cultures can ferment lactose and dextrose. Protein decomposition can, however, be largely inhibited by the presence of sugar, as is illustrated by the fact that gelatin liquefying cultures sometimes do not liquefy gelatin when sugar is added. The presence of air also

favors an alkaline development, while the lack of air favors an acid fermentation. The action of two gram-negative, gelatin-liquefying fluorescent organisms on milk was studied. One produced a bitter putrefaction, the other a soapy condition in milk; both strongly hydrolyzed fat and both produced a putrid odor and ammonia in peptone broth; both grew well at 6° to 8°C.; 7.5 per cent salt was endured by one organism but only 5 per cent by the other. It was stated that *Bacterium fluorescens* and other alkali forming bacteria are important in decomposing the fat and protein in butter, but a low temperature, acid and salt are preserving factors.

Berry (1933) found that *Pseudomonas fluorescens* was one of the organisms capable of splitting fat.

Hiscox (1936) reported that butter from the refrigeration room of a ship was made into "pots" and became permeated with a dark discoloration while being held in cold storage. The discoloration sometimes occurred in the form of small blue-black areas but developed more frequently as large irregular patches that varied from blue-black in the center to violet-red at the edge. The butter contained 0.38 to 0.55 per cent salt. The discoloration could be reproduced in laboratory samples held at cold storage temperature but not at 15°C. or higher. Two gram-negative, motile, non-spore-forming rod-shaped organisms, that were identical except for slight differences in colony form, were isolated

from nutrient agar plates poured with the defective butter and incubated at 1° to 3°C. No growth occurred at 37°C., slight growth at 30°C., optimum growth but no pigment production at 22°C. and slow but abundant growth and pigmentation at 1° to 3°C. Growth on an agar slope was clear, moist and slightly fluorescent in the early stages at 15°C., while at 1° to 3°C. a slow development of violet-black pigmented growth occurred. A brown color diffused through the medium at all temperatures but was less abundant as the temperature increased. Colorless crystals of magnesium phosphate developed on the surface of the medium as a result of the decomposition of peptone and the formation of ammonia. Gelatin was liquefied. Litmus milk was rendered alkaline, then reduced and digested. One to 2 per cent sodium chloride in the media was necessary for growth, while 5 per cent was inhibitive. The source of nitrogen influenced pigment production, protease peptone being inhibitive. The organism was stated to belong to the Pseudomonas or Chromobacter group.

Stark and Scheib (1936) made a study of 486 cultures of lipolytic and caseolytic bacteria isolated from butter prepared and held under known conditions. Among these were 40 cultures that resembled Pseudomonas aeruginosa in physiological properties. Thirty of these cultures produced a blue-green pigment, soluble in water, that turned dark brown with age and became red in the presence of acid. In testing for indol, a red color was produced but it was insoluble in

chloroform. The other 10 cultures were identical in all respects with the above type except that they produced a yellow pigment, slightly soluble in water, the color of which did not change in the presence of acid. Under the same conditions no blue pigment was produced by these cultures. This group was assumed to be a variant of *Pseudomonas aeruginosa*.

Storck (1936) found that alkali forming bacteria constituted an important part of the milk flora during the winter months when the population of acid forming bacteria is low. The *Bacterium fluorescens* group was one of the alkali forming types present in raw milk, and three strains were isolated. All three strains hydrolyzed fat and liquefied gelatin but none formed indol. Two cultures coagulated milk before digestion, while the other culture digested milk without coagulation. Only one culture reduced nitrate. Pasteurization at 63°C. for 30 minutes destroyed all of the alkali producing bacteria in milk except the spore-forming group.

Hansen (1937) added 0.05 per cent of a culture of *Pseudomonas fluorescens* to milk used for cheese making and found that it did not significantly affect the flavor score or the nitrogenous decomposition in the cheese during ripening.

METHODS

Detection of Fluorescent Bacteria on Plates

Fluorescent colonies were detected by observing agar plates under a strong source of relatively pure ultra-violet light in a dark room. The fluorescent colonies were indicated by marking the dish with a wax pencil; certain of these colonies were later picked for purification and study. Beef infusion agar at a pH of 7.0 to 7.2 and an incubation temperature of 20° to 25°C. for 72 hours were employed because these conditions were favorable for production of the fluorescent pigment.

Staining Procedures

Twenty-four hour cultures on freshly prepared agar slopes were used for determining morphology. The cells were stained by the method of gram, while Gray's procedure was followed for staining flagella.

Incubation Temperature

Unless otherwise indicated, all cultures were incubated at room temperature for the time specified. This temperature varied somewhat with the season of the year but was

usually between 20° and 25°C.

Absorption Spectra of Broth Cultures and Solutions of Certain Fluorescent Substances

The adsorption spectra were made on centrifuged beef infusion broth cultures after growing at room temperature for various periods. Similar measurements were also made on dilute solutions of fluorescin, fluorescein, riboflavin and diacetyl in beef infusion broth. The adsorption spectra were measured with a Hilger Spekker Spectrophotometer, using the iron-tungsten spark as a source of radiation.

General Characteristics

Action on litmus milk

Tubes of litmus milk were inoculated with the fluorescent organisms and incubated at room temperature. Observations were made after 5, 10 and 28 days and the changes recorded.

pH and titratable acidity

The pH determinations were made electrometrically with the glass electrode. The milk acidities were determined by titrating 18 gm. samples with N/10 sodium hydroxide and phenolphthalein and calculating as percentage lactic acid.

Fermentation of carbohydrates

The fermentation of carbohydrates was determined by ascertaining the ability of the organisms to produce acid in beef extract-peptone broth containing brom cresol purple and 1 per cent of the test substance. The tubed solutions were heated in the autoclave at 10 pounds pressure for 15 minutes, then held at room temperature for several days to determine sterility. The inoculated tubes were incubated for 14 days and examined at 2 day intervals to note the production of acid.

Proteolysis

The proteolytic ability of the organisms was studied with gelatin and casein. Stab inoculations were made into tubes of plain gelatin and the type of liquefaction noted. Ability to digest casein was ascertained by making spot inoculations on plates poured with beef extract agar containing 5 per cent sterile skimmilk and observing for clearing of the agar around the areas of growth.

Lipolysis

The hydrolysis of fat was determined by the nile blue sulphate procedure of Long and Hammer (1937). Both cottonseed oil and corn oil were used.

Hemolysis

The ability of the organisms to hemolyze red blood cells was determined by spotting the cultures on plates poured with beef infusion agar containing 0.6 per cent sodium chloride and 5 per cent defribrinated ox blood.

Nitrate reduction

Duplicate tubes of beef extract-peptone broth containing 0.1 per cent potassium nitrate were inoculated with each organism and incubated at room temperature. The tubes were examined for the evolution of gas at 12 hour intervals for 48 hours and at each observation 5 ml. of culture was removed and a few drops of the following solutions added: (a) 8 gm. sulphanilic acid in 1 liter of 5N acetic acid; (b) 6 ml. dimethyl-alpha-naphthylamine in 1 liter of 5N acetic acid. The development of a distinct pink color denoted the presence of nitrite. A pinch of zinc dust was added to all samples that were negative after 48 hours to determine whether nitrate was still present; this likewise was indicated by the development of a red color.

Formation of hydrogen sulphide

Two procedures were employed to detect the ability of the organisms to produce hydrogen sulphide. One method consisted of growing the organisms in beef extract-peptone broth

with a strip of lead acetate filter paper (soaked in saturated aqueous lead acetate solution and then dried) suspended in the top of the tube and observing for blackening of the test paper as the culture aged. The other procedure followed was to grow the organisms on tryptone, iron agar (tryptone 20 gm., ferric ammonium citrate 0.5 gm., dipotassium phosphate 1 gm., agar 15 gm. and water 1000 ml.) and note whether darkening of the agar occurred.

Production of indol

The organisms were grown for 5 days in a 1 per cent solution of tryptone, after which the cultures were tested for the presence of indol by the Böhme (1905) technic. Incubation periods both shorter and longer than 5 days were used with some cultures. The Gorè (1921) test was also employed with part of the cultures.

Utilization of urea

The ability of the organisms to decompose urea was determined by ascertaining their ability to grow in a synthetic medium containing urea as the only source of nitrogen. The first medium employed was essentially the Waksman (1932) formula number 1, while the second medium used contained 0.5 gm. magnesium sulphate, 0.5 gm. dipotassium phosphate, 1.0 gm. sodium citrate, 5.0 gm. dextrose and 10.0 gm. urea in 1000 ml. of distilled water. After incubating 2 weeks the cultures

were examined for growth as indicated by turbidity and fluorescence of the broth.

Formation of catalase

Formation of catalase was detected by placing 1 ml. of a 2 day old broth culture in a small agglutination tube and adding 2 drops of 3 per cent hydrogen peroxide. The evolution of gas, which was usually vigorous and easily noted, indicated the presence of catalase.

Production of ammonia

The organisms were grown in beef extract-peptone broth for 5 days, then tested with Nessler's reagent to detect the presence of ammonia.

Growth in Uschinsky's medium

The formula proposed by Uschinsky (1893) was used. The ability of the organisms to grow and produce fluorescence in this medium was noted after incubating 2 weeks.

Diastatic action

Beef extract-peptone agar containing 0.2 per cent soluble starch was poured into petri dishes and, after hardening, spot inoculations were made on the surface. The plates were incubated for 5 days, then flooded with a saturated solution of iodine in 50 per cent alcohol. A clear zone around the

area of growth denoted starch hydrolysis.

Production of acetylmethylcarbinol

The ability of the organisms to produce acetylmethylcarbinol was ascertained by growing for 4 days in 5 ml. of broth composed of 5 gm. proteose peptone, 5 gm. dextrose and 5 gm. dipotassium phosphate in 1000 ml. distilled water. The presence of acetylmethylcarbinol was detected by adding 0.5 ml. of a 5 per cent alcoholic solution of alpha-naphthol, a small grain of creatin and 4 drops of 40 per cent sodium hydroxide, then shaking well and observing for the development of a red color.

Formation of chlororaphine

The ability of the organisms to form chlororaphine was determined by growing in the synthetic medium of Lasseur (1911) and noting whether crystals of this substance appeared in the culture.

Lactose Determination

Lactose was determined by the Shaffer-Somogyi (1933) copper-iodometric method.

Protein Breakdown in Skimmilk

Total nitrogen was determined on the skimmilk serum by

the Kjeldahl method and amino nitrogen by the Van Slyke (1929) manometric method. The nitrogen was fractionated according to the procedure used by Lane and Hammer (1935) and by Long and Hammer (1936). Ammonia was determined by the Folin (1902) aeration method, as modified by Van Slyke and Cullin (1916).

Fat Acidities

The method developed by Breazeale and Bird (1938) was used to determine the acidities of the butterfat. The acidity of the butterfat corresponds to the milliliters of N/10 potassium hydroxide required to neutralize the acid in 10 gm. of fat.

EXPERIMENTAL

Isolation of Fluorescent Bacteria from Dairy Products and Other Materials

Attempts to isolate fluorescent bacteria from fresh milk and cream and freshly made dairy products by plating directly were seldom successful because of the limited numbers of these organisms in such materials. The procedures generally followed were intended to favor enrichment of the fluorescent bacteria in the product concerned and thus facilitate their isolation. It is probable, however, that this did not always occur and that the fluorescent organisms were sometimes overgrown by other types present in certain samples. It is, likewise, probable that the organisms were not obtained from all samples in which they existed because they could not always be detected when growing on agar plates. Overcrowding of the plates with non-fluorescent types frequently obscured the fluorescent colonies since they sometimes were not observed on badly crowded plates but were noted on plates poured with higher dilutions of the same sample. Deep subsurface colonies were not fluorescent because of an insufficient oxygen supply for pigment production; pouring thin layers of agar in the plates overcame this difficulty to some extent.

Samples of raw and pasteurized milk and cream were held at 5° to 7°C. for 3 to 6 days, then plated and, after incubation, representative fluorescent colonies were picked. The results indicated that there was a relative increase in the fluorescent bacteria at these temperatures. Some of the samples that failed to yield the organisms by direct plating often contained them in considerable numbers after holding at low temperatures.

Ice cream samples were allowed to melt at room temperature and plated soon after melting and again after holding at 5° to 7°C. for 5 days; with some samples, 11 ml. of the melted ice cream was also added to 99 ml. of sterile water and held at 5° to 7°C. for 5 days to allow growth to occur before plating. The plates were incubated 72 hours and then examined for fluorescent colonies.

Both salted and unsalted creamery butter made with and without the use of butter culture were examined. Most of the samples were of high quality but some of them showed certain defects. The butter was plated in dilutions from 1:10 to 1:100,000, the plates incubated for 72 hours, then examined for fluorescent colonies and, when found, representative colonies were picked for further study. The number of non-butter culture colonies on the plates were also counted. About 5 ml. of melted butter from each sample was also placed in a tube of litmus milk and shaken; after holding at 5° to 7°C. for 3 to 6 days, the milk was plated and the

plates later examined for fluorescent colonies. This method often yielded fluorescent organisms when direct plating of the sample failed. Most of the butter samples were held at 21°C. for 1 week to determine the keeping qualities. These samples were again plated at the end of the holding period in an effort to obtain fluorescent organisms if they had not been previously isolated from the sample.

An attempt was also made to isolate fluorescent bacteria from samples of water from various sources. Plating the fresh sample was not always successful because of the relatively small numbers of the organisms in certain samples and because of overgrowth with non-fluorescent types in others. The addition of about 5 per cent sterile milk to a water sample with holding at 5° to 7°C. for a few days before plating greatly increased the chances of obtaining the fluorescent bacteria.

Several miscellaneous substances, such as feed, manure and soil, were also examined for fluorescent organisms. A small portion of these materials were placed in bottles of sterile water and held in the refrigerator for a few days, then plated and examined according to the usual procedure.

Distribution of the Fluorescent Bacteria

The prevalence of fluorescent bacteria in the various dairy products and other materials examined is shown in

table 1. Because of the enrichment procedure commonly employed, no attempt was made to determine the numbers of these organisms present, except in a few cases.

Milk

Two hundred and seventy-four samples of milk delivered by producers to plants in Missouri and Iowa were examined and 178 (65.0 per cent) gave positive results. A number of the samples contained mostly fluorescent organisms when plated after aging 3 to 6 days at 5° to 7°C. The off-flavors present in such samples were astringent, bitter, quinine-like, rancid, nutty and stale.

Thirty-five samples of raw bottled milk, obtained from the same localities as the producer samples, were plated and 18 (51.4 per cent) yielded the organisms under investigation. The organisms were also isolated from 11 (44.0 per cent) of 25 samples of bottled pasteurized milk from different dairies in the two states. One sample contained essentially only fluorescent organisms in the aged milk. Since the fluorescent bacteria are not heat resistant, they probably were added from the equipment after pasteurization.

In connection with an investigation on mastitis 580 samples of milk drawn aseptically from the individual quarters of the udder of 145 cows in four dairy herds were plated directly on beef infusion agar and incubated at 37°C. for 48 hours. Fluorescent colonies were obtained from one or more

Table 1. Distribution of the Fluorescent Bacteria

Product	Number of samples examined	Fluorescent bacteria found in	
		Number	Per Cent
Unpasteurized milk - in cans	274	178	65.0
Unpasteurized milk - in bottles	35	18	51.4
Pasteurized milk	25	11	44.4
Unpasteurized sweet cream	149	87	58.4
Unpasteurized sour cream	104	5	4.8
Ice cream	38	7	18.4
Freshly made sweet cream butter	113	39	34.5
Butter not freshly made from sweet cream	72	20	27.8
Water - well and land surface	49	47	95.9
Water - city supplies	12	9	75.0
Miscellaneous materials	16	15	94.0

quarters of four of these cows. The milk from the infected quarters contained the organisms in numbers ranging from 500 to 4,500 per ml. The organisms isolated were later identified as *Ps. aeruginosa*.

Unpasteurized sweet cream

The samples investigated were obtained from the producer deliveries to creamery and ice cream plants in Iowa and Missouri. Eighty-seven (58.4 per cent) of the 149 samples examined gave positive results. Fluorescent bacteria greatly predominated in several of the samples after holding; the off-flavors noted in these samples were cheesy, putrid, bitter, rancid and unclean.

Unpasteurized sour cream

Fluorescent bacteria were seldom found in sour cream. Enrichment procedures were not tried with this product. The organisms were recovered from only 5 (4.8 per cent) of 104 samples examined. The acidities of the cream were not determined but the samples varied from only slightly sour to very high acid.

Ice cream

Thirty-eight samples of ice cream frozen in counter freezers and in commercial plants in Missouri and surrounding states were examined and fluorescent bacteria were secured

from seven samples (18.4 per cent). The comparatively high sugar and total solids contents may have prevented any relative increase in these organisms in the melted ice cream during aging.

Freshly made sweet cream butter

One hundred and thirteen samples of freshly made sweet cream butter from various Iowa creameries were obtained at intervals during the period from January to May of 1938. They were all of high quality and with but few exceptions scored 38 on flavor. Most of the samples were unsalted and were made with or without the use of butter culture. Fluorescent organisms were isolated from 39 (34.5 per cent) of these samples.

Butter not freshly made from sweet cream

Seventy-two samples of butter of miscellaneous types from plants in Iowa, Missouri, Nebraska, and Oklahoma were examined. Fluorescent bacteria were obtained from 20 (27.8 per cent) of the samples. Included in this group were 24 samples of stored, unsalted, sweet cream butter that showed certain defects. Seven of the samples were farm-made and two (28.6 per cent) contained the organisms under investigation. The remaining 41 samples were made from sour cream; 22 were unsalted, and some were prepared with the addition of butter culture; 11 (26.8 per cent) of the samples yielded fluorescent organisms.

Water

Fluorescent bacteria were found in essentially all land surface and well water samples investigated. Forty seven (95.9 per cent) of 49 samples collected from pasture ponds, roadside pools, running streams, rivers, farm wells and the roof of a building yielded these organisms. They were also obtained from 9 (75.0 per cent) of 12 municipal water supplies examined.

Miscellaneous materials

Fluorescent bacteria were isolated from 15 (94.0 per cent) of 16 samples of miscellaneous materials examined. These consisted principally of materials taken from a dairy barn and surroundings and included dust from the air and floor, cow feces, soil, alfalfa, bedding, beet pulp, cottonseed meal, linseed meal, ground corn, mixed grain, wheat bran, green wheat and green barley. The organisms were also obtained from the leaves of wild crab and hawthorne trees. Only one sample of each of the above materials was examined and all but one, a sample of linseed meal, gave positive findings.

Sources of the Cultures Studied in Detail

Five hundred and five cultures of fluorescent bacteria were studied in detail in this investigation. They were isolated from the various sources listed in table 1, with the

exception of nine that were received as identified stock cultures from the Iowa Agricultural Experiment Station. Usually only one culture was isolated from a sample, but two colonies sometimes were picked from plates suspected of containing more than one species of fluorescent organisms. The colonies were picked into sterile water blanks and replated; well isolated colonies that later developed on plates were again picked and replated, the process being continued through at least four purifications before the biochemical characteristics of the cultures were studied.

The cultures isolated from the various sources were numbered as follows:

Milk (220), 1 to 200, 481 to 492 and 495 to 502 inclusive

Cream (100), 201 to 300 inclusive

Butter (90), 301 to 390 inclusive

Ice Cream (10), 391 to 400 inclusive

Water (61), 401 to 457 inclusive; 493, 494, 504 and 505

Miscellaneous materials (15), 458 to 471 inclusive; 503

Iowa Agricultural Experiment Station (9) 472 to 480 inclusive; (Identified cultures of _Pseudomonas_ and _Phytomonas_ species)

Influence of Various Factors on Fluorescence

Composition of the medium

A green, yellowish-green or bluish-green, fluorescent pigment was produced by all of the 505 cultures on beef infusion agar and nutrient gelatin at a pH of 7.0 to 7.2 when incubated 3 days at 20°C. Similar pigments were also formed by all cultures in Uschinsky's medium and by all but a few cultures in Lasseur's medium after 2 weeks incubation at room temperature. The cultures, likewise, all developed fluorescence in litmus milk; proteolytic cultures were strongly fluorescent, the color intensity tending to increase as digestion continued; cultures that developed an alkaline reaction without proteolysis were fluorescent at the surface only, while acid-forming cultures were non-fluorescent after an acid reaction had developed. Fluorescence was not evident in beef extract broth at an initial pH of 7.0 after 3 days incubation at room temperature. After the cultures had aged for several weeks at 1° to 3°C., however, they all showed definite fluorescence, the intensity being rather pronounced in many cultures. During this period the cultures were frequently held at room temperature for several hours and the pH of the medium had increased to about 8.5.

Seventy-three cultures were studied for their ability to produce fluorescence on beef extract-peptone agar and on

beef extract-tryptone-dextrose-skimmilk agar at a pH of 7.0, with and without addition of various substances. The incubation temperature used was 21°C. All cultures produced fluorescence on beef extract-peptone agar but the intensity was very slight with some cultures. The amount of fluorescence produced by most cultures was increased when 0.2 per cent soluble starch was added to this medium. The increase in fluorescence may have been due to phosphorus in the starch since Martin, Naylor and Hixon (1939) found phosphorus in all starch samples examined. Tryptone agar had somewhat greater fluorescigenic properties than beef extract agar; all of the 73 cultures produced slight or moderate fluorescence on this medium. The addition of 0.1 per cent magnesium sulphate or dipotassium phosphate to either beef extract-peptone agar or to beef extract-tryptone-dextrose-skimmilk agar resulted in the development of moderate or strong fluorescence by all cultures. When 0.1 per cent of both of these substances were added to the above agars the intensity of fluorescence produced seemed to be increased slightly, at least with some cultures.

pH of the medium

Cultures incubated 3 to 4 days at room temperature in beef extract-peptone broth at an initial pH of 7.0 showed little or no evidence of fluorescence. When the reaction of

the broth was adjusted so that the pH was 9.2, as determined by the glass electrode after sterilization, all of the 505 cultures produced a moderate or strong fluorescence after 3 to 4 days incubation. In another trial, broth with a pH of 11.2 before sterilization had a pH of 9.6 after sterilization; growth was rather slow in this broth but after 4 days many cultures were strongly pigmented and all were fluorescent to some extent. The cultures showed more of a bluish fluorescence at this high pH than was characteristic when grown at a lower pH.

The ability to develop fluorescence on beef extract-peptone agar and on beef extract-tryptone-dextrose-skimmilk agar, at a temperature of 21°C., when the pH was varied was studied with 73 cultures. Fluorescence was lacking or only slight on beef extract agar at a pH of 7.0. When the pH of the medium was adjusted to 8.5 (after sterilization), moderate fluorescence was produced by all cultures. When the pH of the medium was similarly adjusted to 10.0, all cultures (505) showed definite fluorescence. The 73 cultures were all fluorescent to some extent on beef extract-tryptone-dextrose-skimmilk agar at a pH of 7.0 but the intensity of fluorescence was increased when the pH was raised to 8.5.

Oxygen supply

Subsurface colonies of pure cultures of fluorescent bacteria on beef infusion agar plates were always achromogenic,

while surface colonies of the same cultures were decidedly fluorescent. Suitable dilutions of 10 cultures were made and plated on beef infusion agar. When the agar had hardened, about 15 ml. of additional agar was poured into each plate in order to eliminate surface colonies. After 4 days incubation at room temperature only well isolated, subsurface colonies had developed and all were non-fluorescent.

Twenty cultures were streaked on beef infusion agar slopes and placed in a Novy jar. Carbon dioxide was introduced until a volume of gas equal to the volume of the container was expelled. After holding the jar at room temperature for 2 days, no evidence of growth was apparent on any of the slopes. About one-half of the gas in the container was then replaced with air and the valve again closed. All tubes showed abundant growth after 2 days but no pigmentation was evident. Three days later the tubes were removed from the jar and 12 showed a slight fluorescence when examined under ultra-violet light. Apparently sufficient oxygen was present to permit a slow development of pigment by some of the cultures.

Light

Cultures of fluorescent bacteria grown in the dark on beef infusion agar showed no apparent difference in fluorescent properties from cultures of the same organisms grown in natural light in a room at essentially the same temperature.

Seventy-three cultures were streaked on beef infusion agar plates and the plates held in the diffused daylight on the ledge of a north window. During the night the plates were removed to a refrigerator, then returned to the window ledge during the day. The temperature on the window ledge was approximately 18° to 20°C. After 3 days incubation the cultures all showed strong fluorescence.

Attempts to grow plate cultures in the direct sunlight on the ledge of a south window failed because the temperature in the sunlight was too high for the growth of all but 2 cultures. The temperature of the room was only 23°C. but the temperature on the window ledge was sometimes as high as 40°C., even when the window was partly raised to admit outside air. The cultures that grew were both _Pseudomonas aeruginosa_ and both developed a definite fluorescence.

Incubation temperature

The fluorescigenic ability of 73 cultures on beef infusion agar was compared at 10°, 20°, 30° and 37°C., allowing 3 days incubation. In general, fluorescence was very pronounced at 10°C. and tended to decrease as the incubation temperature was raised. All cultures were definitely fluorescent at 20°C., but at 30°C. fluorescence was rather slight in some cultures while others showed moderate fluorescence. Only 32 of the 73 cultures grew at 37°C. and 14 of these developed slight or moderate fluorescence. Cultures of _Pseudo-_

monas aeruginosa failed to make sufficient growth in 3 days at 10°C. to show any evidence of fluorescence. This species produced fluorescence at the other temperatures and was the only organism that developed fluorescence at 42°C.

Heating at 48.9°C.

Fifty-four 1 day old cultures in skimmilk were heated in sealed tubes in a water bath at 48.9°C. for 30 minutes. After chilling in cold water the tubes were opened and streaked on beef infusion agar plates. Forty-five cultures survived the heat treatment and all produced fluorescence on the agar.

Age of the culture

Pigment formation on beef infusion agar was usually evident after the cultures had incubated 12 hours at 20°C., but maximum fluorescence was apparently not reached until about the third day. Four cultures, grown in beef infusion broth at room temperature, showed increasing density of the absorption spectra for 26 days. Between 26 and 42 days, two cultures showed further increase in density while the other two cultures showed a slight decrease. An increase in density of the absorption spectrum was evidently due to an increase in concentration of the fluorescent pigment.

Agar slope cultures retained their fluorescence during 4 to 5 months at 1° to 3°C. but the agar usually acquired a slight reddish-brown color as the cultures aged. Only three of

the 505 cultures lost their ability to produce fluorescence at 20°C. after being carried on beef infusion agar (with transfers to new slopes every 4 to 5 months) at 1° to 3°C. for 2 years. One of these cultures (348) was weakly fluorescent when isolated, while the other two (478 and 480) were obtained as pure cultures of Phytomonas and had previously been carried on agar for an unknown period. These three cultures were fluorescent, however, when grown at 10°C. The other cultures showed no apparent decrease in their fluorescigenic ability while being carried on beef infusion agar at 1° to 3°C. for periods up to 3 years.

Absorption Spectra of Broth Cultures and Solutions of Certain Fluorescent Substances

Four cultures of fluorescent bacteria with different biochemical characteristics were selected for a study of their absorption spectra. Culture 14 was proteolytic, culture 241 produced a reddish-brown pigment in addition to the green fluorescent pigment, culture 273 was non-proteolytic, while culture 376 produced pyocyanine. The cultures were inoculated into separate flasks of beef infusion broth and the flasks held at room temperature. Approximately 10 ml. of broth was removed aseptically from one flask at the beginning and from each flask after 2, 5, 10, 26 and 42 days incubation and clarified by centrifuging. Absorption spectrum measurements

were then made on each broth with a Hilger Spekker Spectrophotometer which had a variable aperture graduated in terms of density*. It was necessary to increase the dilution of the broths as the cultures aged in order to bring them into the range of the instrument. Accordingly, measurements were tried at different concentrations and cell lengths upon certain broths to ascertain if Beer's Law, $I = I_o e^{-kcl}$ (I_o = incident light intensity, I = transmitted light intensity, k = specific extinction, c = concentration and l = length of cell), was obeyed. Beer's law was found to be obeyed by the peak, so it was possible to multiply the density values by the concentration factor and express them in terms of the undiluted cultures.

The absorption spectra of the 4 cultures at various time intervals are shown in figures 1 to 4. The uninoculated broth (figure 1) showed a rather uniform absorption curve with no peak and the density was lower than the absorption curves of the inoculated broths. All cultures regardless of age had the same type of absorption spectra. An absorption peak occurred at approximately 4,020Å, after which there was a decrease, then an increasing straight line absorption of the ultra-violet waves. Wave lengths longer than 4,800Å were only slightly absorbed. The density of the absorption curves increased as the cultures aged with the exception that cul-

*Density = log Io/I = kcl.

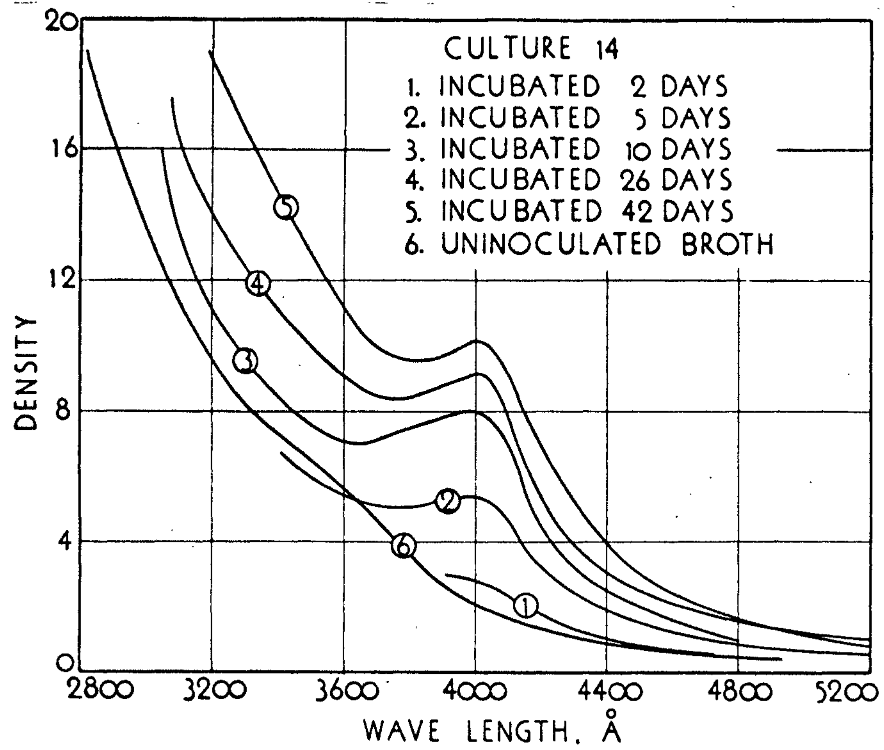

Fig. 1. Absorption spectra of culture 14

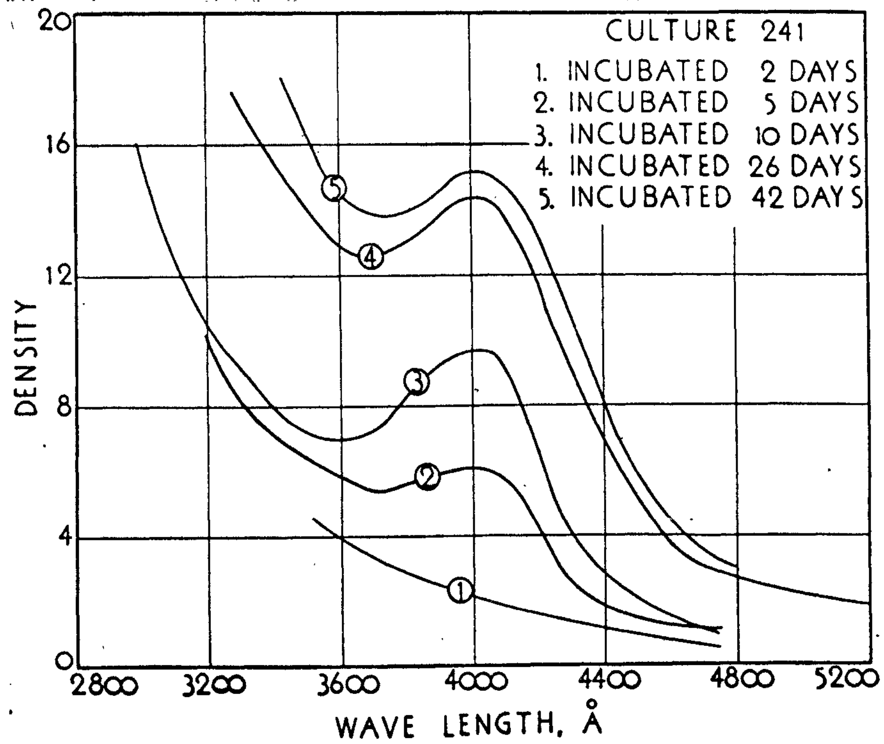

Fig. 2. Absorption spectra of culture 241

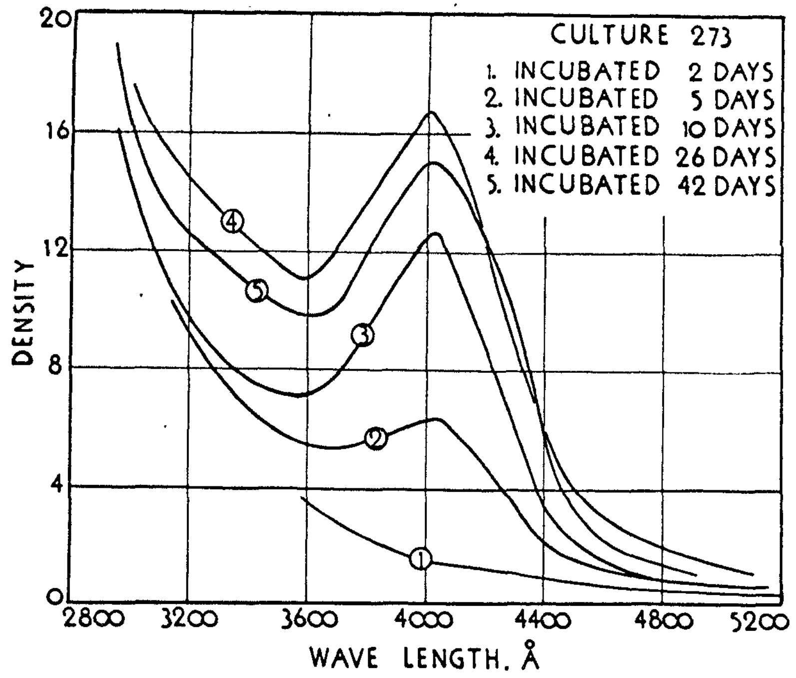

Fig. 3. Absorption spectra of culture 273

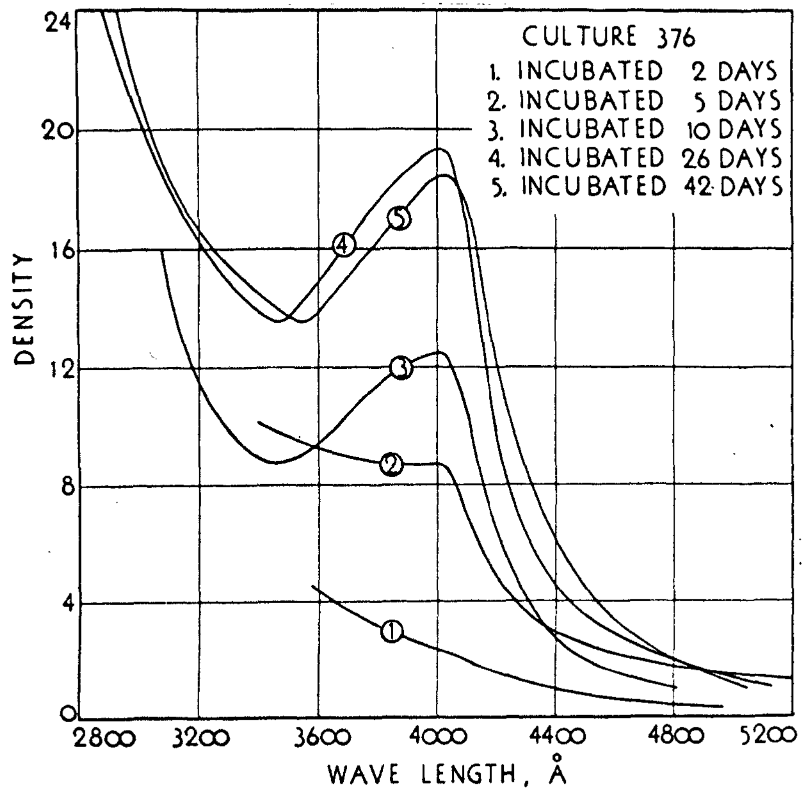

Fig. 4. Absorption spectra of culture 376

tures 273 and 376 had curves of slightly lower density at 42 days than at 26 days. The absorption curves of culture 14 were the lowest while those of culture 376 were the highest in density of the four cultures at the different age intervals.

Figure 5 shows the absorption spectra of the chloroform extract of the broth culture and the broth culture after extraction with chloroform for culture 376. The absorption curves of the chloroform extracts of the 5 and 10 day cultures had slight absorption peaks at about 3,300 and 3,650Å. After the pyocyanine had been removed from the 10, 26 and 42 day broth cultures with chloroform, the absorption curves were similar to the absorption curves of the unextracted broth (figure 4), but the densities were slightly lower.

The pigment produced by the fluorescent bacteria is sometimes referred to as fluorescin and sometimes as flavine. It was thought that a comparison of the absorption spectra of a solution of each of these substances with the absorption spectra of broth cultures of fluorescent bacteria would indicate whether the fluorescent bacterial pigment is the same or similar to either of these compounds. Fluorescein was included since it is fluorescent and differs in composition from fluorescin only in that it contains two less hydrogens in the molecule. Riboflavin was used because it could be obtained in chemically pure form. The absorption spectra

of diacetyl was also determined to ascertain whether the color of broth cultures of fluorescent bacteria might be due to formation of diacetyl. Harden and Norris (1911) observed that a pink color with a green fluorescence developed when 1 drop of a dilute solution of diacetyl was added to a dilute solution of protein made alkaline with potassium hydroxide.

Two mg. of fluorescin, fluorescein or riboflavin or 0.1 ml. of diacetyl was dissolved in 100 ml. of beef infusion broth at pH 8.0 and the absorption spectra determined. The broth was adjusted to pH 8.0 to correspond with the pH of aged broth cultures of fluorescent bacteria. The absorption spectra of these broths are shown in figure 6. The spectra of the broths containing fluorescin or fluorescein were similar with peaks at about 4,900Å. The riboflavin broth had two slight peaks at about 3,700 and 4,650Å. The absorption spectra of the broth that contained diacetyl resembled the absorption curve for the uninoculated broth. The absorption spectra of the above broths in no way resembled the absorption curves of the broth cultures of fluorescent bacteria.

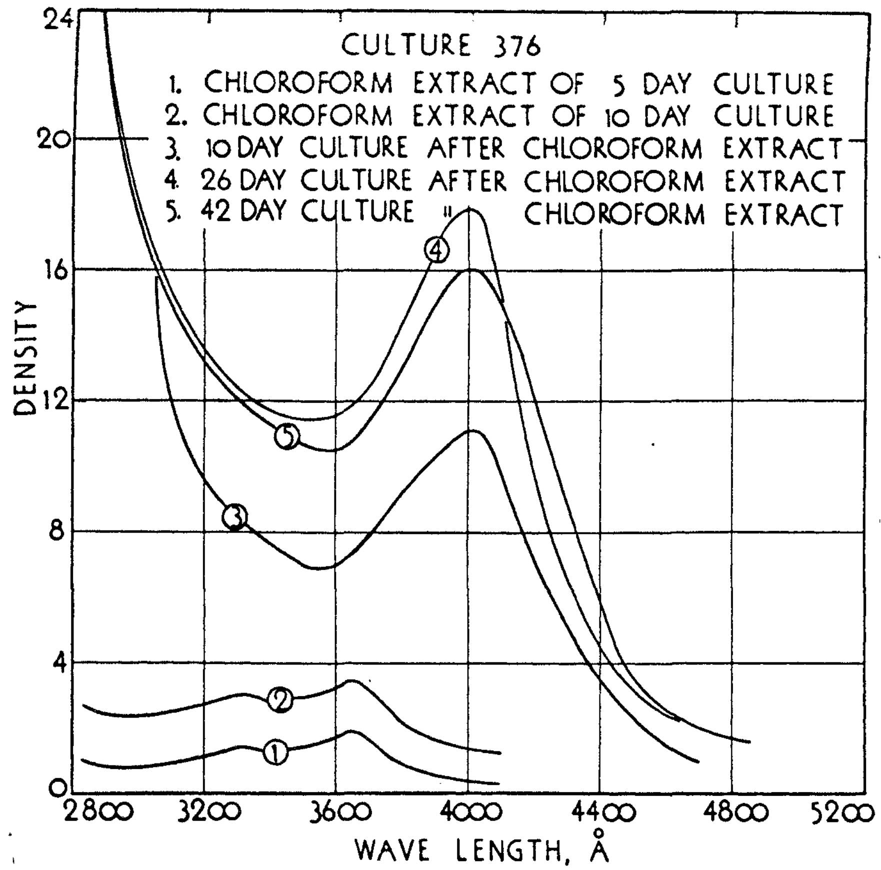

Fig. 5. Absorption spectra of culture 376 extracted with chloroform

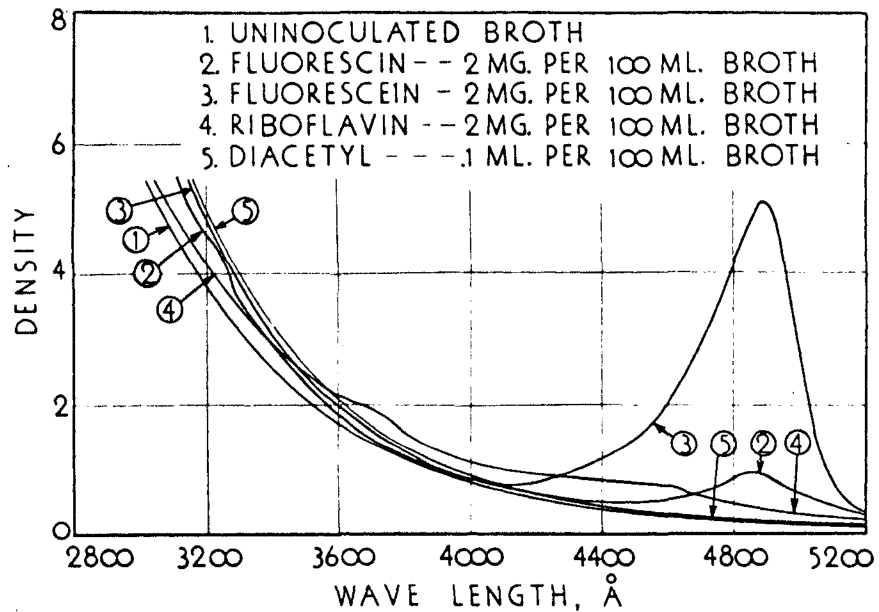

Fig. 6. Absorption spectra of fluorescent substances.

General Characteristics of the Fluorescent Bacteria

Morphology. (Cultures grown 1 day at 20°C. on beef infusion agar)

Form and size: Rods with rounded ends; 0.55 to 1.1 by 1.0 to 3.0 microns.

Arrangement: Singly and occasionally in pairs; long chains formed by 1 culture.

Motility: Motile: 1, 2 or 3 and occasionally 5 polar flagella; flagella sometimes noted on both ends of cell.

Staining reaction: Stained readily with common stains; gram negative.

Spores: None observed.

Growth on beef infusion agar. (Cultures grown 2 days at 20°C.)

Agar slant: Growth characteristics varied with different cultures.

The general types of growth were:

(a) Abundant, filiform, raised, white to grayish-white, shiny and butyrous or viscid. Medium turned green or yellowish-green and fluorescent. After 5 days some cultures developed a reddish-brown on black pigment.

(b) Abundant, spreading, thin, glistening, white to grayish-white, butyrous or viscid. Medium turned green or yellowish-green and fluorescent.

(c) Abundant, spreading, raised, orange-brown and butyrous. Medium turned yellowish-green and fluorescent.

Agar stab: Surface growth with only scanty growth along the line of inoculation.

Agar colonies: Subsurface colonies were non-fluorescent and usually punctiform unless located at

Agar colonies: (continued)	the agar-glass boundary, when they were larger, circular and flat.
	Surface colonies varied somewhat with the different cultures but were usually 2 to 5 mm. in diameter, circular, convex, smooth-surfaced with edges entire, butyrous or viscid and opaque. Some cultures formed colonies that were (a) irregular, thin and spreading, smooth-surfaced, lobate, and translucent, (b) irregular, mucoid and opaque or (c) circular, convex, rough-surfaced, edges wrinkled and grayish. More than one colony type was sometimes formed by certain cultures on the same plate. All colonies were fluorescent.

Growth in beef extract-peptone broth

The organisms regularly produced moderate or heavy clouding in beef extract-peptone broth with a rather abundant, compact or flocculent sediment. Surface growth was either flocculent or membraneous; the pellicle formed varied from friable to firm or leathery. A putrefactive odor was usually produced; some cultures formed an odor suggestive of indol.

Growth temperatures

The minimum and maximum growth temperatures were ascertained by inoculating the cultures into beef-extract-peptone broth and holding at 3°, 7°, 32°, 37°, 42° and 45°C., then later examining for the development of turbidity. An incubation period of 14 days was allowed for the two lowest temperatures and 5 days for the other temperatures. The number of cultures that grew at different temperature ranges

were as follows:

Growth temperature range:	3°-32°C.	3°-37°C.	7°-37°C.	7°-42°C.	7°-45°C.
Number of cultures:	233	208	27	22	15

Four hundred and forty-one of the 505 cultures grew at 3°C., while all cultures grew at 7°C. All cultures likewise grew at 32°C., while 235 cultures grew at 37°C., 22 grew at 42°C. and 15 grew at 45°C.

Growth of selected cultures in skimmilk at 5° and 21°C.

Skimmilk was sterilized in 100 ml. quantities in bottles with a screw cap and then cooled to 5° or 21°C. One bottle at each temperature was inoculated with 0.1 ml. of a 12 hour broth culture of a fluorescent organism, 10 selected cultures being used in the study. Immediately after inoculation each bottle was thoroughly shaken and then plated on beef infusion agar. One set of cultures was placed in a refrigerator with an average temperature of 5°C., while the other set was placed in a 21°C. incubator. At 24 hour intervals the samples were well mixed, plated and the flavor noted. The examination was continued for 1 week or until an objectionable flavor developed. The results obtained are presented in table 2.

Eight of the 10 cultures grew fairly rapidly in sterilized skimmilk at 5°C., and the counts continued to increase during the 7 days that the bacterial analyses were made. Ex-

Table 2. Growth of Selected Cultures in Skimmilk at 5° and 21°C.

Culture	Bacteria per ml. (000 omitted) after							
	0 hr.	24 hr.	48 hr.	72 hr.	96 hr.	120 hr.	144 hr.	168 hr.
				Held at 5°C.				
42	37	840	8,200	32,000	58,000	120,000	170,000	230,000
65	68	3,000	28,800	54,000	144,000	390,000	560,000	620,000*
110	148	280	450	960	1,170	1,450	2,200	3,400
173	26	510	3,100	13,100	28,000	44,000	93,000	105,000
241	27	700	11,500	42,000	83,000	180,000	240,000	370,000
297	14	1,350	15,200	29,000	74,000	96,000	171,000	210,000
335	32	2,300	6,600	24,300	47,000	65,000	140,000	224,000
354	78	2,270	21,700	42,000	165,000	470,000	720,000	830,000*
376	60	65	83	174	360	640	830	1,130
447	84	3,600	29,400	63,000	140,000	230,000	380,000	540,000*
				Held at 21°C.				
42	34	97,000	630,000	780,000	1,250,000*	--		
65	94	310,000	1,870,000*	1,470,000	1,130,000	--		
110	135	82,000	1,400,000	2,230,000*	2,450,000	--		
173	18	14,500	160,000	430,000	950,000	1,250,000		
241	25	72,000	1,100,000	1,600,000	2,140,000*	--		
297	27	211,000	940,000	1,920,000	2,200,000*	--		
335	21	98,000	210,000	490,000	680,000	1,320,000		
354	86	390,000	2,410,000*	2,200,000	1,830,000	--		
376	50	42,000	1,260,000	1,240,000*	990,000	--		
447	112	234,000	5,500,000*	2,100,000	1,760,000	--		

*Denotes when off-flavor first appeared in milk.

cluding cultures 110 and 376, which grew very slowly, the original counts ranged from 14,000 to 84,000 per ml.; after 24 hours the counts ranged from 510,000 to 3,600,000 per ml. and after 168 hours the counts varied from 105,000,000 to 830,000,000 per ml. Three samples developed a slightly bitter flavor at the end of 168 hours.

At 21°C. the counts increased rapidly in all samples during the first 2 days and then slowly decreased in four cultures. Three samples developed a bitter or quinine-like flavor after 48 hours, two after 72 hours and three after 96 hours. Two cultures contained no off-flavor after 120 hours when the counts were about 1,250,000,000 per ml. These cultures (173 and 335) were non-proteolytic and non-lipolytic. The first change usually noted in the samples was a bitter flavor, followed by digestion or sweet curdling, with curdling usually conspicuous. After further holding the flavor became decidedly bitter, quinine-like or astringent.

Action on litmus milk

The changes produced in litmus milk by the fluorescent bacteria were of seven general types:

a. Rapid digestion, without noticeable coagulation, from the top down and usually complete within 5 to 10 days with a white or yellowish-white sediment and a putrid odor; digested portion usually wine colored at first, becoming light or dark amber or green after several days, the color varying

somewhat with the incubation temperature and the amount of aeration (313 cultures).

b. Same as (a) except that the digested portion was amber colored and the odor resembled indol (18 cultures).

c. Alkaline reaction slowly developed, with no apparent proteolysis and no off-odor; color bluish-grey after 10 to 21 days, with a white sediment (75 cultures).

d. Alkaline reaction slowly developed, with slight digestion evident after 10 to 21 days; color grey to bluish-grey (13 cultures).

e. Slight alkaline reaction after 5 days followed by an acid reaction but usually no coagulation after 21 days; no reduction except at base of tube (50 cultures).

f. Formation of an acid ring with acid coagulation from the top down and reduction except at surface; slight proteolysis and a May apple odor sometimes noted (36 cultures).

Fermentation of carbohydrates

The numbers of cultures that fermented the various test materials were as follows:

Arabinose	85 (16.8%)	Lactose	0	Raffinose	0
Dextrose	475 (94.1%	Levulose	25 (5.0%)	Salicin	0
Galactose	376 (74.5%)	Maltose	37 (7.5%)	Sucrose	19 (3.8%)
Glycerol	0	Mannitol	0	Xylose	476 (94.3%)
Inulin	0	Mannose	224 (44.3%)		

Cultures that attacked dextrose usually fermented xylose, but there were some exceptions. Cultures that fermented galactose also fermented dextrose or xylose and usually both of these materials. Levulose and sucrose, when attacked, were only weakly fermented, but the maltose positive cultures usually showed strong acid production.

Several cultures that first produced an acid reaction in certain of the broths later developed an alkaline reaction due to formation of ammonia. Some cultures developed an acid reaction at the surface of the tube only and this sometimes existed for only 1 or 2 days before being replaced by an alkaline reaction; individual cultures often showed considerable variation in the amount of yellow color produced with the same test material in different trials. Observation at frequent intervals, without any agitation of the tubes, was therefore necessary in order to detect the positive cultures with any degree of accuracy. This indicates that the use of indicator broth to detect acid production from carbohydrates is probably not very reliable for slow fermenting cultures that also produce ammonia.

Acid development and utilization of lactose in skimmilk and lactose broth

An attempt was made to ascertain whether cultures that developed an acid reaction in litmus milk but failed to produce an acid reaction in lactose broth containing brom cresol

purple utilized the lactose in these media. Skimmilk and 1 per cent lactose-beef extract-peptone broth were sterilized in 100 ml. quantities in cotton plugged Erlenmeyer flasks. One flask of each medium was inoculated with a selected culture, and after 5, 13 and 23 days at room temperature each culture and an uninoculated flask of each medium were analyzed for lactose by the Shaffer-Somogyi method; the pH also was determined, using the glass electrode. At the end of the incubation period the titratable acidity of the milk was determined.

The data obtained on the skimmilk cultures are shown in table 3, while the data on the lactose broth cultures are presented in table 4. Although all the 15 cultures brought about a decrease in the lactose content of skimmilk, the rate of decrease varied with the different cultures. Appreciable quantities of lactose still remained in all cultures, however, after 23 days, the lowest value being 1606 mg. per 100 ml. compared to 4850 mg. in the control. With exception of one culture (291) the pH of the milk gradually decreased as the cultures aged, the lowest value obtained being 5.28 with culture 246. This organism also developed a titratable acidity of 0.78 per cent and was the only culture that coagulated the milk. The titratable acidities of the other cultures varied from 0.29 to 0.62 per cent after 23 days.

The lactose content of the broth also was progressively reduced as the cultures aged. After 23 days the lactose con-

Table 3. Utilization of Lactose by Fluorescent Bacteria in Skimmilk

Culture	Held 5 days		Held 13 days		Held 23 days		
	Mg. lactose per 100 ml.	pH	Mg. lactose per 100 ml.	pH	Mg. lactose per 100 ml.	pH	Acidity
Control	4854.9	6.56	4850.0	6.55	4850.0	6.54	0.22
42	3439.9	6.32	2726.6	5.97	2624.9	5.85	0.51
95	3639.0	6.57	3469.9	6.33	2939.0	6.29	0.42
124	2867.7	6.31	2624.9	6.13	2354.0	5.78	0.62
219	3643.3	6.43	2648.3	6.11	2590.9	5.84	0.50
234	3418.3	6.39	2878.9	6.29	2838.3	6.28	0.31
246	2448.3	5.88	2182.9	5.48	1606.5	5.28	0.78
253	3988.5	6.49	3048.3	6.17	1713.4	6.12	0.57
266	3639.9	6.54	3078.3	6.49	2868.3	6.45	0.55
291	3418.3	6.53	3038.4	6.54	2940.0	6.55	0.29
303	4763.3	6.41	2741.7	6.38	2629.9	6.02	0.42
323	2403.3	6.07	2116.7	5.90	2035.4	5.61	0.57
332	2720.0	6.46	2263.4	5.96	2049.6	5.67	0.55
356	3843.3	6.50	3209.9	6.43	3116.7	6.36	0.30
387	3164.9	6.12	2659.0	5.92	2360.7	5.68	0.55
492	3988.5	6.58	3599.0	6.51	2569.7	6.13	0.37

Table 4. Utilization of Lactose by Fluorescent Bacteria in Lactose Broth

Culture	Held 5 days		Held 13 days		Held 23 days	
	Mg. lactose per 100 ml.	pH	Mg. lactose per 100 ml.	pH	Mg. lactose per 100 ml.	pH
Control	1026.3	6.64	1025.0	6.65	1026.0	6.63
42	661.8	7.55	237.1	8.40	97.4	8.35
95	330.9	8.18	122.2	8.71	38.5	8.72
124	882.5	8.07	95.5	8.63	67.5	8.59
219	551.4	7.90	66.9	8.59	13.4	8.54
234	441.3	8.20	42.0	8.72	12.1	8.70
246	661.8	8.06	85.6	8.54	13.0	8.52
253	242.7	7.93	120.9	8.61	85.5	8.57
266	882.5	8.19	596.7	8.53	51.4	8.55
291	121.3	7.44	87.7	8.40	12.9	8.39
303	860.4	7.62	110.5	8.54	78.2	8.51
323	769.2	7.73	74.2	8.52	18.5	8.52
332	507.4	8.19	461.7	8.58	67.5	8.59
356	551.5	8.38	237.0	8.67	62.4	8.64
387	705.9	7.64	79.4	8.50	11.1	8.44
492	441.3	7.87	169.7	8.63	44.8	8.63

tents of the different cultures varied from 11 mg. to 97 mg. per 100 ml., compared to 1026 mg. in the control. All cultures showed a decided increase in pH after 5 days incubation and an additional small increase during the next 8 days, after which no further change in pH occurred. The pH of the different cultures ranged from 7.55 to 8.38 after 5 days and from 8.40 to 8.72 after 13 days.

Proteolysis

Of the 505 cultures, 384 liquefied gelatin. All cultures that liquefied gelatin also showed a clearing around the area of growth on milk agar plates and vice versa. The number of cultures that produced various types of gelatin liquefacation are given in the following summary:

No. of:	Type of gelatin liquefaction				
cul-	None	Crateriform	Infundibuliform	Napiform	Stratiform
tures	121	46	185	18	135

In general, cultures that produced infundibuliform or stratiform liquefacation were strongly proteolytic and usually completely liquefied the gelatin in 4 or 5 days. On the other hand, cultures that produced only crateriform or napiform liquefacation were usually weakly proteolytic and liquefied only the upper portion of the gelatin tube during the observation period of about 1 week.

Lipolysis

The number of cultures that hydrolyzed corn oil and cottonseed and the extent of lipolysis are as follows:

Fat	Extent of fat hydrolysis			
	−	±	+	++
	Number of cultures			
Corn oil	191	21	155	138
Cottonseed oil	191	30	151	133

− = fat not hydrolyzed (fat droplets red colored)

± = fat partly hydrolyzed (both red and blue colored fat droplets underneath area of growth)

\+ = fat hydrolyzed (only blue colored fat droplets underneath area of growth)

++ = fat hydrolyzed in medium surrounding area of growth

Considerable variation existed in the lipolytic ability of the different cultures. In general cultures that attacked one oil also attacked the other, but slight variations existed in the extent to which the two fats were hydrolyzed by a few organisms.

Hemolysis

Fifty-nine of the 505 cultures hemolyzed bovine red blood cells. Eleven cultures produced a narrow zone of hemolysis that extended only about 1 mm. beyond the area of growth, while 48 cultures formed a broad zone of hemolysis that extended from 1 to several mm. beyond the colony border.

Nitrate reduction

Potassium nitrate was reduced by 168 of the 505 cultures; 94 of these cultures formed nitrites only, while the other 74 cultures extended the reduction to the liberation of nitrogen gas. It was necessary to test for nitrite at comparatively short intervals (12 hours) because, when produced, this substance evidently was utilized by some cultures and disappeared from the medium after a time. This was particularly true of cultures that liberated nitrogen gas; frequent checking of these cultures, however, showed that they all reduced the nitrate to nitrite before gas was produced.

Formation of hydrogen sulphide

None of the cultures gave a positive test for hydrogen sulphide when grown in beef extract-peptone broth with lead acetate paper strips in the top of the tube. When streaked on tryptone, iron agar, however, 30 cultures gave a strong test for hydrogen sulphide and 11 cultures gave a moderately positive reaction.

Production of indol

Several cultures showed a slight red color in the Böhme test, but the reactions were not very definite. With the Gorè method one culture gave a slightly positive reaction; when retested several months later this culture also was

negative. Sandiford (1937) pointed out that the Böhme test for indol generally gives a false positive reaction with cultures of Ps. aeruginosa because of the acid in the reagents used. Apparently the odor suggestive of indol that is produced in various media (including milk) is not due to this compound.

Utilization of urea, formation of catalase, production of ammonia and growth in Uschinsky's medium

All the 505 cultures utilized urea as the only source of nitrogen, formed catalase in liquid media, produced ammonia in beef extract-peptone broth and grew in Uschinsky's medium with the production of a green fluorescence. The development of fluorescence in Uschinsky's medium denoted that the organisms were able to produce a fluorescent pigment in a medium containing magnesium, sulphate and phosphate ions and a suitable source of nitrogen.

Diastatic action, production of acetylmethylcarbinol and formation of chlororaphine.

Starch was not hydrolyzed by any of the cultures under the conditions employed. All cultures, likewise, gave a negative or only a faintly positive test for acetylmethylcarbinol. Chlororaphine was not formed by any of the cultures in Lasseur's medium, but a few white crystals of an unknown substance developed in some cultures.

General Resistance of Fluorescent Bacteria

Viability in culture media

The individual cultures of fluorescent bacteria varied considerably in the period that they remained alive in or on laboratory media without being transferred to fresh media. In trials with selected cultures, the cultures of *Ps. aeruginosa* were the least resistant, while certain cultures that produced an alkaline reaction in milk without digestion were intermediate in this respect. The *Ps. aeruginosa* cultures seldom survived longer than 4 to 5 months on beef infusion agar slopes at 1° to 3°C.; the remaining cultures, with the exception of a few of the non-proteolytic ones, usually remained viable for 6 months or longer. Forty-three agar slope cultures (258 to 300 inclusive) were alive after being held 10 months at 1° to 3°C.

The 505 cultures all survived in beef extract-peptone broth for 3 months at 1° to 3°C., but 30 of the cultures were dead at the end of 4 months. The cultures that died included all of the 17 *Ps. aeruginosa* cultures in the group, while the others were non-proteolytic types; the remaining cultures were still viable at the end of 6 months. Twenty-one litmus milk cultures held at room temperature and at 1° to 3°C. were all alive after 6 months; the original 10 ml. of milk in the tubes held at room temperature had evaporated to 1 to 2 ml.

after 6 months and was thick, dark and foul smelling.

Seven samples of unsalted butter made from sterilized cream to which cultures of fluorescent bacteria were added before churning still contained viable fluorescent organisms after 6 months at 1° to 3°C. Nine other samples of butter prepared as above except that two were salted yielded fluorescent bacteria when examined after 4 months at 21°C.

Resistance to heat

The thermal death points of 90 cultures were determined as follows: 0.1 ml. of a 1 day milk culture of each organism grown at room temperature was added to 10 ml. of milk and 2 ml. of each suspension was placed in each of three agglutination tubes; the tubes were then sealed and exposed in a water bath at temperatures of 51.7°, 54.4° or 57.2°C. for 30 minutes; after cooling in ice water, the tubes were opened and the contents inoculated into beef extract-peptone broth and streaked on beef infusion agar to determine sterility.

Forty-three of the 90 cultures survived an exposure of 51.7°C. for 30 minutes while 20 cultures survived at 54.4°C.; at 57.2°C., however, all cultures were destroyed. Fifty-four of the 90 cultures were also exposed at a temperature of 48.9°C. for 30 minutes and nine were killed. A culture isolated from a bottle of pasteurized milk in which fluorescent bacteria predominated was destroyed at 51.7°C. for 30 minutes.

The heat resistance of several cultures was also determined using 2 day milk cultures grown at room temperature and 2 or 3 day milk cultures grown at room temperature for 1 day and then held at 1° to 3°C. for 1 or 2 days. The heat resistance of these cultures was essentially the same as that of the 1 day cultures.

Resistance to chlorine

The resistance to chlorine of eight selected cultures of fluorescent bacteria was determined in the following manner: Chlorine solutions containing 2.5, 5, 10 or 15 parts per million of available chlorine were freshly prepared from a stock solution of sodium hypochlorite and sterile distilled water at room temperature and 100 ml. quantities were placed in sterile dilution bottles. Five ml. of a 12 hour broth culture of each organism, grown at 21°C., were placed in 100 ml. of sterile distilled water and 1 ml. of this suspension was added to a bottle of each chlorine solution and well agitated. At the end of 2.5, 5, 7.5, 10 and 12.5 minutes, 1 ml. was removed from each bottle and added to a 99 ml. sterile water blank for dilution before plating or (according to the chlorine strength and period of exposure) placed directly in a petri dish containing sufficient sterile sodium thiosulfate solution to neutralize the available chlorine present (0.3 ml. of a 1 per cent solution was added for solutions containing 2.5 or 5 parts per million of available chlorine and 0.5

ml. for solutions containing 10 or 15 parts per million). Beef infusion agar was immediately added to the plates and after 3 days at 21°C. the colonies were counted and the number of organisms per ml. of chlorine solution at the different time intervals was computed. The numbers of organisms per ml. in the chlorine solution at the beginning of the exposure were estimated by determining the number of organisms in the original suspension.

The data are given in table 5. The numbers of organisms in the chlorine solutions at the beginning of the exposures ranged from 73,000 to 960,000 per ml. In general, the fluorescent bacteria were readily destroyed by chlorine and there was no noticeable difference in the resistance of the eight cultures used. A concentration of 2.5 parts per million of available chlorine was slowly destructive, but the organisms usually were not completely destroyed at the end of 12.5 minutes. The rate of destruction in solutions containing 5 parts per million of available chlorine was fairly rapid but a few viable cells generally remained after 12.5 minutes exposure. The organisms were usually destroyed within 5 minutes in solutions containing 10 parts per million and within 2.5 minutes in solutions containing 15 parts per million of available chlorine, but there were a few exceptions.

Tolerance of sodium chloride

The amount of sodium chloride in the medium required to

Table 5. The Resistance of Fluorescent Bacteria to Chlorine.

Available chlorine (p.p.m.)	Bacteria per ml. after 0 min.*	2.5 min.	5 min.	7.5 min.	10 min.	12.5 min.
			Culture 14			
2.5	310,000	92,000	18,600	520	40	1
5	310,000	50	10	10	10	2
10	310,000	10	10	1	1	-
15	310,000	10	1	1	-	-
			Culture 69			
2.5	200,000	46,000	6,000	1,650	70	1
5	200,000	40	10	20	10	1
10	200,000	10	10	1	1	-
15	200,000	10	1	1	--	-
			Culture 110			
2.5	960,000	1,200	360	20	20	1
5	960,000	140	70	20	10	1
10	960,000	40	40	6	1	-
15	960,000	10	8	1	--	-
			Culture 142			
2.5	120,000	1,500	30	30	50	2
5	120,000	110	10	30	10	3
10	120,000	50	70	27	3	-
15	120,000	10	5	1	--	-

Table 5. (continued)

Available chlorine (p.p.m.)	0 min.*	2.5 min.	5 min.	7.5 min.	10 min.	12.5 min.
	Bacteria per ml. after					
			Culture 241			
2.5	73,000	2,270	760	130	30	1
5	73,000	90	30	70	10	1
10	73,000	60	30	5	1	-
15	73,000	40	1	1	--	-
			Culture 273			
2.5	430,000	2,700	300	70	20	11
5	430,000	1,950	180	50	30	2
10	430,000	10	10	4	1	-
15	430,000	10	1	1	--	-
			Culture 354			
2.5	215,000	7,300	440	290	120	10
5	215,000	70	50	80	10	1
10	215,000	20	20	1	1	-
15	215,000	10	1	1	--	-
			Culture 376			
2.5	147,000	24,000	510	170	40	3
5	147,000	8,500	320	210	50	7
10	147,000	10	30	7	2	-
15	147,000	10	1	1	--	-

*Approximate number of bacteria estimated by determining the number of organisms in the original suspension.

inhibit growth of the fluorescent bacteria was determined with beef extract-peptone agar and with beef extract-peptone broth. The liquid medium proved to be preferable for the trials, however, because slight growth could be more readily detected in broth than on agar plates.

Spot inoculations were made on the surface of solidified agar in petri dishes and the plates incubated for 5 days. The 505 cultures all grew on agar containing 4 per cent sodium chloride, but 40 cultures failed to grow when the sodium chloride content was increased to 6 per cent. No higher concentrations of sodium chloride were tried with the solid medium. The cultures all grew in broth containing 4 per cent sodium chloride but 75 cultures failed to grow in broth with a salt content of 6 per cent; only nine cultures showed evidence of growth in 8 per cent sodium chloride broth after 10 days, and with five of these cultures the turbidity produced was slight.

Ability to grow in alkaline and acid media

The ability of the fluorescent bacteria to grow in or on media with a strongly alkaline or strongly acid reaction was determined by streaking the cultures on the surface of beef extract-peptone agar plates and by inoculation into beef extract-peptone broth. The pH of the medium was always adjusted or determined after sterilization. The 505 cultures all made abundant growth on agar at a pH of 8.5; the cultures,

likewise, all made moderate growth on agar at a pH of 10.0 when the plates were poured 24 hours before inoculating, but growth was often negative when the cultures were streaked on the medium soon after the plates were poured. All cultures grew fairly rapidly in beef extract-peptone broth at a pH of 9.2; the cultures, likewise, all grew in the broth at a pH of 9.6, but growth was not apparent in some cultures until 2 to 3 days incubation. The reaction of the medium decreased to a pH of about 8.6 after considerable growth had occurred.

Irregular and inconsistent results were obtained when the cultures were streaked on agar at a pH of 4.5 to 5.0, and for that reason the data are not included. The number of cultures that grew in beef extract-peptone broth at a pH of 4.0, 4.5, 5.0 or 5.5 were 4, 359, 448 and 505 respectively. Growth usually was not evident in broth at a pH of 5.0 or below until after 2 to 3 days; it was initiated at the surface and then gradually extended downward as the cultures aged. The pH of the medium increased considerably during the first few days as is illustrated by the pH measurements made on a culture (230) growing in broth with an original pH of 4.5; when growth was first clearly evident after 2 days the pH of the medium was 4.95, while 2 days later the broth was cloudy and the pH had increased to 6.85.

Ten cultures were also inoculated into acidified milk to ascertain whether they would grow in a well buffered medium at a low pH. Skimmilk was sterilized in 2 liter Erlenmeyer

flasks and, after cooling, the milk in individual flasks was adjusted to a pH of 6.0, 5.5 or 5.0 by the addition of sterile N/1 lactic acid. About 1 hour was allowed to elapse after the first acid was added before the final adjustment in pH was made, to permit equilibrium to occur. The milk that was adjusted to a pH of 5.0 was slightly coagulated when acidification was completed. After acidification, 100 ml. of milk from each flask was pipetted into sterile screw cap dilution bottles, and 0.1 ml. of a 12 hour broth culture was added. The samples were shaken and plated on beef infusion agar, then held at 21°C. and again plated after 48 and 96 hours.

The results are shown in table 6. The counts increased fairly rapidly in all cultures but in general the rate of growth was slower as the acidity increased. The original counts of all samples varied from 22,000 to 41,000 bacteria per ml. After 48 hours the counts in milk at pH 5.0 originally varied from 31,000,000 to 175,000,000 per ml., while after 96 hours the variation in counts in the same samples ranged from 250,000,000 to 970,000,000 per ml. At the end of 96 hours the pH of the cultures with an original value of 5.0 ranged from 5.25 to 5.66. Six cultures developed a bitter flavor in milk at a pH of 5.0 after 96 hours and four of these coagulated the milk.

Table 6. Growth of Fluorescent Bacteria in Milk at Different pH Values

Hours held at 21°C.	Bacteria per ml. (000 omitted) at pH 6.0	5.5	5.0	Hours held at 21°C.	Bacteria per ml. (000 omitted) at pH 6.0	5.5	5.0
		Culture 42				Culture 297	
0	114	98	126	0	204	236	242
48	242,000	111,000	54,000	48	580,000	160,000	94,000
96	1,890,000	1,130,000	820,000	96	2,400,000	550,000	470,000
		Culture 65				Culture 335	
0	151	165	147	0	23	29	22
48	540,000	480,000	163,000	48	390,000	195,000	35,000
96	1,920,000	1,320,000	650,000	96	1,380,000	1,150,000	560,000
		Culture 110				Culture 354	
0	270	314	295	0	68	55	77
48	750,000	160,000	67,000	48	910,000	740,000	390,000
96	2,050,000	880,000	520,000	96	1,950,000	1,260,000	970,000
		Culture 173				Culture 376	
0	55	63	42	0	360	390	340
48	490,000	95,000	43,000	48	710,000	165,000	31,000
96	1,850,000	420,000	250,000	96	1,350,000	1,170,000	650,000
		Culture 241				Culture 447	
0	73	68	81	0	380	320	410
48	360,000	142,000	51,000	48	2,300,000	540,000	175,000
96	1,360,000	910,000	330,000	96	6,100,000	1,170,000	540,000

Protein Breakdown in Skimmilk

The protein breakdown in skimmilk by fluorescent bacteria was studied with five representative cultures. Culture 273 developed an alkaline reaction in milk and was non-proteolytic, culture 142 produced an acid reaction in milk with no evidence of proteolysis, culture 376 digested milk slowly and developed an indol-like odor, culture 241 digested milk fairly rapidly and produced a brown color on nutrient agar, while culture 354 was actively proteolytic and was representative of the cultures that rapidly digested milk.

Approximately 125 ml. quantities of milk were sterilized in 6 ounce bottles and after cooling the weight of each bottle and its contents was recorded. Several bottles were then inoculated with each culture and incubated at 21°C. (and 5°C. with culture 354) for different periods. After incubation, the weight of each bottle was restored with distilled water, 2 ml. of glacial acetic acid was added and the bottle heated in boiling water for 10 minutes, with frequent agitation. The culture was then cooled and filtered through paper after which the following determinations were made on the serum: Total nitrogen, amino nitrogen, ammonia nitrogen (on certain cultures) and the nitrogen soluble or insoluble in trichloracetic acid, ethyl alcohol or phosphotungstic acid. The values were expressed as milligrams of nitrogen in 5 ml. of serum. The data, except for cultures 142 and 273, are presented in table 7.

Table 7. Protein Breakdown in Skimmilk.

Culture	Days held	Mg. nitrogen in 5 ml. serum: Total nitrogen	Nitrogen fractionated into sol. & insol. portions with: Trichloracetic acid: Sol.	Trichloracetic acid: Insol.	Ethyl alcohol: Sol.	Ethyl alcohol: Insol.	Phosphotungstic acid: Sol.	Phosphotungstic acid: Insol.	Amino nitrogen	NH_3 nitrogen
					Held at 21°C.					
Check	2	5.4	3.6	1.8	0.6	4.7	1.0	4.3	0.274	--
Check	14	5.3	3.6	1.6	0.5	4.7	1.0	4.3	0.267	--
241	2	5.5	3.8	1.6	0.5	4.9	0.9	4.4	0.275	--
241	5	5.6	4.0	1.4	0.7	4.8	1.1	4.6	0.299	--
241	14	5.6	4.3	1.2	0.9	4.7	1.4	4.1	0.719	.277
241	21	5.7	4.4	1.2	1.1	4.6	1.5	4.3	1.228	.701
354	2	5.9	4.1	1.9	0.9	4.8	1.2	4.6	0.612	--
354	5	6.2	4.7	1.4	0.8	5.3	1.4	4.7	3.639	--
354	14	6.8	5.2	1.4	1.0	5.7	1.9	4.8	3.222	.923
354	21	7.2	5.7	1.4	1.2	5.9	2.2	5.0	4.117	1.958
376	2	5.4	3.5	1.9	0.7	4.7	0.8	4.5	0.275	--
376	5	5.5	3.7	1.9	0.8	4.6	0.9	4.5	0.331	--
376	14	5.7	4.0	1.7	0.9	4.7	1.2	4.5	0.517	.270
376	21	5.9	4.1	1.7	1.1	4.8	1.6	4.3	0.686	.489
					Held at 5°C.					
Check	14	5.4	3.6	1.7	0.5	4.8	1.0	4.4	0.285	.194
Check	21	5.4	3.6	1.8	0.5	4.7	1.0	4.3	0.231	.174
354	5	6.0	4.2	1.6	1.2	4.8	1.2	4.8	0.245	--
354	14	6.3	4.6	1.6	1.4	4.9	1.7	4.5	0.743	.195
354	21	6.7	4.9	1.7	1.8	4.8	2.0	4.7	1.511	.597

As would be expected, cultures 142 and 273 produced no significant changes in the nitrogen fractions of the milk serum during 21 days incubation at 21°C. Cultures 241, 354 and 376 produced similar changes in the nitrogen fractions at 21°C., but the changes were the most extensive with culture 354. The changes produced were an increase in total nitrogen, amino nitrogen, ammonia nitrogen and the nitrogen fractions soluble in the three reagents used. Increases in total nitrogen, amino nitrogen and ammonia nitrogen were the most significant changes produced by the three organisms at 21°C. and with culture 354 these changes (except for ammonia nitrogen which was not determined) were very evident after 2 days. At 5°C., culture 354 showed similar, but less extensive changes in the nitrogen fractions.

Action on Butter of Selected Cultures of Fluorescent Bacteria

The general action of 52 cultures of fluorescent bacteria on butter was studied as follows: Five hundred ml. of sterilized cream to which 10 ml. of a milk culture of the test organism had been added was churned, washed and worked in sterile equipment using sterile water. Two per cent of sterile salt was added to a portion of the butter from each churning. With each test organism a churning was also made after adding 10 per cent butter culture to the cream, but the butter was

unsalted. All the samples of butter were placed in sterile petri dishes and held at 21°C. and the changes in flavor noted during 7 days; the unsalted butter made without the use of butter culture was also held at 1° to 3°C. and the changes in flavor noted during 28 days. The acidity of the fat in the unsalted samples prepared without the use of butter culture was determined at once and after 7 days at 21°C. The acidity corresponds to the milliliters of N/10 potassium hydroxide required to neutralize the acid in 10 gm. of fat. The data are given in table 8.

In unsalted butter made without butter culture, 47 of the 52 cultures produced some type of flavor defect during 7 days at 21°C. The off-flavors were usually evident after 2 days but sometimes did not appear until near the end of the holding period. At 1° to 3°C., off-flavors were produced by 21 cultures during the 28 days that the samples were held. The addition of butter culture to the cream or the addition of salt to the butter prevented the development of flavor defects in the butter at 21°C. by some organisms but not by others. The off-flavors did not develop as rapidly and were usually not as pronounced, however, when butter culture or salt was used as when the butter was made without these ingredients. When butter culture was added to the cream, 36 of the 52 cultures were detrimental to the flavor of the butter during 7 days holding at 21°C., while in salted butter 25 cultures produced flavor defects.

Table 8. Action on Butter of Selected Cultures of Fluorescent Bacteria.

Culture	Flavor of butter after holding				Acidity of fat	
	28 days at 1° to 3°C.	7 days at 21°C.			Original butter	Butter held 7 days at 21°C.
			10% butter culture in cream	2% salt in butter		
1	good	putrid	sl. putrid	good	0.70	2.20
5	sl. putrid	putrid, rancid	sl. putrid	good	0.70	3.40
10	good	sl. putrid	unclean	good	0.70	0.75
20	good	cheesy	sl. rancid	good	0.70	1.30
23	good	putrid, rancid	sl. putrid	good	0.70	3.10
29	good	cheesy	unclean	good	0.70	1.60
36	good	putrid, rancid	sl. putrid	good	0.70	2.70
61	sl. rancid	rancid	sl. rancid	sl. rancid	0.80	7.60
69	good	sl. putrid	good	good	0.80	1.00
83	sl. putrid	putrid	putrid	sl. putrid	0.80	2.30
99	sl. rancid	rancid	rancid	rancid	0.80	4.40
109	sl. putrid	putrid	putrid	putrid	0.80	2.50
120	good	putrid	good	good	0.80	0.80
134	sl. rancid	rancid	sl. rancid	sl. rancid	0.33	4.60
142	good	unclean	good	good	0.33	0.35
151	sl. putrid, sl. rancid	fruity, rancid	good	sl. putrid, sl. rancid	0.33	1.65
160	unclean	putrid, fruity	unclean	unclean	0.33	0.35
174	good	unclean, fruity	unclean	unclean	0.33	0.60
187	rancid	rancid	sl. rancid	sl. rancid	0.33	10.35
196	rancid	rancid	rancid	rancid	0.33	16.90
205	rancid	rancid	sl. rancid	sl. rancid	0.33	8.40
217	good	fruity	fruity	fruity	0.80	0.90
223	sl. putrid	putrid	good	good	0.80	2.70
241	rancid	rancid	sl. rancid	sl. rancid	0.80	10.60
252	good	rancid	good	good	0.80	3.00

Table 8 (Continued)

Culture	Flavor of butter after holding: 28 days at 1° to $3^{o}C$.	Flavor of butter after holding: 7 days at $21^{o}C$.	7 days at $21^{o}C$.: 10% butter culture in cream	7 days at $21^{o}C$.: 2% salt in butter	Acidity of fat: Original butter	Acidity of fat: Butter held 7 days at $21^{o}C$.
259	rancid	rancid	sl. rancid	sl. rancid	0.80	12.00
262	good	rancid	rancid	good	0.33	4.00
266	good	cheesy, rancid	good	good	0.33	2.30
268	good	fruity, rancid	fruity,rancid	good	0.33	1.05
270	good	good	good	good	0.33	0.54
281	sl. rancid	fruity,putrid, rancid	putrid,rancid	sl. putrid, sl. rancid	0.33	1.95
285	good	good	good	good	0.33	0.30
291	fruity	putrid,fruity	sl. putrid	sl. putrid	0.33	0.32
299	good	putrid,rancid	putrid,rancid	rancid	0.43	3.75
303	good	putrid,bitter	good	sl. rancid	0.43	0.95
309	rancid	rancid	rancid	rancid	0.43	11.95
313	good	good	good	good	0.43	0.45
324	good	good	good	good	0.43	0.45
328	good	good	good	good	0.43	0.55
335	good	fruity	fruity	good	0.43	0.45
346	good	putrid	good	good	0.43	0.65
354	sl. putrid, sl. rancid	putrid,rancid	sl. bitter	good	0.43	1.70
363	sl. rancid	putrid,bitter	sl. putrid	sl. putrid	0.43	2.70
374	good	putrid,bitter	sl. putrid	sl. putrid	0.43	0.45
380	putrid	putrid,bitter	putrid	putrid	0.43	0.45
386	good	putrid,bitter	putrid	putrid	0.40	0.50
398	putrid,rancid	putrid,rancid	sl. rancid	good	0.40	2.20
453	good	rancid	sl. rancid	sl. rancid	0.40	5.20
481	good	rancid	sl. rancid	sl. rancid	0.40	2.70
490	good	putrid,bitter	sl. putrid	good	0.40	1.40
503	good	sl. cheesy	good	good	0.40	0.80
508	good	unclean	good	good	0.40	0.40

The flavors that developed in the samples of unsalted butter made without the addition of butter culture and held at 21°C. were putrid, cheesy or unclean (13), putrid and bitter (6), putrid and rancid (7), rancid (13), fruity and rancid, putrid or unclean (6) and fruity (2). More than one off-flavor was sometimes evident in individual butter samples and the flavors present often changed as the samples aged. Cheesy or slightly putrid flavors were present in certain samples when fairly fresh, but upon further aging only a rancid flavor was evident. The samples that developed a decided putrid flavor, however, continued to show this defect throughout the holding period even when rancidity was later produced. The putrid or putrid and bitter flavors were quite pronounced in some samples and were very offensive. Seven cultures that hydrolyzed corn oil and cottonseed oil and increased the acidity of the fat developed only a putrid flavor in the butter; presumably, the flavor from the free fatty acids was submerged by the pronounced putrid flavor. The rancid flavor was quite intense in the 13 samples that developed only this type of defect and was correlated with a high acid number of the fat. A fruity flavor occurred in several samples along with other flavors but two samples developed only this flavor.

The acidity of the fat was below 1.0 in 20 samples (none of which were rancid) between 1.0 and 2.5 in 13 samples (6 of which were slightly rancid), between 2.6 and 5.0 in 11 samples

(9 of which were rancid), between 5.1 and 10.0 in 3 samples (all of which were rancid), and above 10.0 in 5 samples (all of which were rancid).

Culture 291 produced a bluish-purple color on the surface of unsalted butter within 5 days at 21°C. The color darkened somewhat upon aging, becoming bluish-black after 2 to 3 weeks. The coloration did not extend more than 2 to 3 millimeters in the butter, but the entire surface of the butter in the container was affected.

Culture 346 developed a salmon-pink color in unsalted butter within 1 week at 21°C. The color extended throughout the entire butter mass and did not change as the sample aged.

Keeping Qualities of Fresh Sweet Cream Butter Containing Fluorescent Bacteria

One hundred and thirteen samples of freshly made sweet cream butter representing different churnings from several Iowa creameries were obtained at monthly intervals from January to May of 1938. Both unsalted and lightly salted samples, made with and without the use of butter culture, were included. The samples were collected by a marketing organization in sterile one-half pint milk bottles and sent to the laboratory. Soon after the samples arrived part of the butter in each bottle was removed for bacterial analysis and the remaining portion was examined for flavor. The butter was then

held at 21°C. and the flavor noted at 48 hour intervals for 6 to 8 days. At the end of the holding period an effort was again made to isolate fluorescent bacteria from the samples that had not previously yielded them.

Fluorescent bacteria were obtained from 39 (34.5 per cent) of the 113 samples examined; the organisms were isolated directly from 22 of the original butter samples while they were obtained only by enrichment procedures from the other 17 samples. The changes in flavor during the holding of the samples that yielded fluorescent bacteria and the numbers of non-butter culture organisms in the original butter are shown in table 9.

Nineteen of the 39 samples that contained fluorescent bacteria developed flavor defects at 21°C. during 6 or 8 days holding. With six samples, the off-flavors present (musty, stale, cheesy, bitter and unclean) were slight and were not evident until near the end of the holding period. One sample had a phenol flavor after 2 days which persisted during the remainder of the observation period but it is probable that this was not of bacterial origin. The flavor defects in the remaining 12 samples were evident after 2 or 4 days and were quite pronounced after 6 or 8 days. Three samples developed both a skunk odor and a rancid flavor but the skunk odor was found to be caused by a non-fluorescent organism. A pronounced rancid flavor also appeared in seven other samples; in four of these samples a cheesy flavor was evident after 2 to 4 days

Table 9. Keeping Qualities of Fresh Sweet Cream Butter Containing Fluorescent Bacteria.

Sample	No. non-butter culture org's. per ml.	Flavor of original butter	Flavor after holding at 21°C. for 2 days	4 days	6 days	8 days
1	21,100	butter culture	good	good	good	good
2	1,770	sl. coarse	good	good	good	good
3	9,000	good	good	good	good	good
4	17,500	butter culture	good	good	good	good
5	720	mild	good	good	good	good
6	150	coarse	good	good	good	good
7	1,900,000	sweet,unsalted	cheesy	rancid	rancid	rancid
8	14,500	good	good	good	good	good
9	780	good	good	good	good	sl. musty
10	85,000	sweet,unsalted	good	good	good	stale
11	150	good	good	good	good	good
12	1,400,000	sweet,unsalted	cheesy,rancid	rancid	rancid	--
13	5,800	sweet,sl. feed	phenol	sl.phenol	sl.phenol	--
14	2,500	butter culture	good	good	good	--
15	3,200	coarse	good	good	good	--
16	2,100,000	sweet,unsalted	good	good	good	good
17	1,800,000	sweet,unsalted	skunk	skunk	skunk,rancid	skunk,rancid
18	9,300,000	cooked, coarse	good	skunk	sl. rancid	rancid
19	2,500,000	cooked, coarse	good	skunk	skunk	skunk,rancid
20	4,900,000	sweet,unsalted	good	sl.cheesy	sl.cheesy	cheesy
21	4,200,000	sweet,unsalted	good	good	good	good
22	17,600,000	sweet,unsalted	good	good	good	good
23	12,000,000	feed	sl. rancid	sl.rancid	rancid	rancid

Table 9 (Continued)

Sample	No. non-butter culture org's. per ml.	Flavor of original butter	Flavor after holding at 21°C. for			
			2 days	4 days	6 days	8 days
24	12,000,000	feed	sl. rancid	rancid	rancid	rancid
25	9,500,000	sweet,unsalted	good	good	good	sl. cheesy
26	12,000,000	sweet,unsalted	sl. cheesy	rancid	rancid	rancid
27	23,000,000	sweet,unsalted	good	sl.bitter	sl.bitter	sl.bitter
28	18,000,000	sl. cooked	good	good	sl.bitter	--
29	3,100,000	sl. cooked	good	good	good	--
30	52,000,000	good	good	good	good	--
31	26,000,000	sl. cooked	sl. unclean	sl.unclean	sl.unclean	--
32	43,000	ripened;fair	good	good	good	--
33	31,000,000	good	sl. cheesy	cheesy; rancid	cheesy, rancid	--
34	27,500,000*	gross	cheesy,rancid	rancid	rancid	--
35	16,800,000	gross	good	good	good	--
36	230,000	fair	good	good	good	--
37	4,100,000	good	good	good	good	--
38	35,000,000	fair	good	sl.unclean	sl.unclean	--
39	44,000,000	good	good	good	good	--

*mostly fluorescent bacteria.

but was later replaced by the rancid flavor. A pronounced cheesy flavor unaccompanied by rancidity was noted in only one sample. The results indicate that rancidity is the most important defect produced in butter by the fluorescent bacteria.

The number of non-butter culture organisms in the 12 samples that developed pronounced flavor defects on holding ranged from 1,400,000 to 31,000,000 per ml. in the original butter; similar counts on the samples that developed only slight flavor defects ranged from 780 to 35,000,000 bacteria per ml. One sample that developed a cheesy and rancid flavor contained 27,500,000 non-butter culture organisms per ml. in the fresh butter, most of which were fluorescent.

Growth of Fluorescent Bacteria in Water on Cottage Cheese

Cottage cheese is frequently placed in milk cans at dairy plants and covered with water, then held in this condition at a relatively low temperature until needed for market. Since fluorescent bacteria often are present in water supplies and usually grow at 3° to 6°C., they may be a factor in the deterioration of the cheese when it is held in water.

Samples of freshly made cheese were obtained from six different dairy plants and held under water in a refrigerator

for several days to ascertain whether the fluorescent bacteria grew and contributed to the deterioration of the cheese. The cheese was placed in gallon containers and covered with water from the plant where it was made, since each plant had a different water supply. The flavor of the cheese was examined when first received and after 3, 6 and 10 days at 3° to 6°C. The water on the cheese was plated on beef infusion agar originally and after 3, 6 and 10 days; the plates were incubated 3 days at 21°C. and the numbers of non-butter culture organisms and fluorescent bacteria counted. The pH of the water on the cheese samples was determined at the beginning and after 10 days holding. The data are given in table 10.

Five samples of the cheese developed a bitter, astringent or putrid flavor after 3 or 6 days but one sample showed no off-flavor even after 10 days. Fluorescent bacteria were found in the water on five samples each time that a bacterial analysis was made, but in only two samples did they increase to any great extent during the holding. The numbers of fluorescent bacteria in the water on the fresh samples that contained these organisms ranged from 10 to 100 per ml., while after 10 days holding at 3° to 6°C., the fluorescent counts ranged from 2,500 to 1,600,000 per ml. Because of the relatively small numbers present, it seems probable that the fluorescent bacteria were not a factor in the deterioration of the cheese, except possibly in samples 4 and 5 where they may have been partly responsible for the off-flavors that de-

Table 10. Growth of Fluorescent Bacteria in Water on Cottage Cheese.

Sample	Days held at 3°-6°C.	Flavor of cheese	pH of water on cheese	Bacteria per ml. in water on cheese: Non-butter culture	Fluorescent
1	0	good	5.26	3,100	100
	3	good		3,000	600
	6	sl. bitter		1,600,000	23,000
	10	putrid, sl. bitter	5.50	13,200,000	64,000
2	0	good	5.09	300	0
	3	good		300	0
	6	good		900	0
	10	good	5.10	95,000	0
3	0	good	4.60	3,400	10
	3	sl. bitter		3,300	30
	6	sl. bitter		13,700	760
	10	putrid, sl. bitter	4.45	250,000	11,000
4	0	good	5.63	5,000	50
	3	astringent		6,000	8,000
	6	astringent		370,000	42,000
	10	bitter,putrid	5.30	29,000,000	1,500,000
5	0	good	5.47	12,000	100
	3	good		380,000	1,300
	6	sl. putrid		1,420,000	54,000
	10	putrid	5.48	19,500,000	1,600,000
6	0	good	4.41	11,000	100
	3	sl. bitter		20,000	300
	6	bitter		29,000	1,200
	10	astringent	4.42	42,000	2,500

veloped. The pH of the water on the cheese samples varied from 4.41 to 5.63 when the samples were fresh and from 4.42 to 5.48 after 10 days at 3° to 6°C. The relatively low pH of the water may have been responsible for the comparatively slow growth of the fluorescent bacteria.

Species of Fluorescent Bacteria

An attempt was made to identify, on a species basis, the 496 cultures of fluorescent bacteria isolated from various dairy products, water and miscellaneous sources. The organisms were all placed in the genus *Pseudomonas*, although it is possible that some of them would have been placed in the genus *Phytomonas* if their pathogenicity for plants had been determined and considered in the classification. Several investigators (Burkholder, 1930; Smith and Fawcett, 1930; Lacey, 1931, 1932; Clara, 1934; Harris, Naghski, Farrell and Reid, 1939; and Dowson, 1939) have called attention to the close similarity between certain fluorescent species in the genus *Phytomonas* and certain fluorescent species in the genus *Pseudomonas*. The phytopathogenic properties of the organisms are of no significance, however, from the standpoint of their importance in the dairy industry and the problem of classification is simplified when they are all placed in one genus.

Considerable difficulty was encountered in identifying many of the cultures from the descriptions given of the var-

ious species by Bergey, Breed, Murray and Hitchens (1939). The ability to produce indol is an important characteristic for separating the different species according to the classification system used by these investigators. However, 495 of the 496 cultures isolated in this study failed to form indol when a reliable method was used for its detection. Sandiford (1937) called attention to the fact that certain reagents sometimes give a false positive test for indol, particularly with certain organisms and showed that Ps. aeruginosa does not produce this compound. It is possible that other species in the genus Pseudomonas that are considered as indol positive are, likewise, indol negative. The description given certain species is also inadequate for careful identification and certain species listed show such close similarities that it is difficult to distinguish between them. The cultures isolated and studied in this investigation were identified as carefully as it was possible from the information given in "Bergey's Manual of Determinative Bacteriology" (1939), but there might be some doubt as to whether certain cultures were placed in the proper species.

Sixteen cultures conformed to the general description of Pseudomonas aeruginosa except that acid was produced from dextrose (16), xylose (16), galactose (16), mannose (16), and arabinose (9). Indol was not produced; nitrates were reduced to nitrites by some cultures and to nitrogen by others; all cultures were hemolytic; a green fluorescent pig-

ment and a blue, chloroform soluble, non-fluorescent pigment (pyocyanine) was produced on suitable media; litmus milk was rapidly digested with the development of an amber colored solution and an indol-like odor; the cultures grew at 7° and at 45° but not at 3°C.

Seven cultures closely resembled the characteristics of Pseudomonas schuylkilliensis except that they did not produce indol and one culture grew at 37°C. Litmus milk first developed an alkaline reaction with a pink ring above and partial reduction below followed by slow digestion; nitrates were not reduced; a slight brown color was produced by some cultures on agar slopes; one culture was hemolytic; and acid was produced from dextrose (7), xylose (6), galactose (3), mannose (4) and arabinose (1).

Seventy-three cultures were identified as Pseudomonas fluorescens. Litmus milk was rapidly digested; nitrates were reduced to nitrites; all cultures grew at 3° and at 32° but only three grew at 37°C.; one culture was hemolytic; and acid was produced from dextrose (72), xylose (71), galactose (62), mannose (23), sucrose (6), levulose (6), and arabinose (4).

Twenty-five cultures were identified as Pseudomonas rugosa. Gelatin was not liquefied; nitrates were not reduced; litmus milk was acid coagulated; red blood cells were not hemolyzed; and acid was produced from dextrose (25), xylose (24), galactose (22), maltose (14), mannose (11),

arabinose (7), and sucrose (1). Ten additional cultures conformed to the above characteristics except that they slowly liquefied gelatin. Acid was produced from dextrose (10), xylose (1), galactose (9), mannose (9), maltose (3), and arabinose (1).

Forty-four cultures closely resembled the characteristics of Pseudomonas incognitus except that they did not reduce nitrates which conforms to the original designation of this species by Wright (1895), but does not conform to the characteristics given by Bergey, Breed, Murray and Hitchens (1939). Gelatin was not liquefied; nitrates were not reduced; red blood cells were not hemolyzed; litmus milk slowly developed an acid reaction without coagulation and was reduced only at the base of the tube; and acid was produced from dextrose (44), xylose (44), galactose (43), mannose (29), maltose (18) and arabinose (10). Five other cultures resembled the above 44 cultures except that they reduced nitrates to nitrites. These cultures produced acid from dextrose (5), xylose (5), galactose (5), mannose (1) and arabinose (1).

Five cultures were identified as Pseudomonas putida but they did not agree in all respects with the description of this species. Litmus milk first showed an alkaline reaction and later slight digestion; gelatin was slowly liquefied; red blood cells were not hemolyzed; nitrates were reduced to nitrites or nitrogen; and acid was produced from

dextrose (5), xylose (5), galactose (4) and mannose (1).

Four cultures resembled the characteristics of Pseudomonas segne rather closely except that they did not soften agar and the color of the growth on agar slants was orange-brown, instead of orange-yellow. Gelatin was not liquefied; litmus milk developed an alkaline reaction; nitrates were not reduced; and acid was produced from dextrose (3), xylose (3), galactose (3), arabinose (3) and mannose (2).

Two cultures were identified as Pseudomonas ureae. Litmus milk was digested; hydrogen sulphide was formed; nitrates were reduced to nitrogen; red blood cells were not hemolyzed; growth occurred at 1° to 3°C.; and acid was produced from dextrose (2) and galactose (2).

Three cultures conformed to the description of Pseudomonas denitrificans. Gelatin was not liquefied; nitrates were reduced to nitrogen; red blood cells were not hemolyzed; litmus milk developed an alkaline reaction; and acid was produced from dextrose (3), galactose (3), xylose (2) and arabinose (2).

Sixty-eight cultures conformed to the description of Pseudomonas non-liquefaciens except that the cells were motile. Gelatin was not liquefied; nitrates were not reduced; red blood cells were not hemolyzed; litmus milk slowly developed an alkaline reaction; and acid was produced from dextrose (67), xylose (63), galactose (52), mannose (40), arabinose (15), maltose (2), levulose (2) and sucrose (1).

Two hundred and thirty-four cultures could not be identified on the basis of the species described by Bergey, Breed, Murray and Hitchens (1939). All but three of these cultures resembled <u>Ps. fluorescens</u> in their action on litmus milk but differed from this species in their action on potassium nitrate and in their ability to hemolyze red blood cells. The other three cultures digested milk and produced a water soluble, brown pigment and presumably should be placed in a separate species. The cultures were divided into four groups but no attempt was made to give them a species name.

Sixty-one cultures were placed in Group I. Litmus milk was rapidly digested; nitrates were reduced to nitrogen; one culture was hemolytic; hydrogen sulphide was not produced; and acid was produced from dextrose (60), xylose (57), galactose (44), mannose (19), arabinose (16) and levulose (2).

One hundred and thirty-four cultures were placed in Group II. Litmus milk was rapidly digested; nitrates were not reduced; hydrogen sulphide was produced by nine cultures; red blood cells were not hemolyzed; and acid was produced from dextrose (119), xylose (132), galactose (84), mannose (55), levulose (8), sucrose (8), arabinose (4) and maltose (1).

Thirty-six cultures were placed in Group III. Litmus milk was rapidly digested; nitrates were not reduced; red blood cells were hemolyzed; hydrogen sulphide was produced

by two cultures; and acid was produced from dextrose (30), xylose (35), galactose (26), mannose (8), arabinose (1), sucrose (1) and levulose (1).

Three cultures that produced a water-soluble, brown pigment were placed in Group IV. Litmus milk was rapidly digested; nitrates were not reduced; red blood cells were not hemolyzed; hydrogen sulphide was produced by one culture; and acid was not produced from dextrose (3), xylose (3), galactose (1), mannose (1) and arabinose (1).

Key to the Identification of Fluorescent Bacteria

A. Milk rapidly digested

a. Nitrates reduced to nitrites or nitrogen
b. Pyocyanine produced
Pseudomonas aeruginosa
bb. Pyocyanine not produced
c. Nitrates reduced to nitrites
Pseudomonas fluorescens
cc. Nitrites reduced to nitrogen
d. Hydrogen sulphide produced
Pseudomonas ureae
dd. Hydrogen sulphide not produced

Group I

aa. Nitrates not reduced
b. Brown pigment produced

Group IV

bb. Brown pigment not produced
c. Red blood cells hemolyzed

Group III

cc. Red blood cells not hemolyzed

Group II

B. Milk slightly alkaline followed by slow digestion

a. Nitrates reduced to nitrites or nitrogen
Pseudomonas putida
aa. Nitrates not reduced
Pseudomonas schuylkilliensis

C. Acid reaction developed in milk

a. Milk coagulated
Pseudomonas rugosa
aa. Milk not coagulated
Pseudomonas incognitus

D. Alkaline reaction without digestion developed in milk

a. Orange-brown pigment produced
Pseudomonas segne
aa. Orange-brown pigment not produced
b. Nitrates reduced to nitrogen
Pseudomonas dentifricans
bb. Nitrates not reduced
Pseudomonas non-liquefaciens

SUMMARY

The attempts to isolate fluorescent bacteria from dairy products were much more successful when enrichment procedures, which involved holding the samples at a low temperature for several days before plating, were followed instead of plating the fresh products.

Fluorescent bacteria were widely distributed in all dairy products examined, except sour cream and ice cream. The organisms likewise were commonly found in water from various sources and in miscellaneous materials, including common dairy feeds.

The fluorescent organisms isolated from dairy products and other sources were regularly gram-negative, non-spore-forming rods with polar flagella.

The fluorescigenic ability of the cultures studied was affected by a number of factors, the most important being the composition and pH of the medium, the oxygen supply and the incubation temperature.

Absorption spectra studies indicated that the fluorescent pigment produced by bacteria is not fluorescin, fluorescein, or riboflavin, and that fluorescence in alkaline culture media is not due to the formation of diacetyl.

All the cultures of fluorescent bacteria grew at 3° to 7°C. and at 32°C., but many cultures failed to grow at 37°C., al-

though a few grew at 45°C.

The types of changes produced in litmus milk by the various fluorescent bacteria were (a) rapid proteolysis accompanied by a putrid odor, (b) rapid proteolysis with development of an amber colored solution and an indol-like odor, (c) alkaline reaction without proteolysis, (d) slight alkaline reaction followed by slow proteolysis, (e) slight alkaline reaction followed by an acid reaction but usually no coagulation and no reduction except at the bottom of the tube, and (f) formation of an acid ring with acid coagulation from the top down and reduction except at the surface; slight proteolysis and a May apple odor sometimes noted.

The cultures of fluorescent bacteria tested were still alive after 6 months in skimmilk at room temperature and after 6 months in unsalted butter at 1° to 3°C.

Milk cultures of fluorescent bacteria were regularly destroyed at a temperature of 57.2°C. for 30 minutes. The organisms, likewise, were not resistant to chlorine; in general they were destroyed within 5 minutes in water containing 10 parts per million available chlorine and within 2.5 minutes in water containing 15 parts.

Six per cent sodium chloride in beef extract-peptone broth inhibited the growth of some cultures of fluorescent bacteria but not of others; only a few cultures grew in broth containing 8 per cent sodium chloride.

All the cultures of fluorescent bacteria grew in beef extract-peptone broth over a pH range from 5.5 to 10.0 (the highest tried), while many cultures grew at a pH of 4.5 and four grew at a pH of 4.0. When the pH of the original broth was high the organisms tended to decrease it and when the pH was low they tended to increase it. The ten cultures tried, likewise, grew fairly rapidly in sterilized skimmilk adjusted to a pH of 5.0 and six of the cultures developed a bitter flavor in the milk after 96 hours at 21°C.

In skimmilk, the most proteolytic culture studied produced considerable increases in total nitrogen and amino nitrogen after 2 days at 21°C. and a considerable increase in ammonia nitrogen after 14 days, the first time that the ammonia content was determined. This culture also produced significant increases in total nitrogen and amino nitrogen within 14 days at 5°C.

In 7 days at 21°C., 47 of 52 cultures produced some type of flavor defect in unsalted butter churned (without butter culture) from sterile cream to which the organisms were added; at 1° to 3°C., 21 cultures produced flavor defects within 28 days. When butter culture was added to the cream before churning, flavor defects were produced in the butter held at 21°C. by 36 cultures, while when 2 per cent salt was added to butter made without butter culture, 25 cultures produced flavor defects. The off-flavors that developed in the unsalted butter made without the use of butter culture

were unclean, cheesy, putrid, bitter, rancid and fruity; a combination of two or more of these flavors occurred in some butter samples. Two cultures produced color defects in the unsalted butter made without butter culture within 5 to 7 days at 21°C., one causing a bluish-black and one a salmon-pink color.

Fluorescent bacteria were isolated from 39 (34.5%) of 113 samples of fresh sweet cream butter obtained from several different plants. Nineteen of the samples that contained fluorescent organisms developed flavor defects during 6 to 8 days at 21°C.; with 12 samples the off-flavors were pronounced and were evident after 2 to 4 days. Rancidity was the most important defect that developed. Both rancid and cheesy flavors occurred in some samples but a pronounced cheesy flavor unaccompanied by rancidity developed in only one sample.

Five out of six samples of cottage cheese, obtained from different plants and held in water at 3° to 6°C., contained fluorescent bacteria, but the organisms grew sufficiently in only two samples to be a possible factor in the deterioration of the cheese during 6 to 10 days.

Two hundred and sixty-two of the 496 cultures of fluorescent bacteria isolated were identified as belonging to 10 different species in the genus *Pseudomonas* but the other 234 cultures could not be identified on the basis of the

species described by Bergey, Breed, Murray and Hitchens (1939). Two hundred and thirty-one of the unidentified cultures resembled <u>Ps. fluorescens</u> except in their nitrate reducing and hemolytic abilities; the other three cultures were also proteolytic but produced a water soluble, brown pigment. The unidentified cultures were divided into Group I, Group II, Group III, and Group IV, but were not given species names.

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. B. W. Hammer for his counsel and suggestions in planning and directing the work herein reported and in the preparation of the manuscript; to Iowa State Brand Creameries, Inc., Mason City, Iowa for supporting the fellowship under which the work was partly conducted; to Dr. R. T. Major, Merck and Company for the riboflavin supplied; and to Dr. Victor R. Ells, Physics Department, University of Missouri for making the spectographic analysis.

LITERATURE CITED

Abbott, A. C. and Gildersleeve, N. 1903 A study of the proteolytic enzymes and of the so-called hemolysis of some of the common saprophytic bacteria. J. Med. Res. 10:42-62.

Arloing, F., Policard, A. and Langeron, L. 1925 Aspects offerts par diverses cultures microbiennes sous l'action des radiations émises par la lampe à arc au mercure aprés filtration par des écrans de wood. Compt. rend. Soc. biol. 92:261-263.

Barthel, C. 1910 Milk and Dairy Products. The Macmillan Co., New York, pp. 229-239.

Berger, Franz. 1926 Eine bakterielle Erkrankung bei Aquarienfischen durch Vertreter der Fluorescens-Gruppe. Centr. f. Bakt., Abt. 2, 69:1-3.

Bergey, D. H., Breed, Robert S., Murray, E. G. D. and Hitchens, A. Parker. 1939 Bergey's Manual of Determinative Bacteriology, 5th Ed. The Williams and Wilkins Co., Baltimore.

Berry, J. A. 1933 Detection of microbial lipase by copper soap formation. J. Bact. 25:433-434.

Bierema, Steven. 1909 Die Assimilation von Ammon,-Nitrat- und Amidstickstoff durch Mikroorganismen. Centr. f. Bakt., Abt. 2, 23:672-726.

Blanc, J. 1923 Transformation de Bacilles pyocyaniques en bacilles sans pigments. Essai d'interpretation. Compt. rend. Soc. biol. 88:52-54.

Blanchetière, A. 1917 Action du bacille fluorescent liquéfiant de Flügge sur l'asparagine en milieu chimiquement défini. Ann. Inst. Pasteur 31:290-312.

Blanchetière, A. 1920 Action du bacille fluorescent liquéfiant de Flügge sur certains amino-acids en milieu chimiquement défini. Bul. Soc. Chim. biol. 2:29-36.

Böhme, A. 1905-1906 Die Anwendung der Ehrlichschen Indolreaktion für bakteriologische Zwecke. Centr. f. Bakt., Abt. 1, 40:129-133.

Bonjean, Ed. 1899 The Bacille pyocyanique dans les eaux d'alimentation. Ann. hyg. publ. méd. légale, Series III, 42:28-51.

Breazeale, D. F. and Bird, E. W. 1938 A study of methods for the determination of acidity in butterfat. J. Dairy Sci. 21:335-344.

Breymann, Margarete. 1902 Ueber Stoffwechselprodukte des Bacillus pyocyaneus. Centr. f. Bakt., Abt. 1, 31: 481-502.

Burge, W. E. and Neill, A. J. 1915 The comparative rate at which fluorescent and non-fluorescent bacteria are killed by exposure to ultra-violet light. Am. J. Physiol. 38: 399-403.

Burkholder, W. H. 1930 The Genus Phytomonas. Phytopathology 20:1-23.

Callow, Anne Barbara. 1926 The heat-stable peroxidase of bacteria. Biochem. J. 20:247-252.

Charrin, M. A. et Dissard, A. 1893 Les propriétés du Bacille pyocyanogène en fonction des qualités nutritives du milieu. Compt. rend. Soc. biol. 45:182-186.

Charrin, M. A. et Phisalix 1892 Abolition persistante de la fonction chromogène du Bacillus pyocyaneus. Compt. rend. Soc. biol. 44:576-579.

Cherrington, V. A. and Gildow, E. M. 1931 Bovine mastitis caused by Pseudomonas aeruginosa. J. Am. Vet. Med. Assoc. 79:803-808.

Chester, F. D. 1901 A Manual of Determinative Bacteriology. The Macmillan Co., New York.

Clara, F. M. 1934 A comparative study of the green-fluorescent bacterial plant pathogens. Cornell Univ. Agr. Exp. Sta. Memoir 159.

Cluzet, J., Rochaix, A. et Kofman, T. 1921 Contribution à l'étude spectrophotographique des pigments du Bacille pyocyanique. J. physiol. path. gèn. 19:378-382.

Cone, J. Frank. 1938 Pseudomonas aeruginosa in bovine mastitis. J. Agr. Res. 58:141-147.

Crabill, C. H. and Reed, H. S. 1915 Convenient methods for demonstrating the biochemical activity of microorganisms, with special reference to the production and activity of enzymes. Biochem. Bul. 4:30-44.

Danielsen, Ernst. 1929-1930 Zur Unterscheidung von Bakterienkulturen mit Hilfe der Quarzlampe. Centr. f. Bakt., Abt. 1, 115:225-228.

Dewey, Bartlett T. and Poe, Charles F. 1938 Action of ultra-violet on members of the Pseudomonas fluorescens group. Proc. Soc. Exp. Biol. Med. 38:176-177.

Dowson, W. J. 1939 On the systematic position and generic names of the gram negative bacterial plant pathogens. Centr. f. Bakt., Abt. 2, 100:177-193.

Ducamp et Planchon. 1894 Note sur un bacille fluorescent et liquéfiant des eaux d'alimentation de Montpellier. Compt. rend. Soc. biol. 10:266-267.

Dupaix, A. 1930 Contribution à l'étude de B. caryocyaneus Beijerinck-Dupaix. Trav. lab. Microbiol. faculté de pharmacie Nancy 3:25-32.

Eicholz, W. 1902 Erdbeerbacillus (Bacterium fragi). Centr. f. Bakt., Abt. 2, 9:425-428.

Eijkman, C. 1901 Ueber Enzyme bei Bakterien und Schimmelpilzen. Centr. f. Bakt., Abt. 1, 29:841-848.

Elema, B. 1931 Studies on the oxidation-reduction of pyocyanine. Part II. Redox potentials of pyocyanine. Rec. trav. chim. 50:807-826.

Elema, B. 1933 Oxidation-reduction potentials of chlororaphine. Rec. trav. chim. 52:569-583.

Emmerling, O. und Reiser, O. 1902 Zur Kenntniss eiweissespaltender Bacterien. Ber. deut. Chem. Ges. 35:700-703.

Escherich, T. 1886 Die Darmbakterien des Saüglings und ihre Beziehungen zur physiologie der verdauung. Ferdinand Enke, Stuttgart.

Fermi, Claudio and Montesano, Giuseppe. 1895 Die von den Mikroben bedingte Inversion des Rohrzuckers. Centr. Bakt., Abt. 2, 1:482-487.

Flügge, C. 1886 Die Mikroorganismen. Vogel, F. C. W., Leipzig.

Folin, Otto. 1902-1903 Eine neue Methode zur Bestimmung des Ammoniaks im Harne und anderen thierischen Flüssigkeiten. Z. f. physiol. chem. 37:161-176.

Fordos, M. 1860 Recherches sur la matières colorantes des suppuration bleues:pyocyanine. Compt. rend. Acad. Sci. 51:215-217.

Fordos, M. 1863 Recerches sur les matières colorantes des suppuration bleues:pyocyanine et pyoxanthose. Compt. rend. Acad. Sci. 56:1128-1131.

Frost, William Dodge. 1904 The antagonism exhibited by certain saprophytic bacteria against the Bacillus typhosus Gaffky. J. Infect. Dis. 1:599-640.

Fuller, George W. and Johnson, George A. 1899 On the differentiation and classification of water bacteria. J. Exp. Med. 4:609-626.

Gassul, R. und Žolkevič, A. 1927 Ueber Differenzierungsversuche an Bakterienkulturen mit Hilfe der sogen. Woodschen Strahlen. Centr. f. Bakt., Abt. 1, 104:503-507.

Georgia, F. R. and Poe, Charles F. 1931 Study of bacterial fluorescence in various media. I. Inorganic substances necessary for bacterial fluorescence. J. Bact. 22: 349-361.

Georgia, F. R. and Poe, Charles F. 1932 Study of bacterial fluorescence in various media. II. The production of fluorescence in media made from peptones. J. Bact. 23: 135-145.

Gessard, M. C. 1882 Sur les colorations bleue et verte des linges à pansements. Compt. rend. Acad. Sci. 94:536-538.

Gessard, M. C. 1892 Sur la fonction Fluorescigène des microbes. Ann. Inst. Pasteur 6:801-823.

Gessard, M. C. 1901 Variété melanogène du B. pyocyanique. Ann. Inst. Pasteur 15:817-831.

Gessard, M. C. 1917 Variété erythrogène du Bacille pyocyanique. Compt. rend. Acad. Sci. 165:1071-1076.

Gessard, M. C. 1919 Variété achromogène du Bacille pyocyanique. Compt. rend. Acad. Sci. 168:1066-1067.

Gessard, M. C. 1919 Classement des germes pyocyaniques par les pigments. Compt. rend. Soc. biol. 82:795-798.

Gessard, M. C. 1920 Technique d'identification des germes pyocyaniques. Ann. Inst. Pasteur 34:88-97.

Gessard, M. C. 1920 Sur les Bacilles pyocyanoïdes. Compt. rend. Acad. Sci. 170:298-300.

Gessard, M. C. 1925 Bacilles pyocyanoïdes de la variété melanogène. Compt. rend. Acad. Sci. 180:972-974.

Gorbach, G. und Pirch, E. 1936 Ueber die Sekretion und die pH-Abhängigkeit der Bakterienproteasen. Enzymologia 1: 191-199.

Gorbach, G. und Pirch, E. 1937 Zur Frage der Proteinase-Sekretion durch gelatine-verflüssigende Bakterien. Enzymologia 2:92-95.

Goré, S. N. 1921 The cotton-wool plug test for indol. Indian J. Med. Res. 8:505-507.

Gruber, T. 1902 Pseudomonas fragariae. Eine Erdbeergeruch erzeugende Bakterie. Centr. f. Bakt., Abt. 2, 9:705-712.

Gruber, T. 1905 Eine weiterer Beitrag zur Aromabildung, speziell zur Bildung des Erdbeergeruches in der Gruppe "Pseudomonas." Pseudomonas fragariae II. Centr. f. Bakt., Abt. 2, 14:122-123.

Gubitz, H. 1928 Kältebakterien, mit besonderer Berücksichtigung ihrer Bedeutung für die Milchwirtschaft. Milchw. Forsch. 5:407-456.

Guignard et Sauvageau. 1894 Sur un nouveau microbe chromogène, le Bacillus chlororaphis. Compt. rend. Soc. biol. 46:841-843.

Haag, E. F. 1928 Die Zersetzung der Fette durch Bakterien. Arch. f. Hyg. 100:271-308.

Hansen, H. C. 1937 The influence of certain bacteria on the ripening of cheddar cheese made from pasteurized milk. B. W. Hammer Panegyric, Iowa State College Press Co., pp. 123-132.

Harden, Arthur and Norris, Dorothy. 1911 The diacetyl reaction for proteins. J. Physiol. 42:332-336.

Harris, R. G., Naghski, J., Farrell, M. A. and Reid, J. J. 1939 The relation of the soluble specific substances to virulence and specificity in bacterial leafspot organisms. J. Bact. 38:235-236.

Henneberg, W. 1931 Spoilage organisms in milk, butter and cheese most common in Germany. Ninth Int. Dairy Congress, Denmark. Report of the Delegation of the United States to the Secretary of State, p. 52.

Henneberg, W. 1931-1932 Zur Kenntnis der Alkalibildner in der Milch. Milchw. Forsch. 12:222-235.

Hiscox, E. R. 1936 A pigment-producing organism (Pseudomonas sp.) isolated from discolored butter. J. Dairy Res. 7:238-243.

Hueppe, Ferdinand. 1884 Untersuchungen über die Zersetzungen der milch durch Mikroorganismen. Mitt. aus dem Kaiserlichen Gesundheitsamte 2:309-371.

Hussong, R. V., Long, H. F. and Hammer, B. W. 1937 Classification of the organisms important in dairy products. II. Pseudomonas fragi. Iowa Agr. Exp. Sta. Res. Bul. 225.

Jirou, J. M. 1901 Sur les bacilles fluorescents et le pyocyanique. J. physiol. path. gèn. 3:188-199.

Jong, L. E., den Dooren de. 1926 Beijdrage tot de Kennis van het Mineral-process. Delft. Cited by Buchanan, R. E. and Fulmer, Ellis, I., Physiology and Biochemistry of Bacteria 2:436-438, 446-449; 3:223, 241, 244, 245, 266. The Williams and Wilkins Co., Baltimore, 1930.

Jordan, Edwin O. 1897 Bacillus pyocyaneus and its pigments. J. Exp. Med. 4:627-647.

Jordan, Edwin O. 1897-1899 The production of fluorescent pigment by bacteria. Trans. Chicago Path. Soc. 3:507.

Jordan, Edwin O. 1903 The connection between the alkalinity of certain bacterial filtrates and their hemolytic power. J. Med. Res. 10:31-41.

Kendall, A. I., Day, A. A. and Walker, A. W. 1914 Studies in bacterial metabolism. XXXIV. The metabolism of the Subtilis mesentericus group and B. pyocyaneus in milk. J. Am. Chem. Soc. 36:1947-1950.

Kendall, A. I., Day, A. A. and Walker, A. W. 1914 Studies in bacterial metabolism. XXXVII. The metabolism of certain bacteria in skimmed milk, whole milk and cream. J. Am. Chem. Soc. 36:1956-1962.

Kendall, A. I., Day, A. A. and Walker, A. W. 1914 Studies in bacterial metabolism. XXXVIII. Observation on fat-splitting in milk by bacterial lipase. J. Am. Chem. Soc. 36:1962-1966.

Kögel, F. and Postowsky, J. J. 1930 Über das grüne Stoffwechselprodukt des Bacillus chlororaphis. Ann. der Chemie 480:280-297.

Kraaij, G. M. and Wolff, L. K. 1923 Over de splitsing van lipoïden door bacteriën. Versl. K. Akad. Wetensch., Amsterdam 32:624-625.

Krueger, R. 1890 Bakteriologisch-chemische Untersuchung käsiger Butter. Centr. f. Bakt., Abt. 1, 7:425-430, 464-469, 493-496.

Kruyff, E. De. 1908 Les bactéries hydrolysant et oxydant les graisses. Centr. f. Bakt., Abt. 2, 20:610-611. Bul. Dutch East Indies Landbouw. Dept. 9:1-12, 1907.

Labrousse, F. 1934 The function fluorescigène chez les Bacteries phytopathogènes. Compt. rend. Acad. Sci. 194: 2173-2175.

Lacey, Margaret S. 1931 Studies in bacteriosis. XIX. Researches on the group of green-fluorescent bacteria, Part I: Bacterium trifoliorum (Jones et al.) as the cause of a disease of Vicia faba. Ann. Applied biol. 18: 180-186.

Lacey, Margaret S. 1932 Studies in bacteriosis. XIX. Researches on the group of green-fluorescent bacteria. Part II: On some plant diseases caused by bacteria of the green-fluorescent group, and a comparison and discussion of various cultural characteristics of certain members of this group. Ann. Applied biol. 19:190-203.

Lafar, Franz. 1891 Bakteriologische Studien über Butter. Arch. f. hyg. 13:1-39.

Lagrange, E. 1927 Production expérimentale de la Gomme du Bacille fluorescent. Compt. rend. Soc. biol. 97: 1047-1049.

Lagrange, E. 1928 Dissociation microbienne des bacilles fluorescents et production expérimentale de ces variations. J. Hyg. 28:133-138.

Lane, C. B. and Hammer, B. W. 1935 Bacteriology of Cheese. I. Effect of pasteurizing the milk on the nitrogenous decomposition in cheddar cheese. Iowa Agr. Exp. Sta. Res. Bul. 183.

Lartigau, August Jerome. 1898 A contribution to the study of the pathogenesis of the Bacillus pyocyaneus, with special reference to its relation to an epidemic of dysentery. J. Exp. Med. 3:595-609.

Lasseur, Ph. 1909 Le Bacillus chlororaphis et la chlororaphine. Compt. rend. Soc. biol. 66:272-273.

Lasseur, A. Phillippe. 1911 Contribution à l'étude de Bacillus chlororaphis G. et S. Thesis, Univ. of Nancy.

Lasseur, Ph. 1911 Le Bacillus chlororaphis. Influence du fer sur la production de la chlororaphine. Compt. rend. Soc. biol. 70:154-156.

Lasseur, Ph., Dompray, P. et Dupaix, A. 1931 Relation entre la concentration en ions hydrogène des milieus de culture, la végétation et la chromogènése bacteriénne. Trav. lab. Microbiol. faculté pharmacie Nancy 4:13-30.

Lasseur, Ph. et Dupaix-Lasseur, A. 1934 Colonies "R" et colonies "S" chez. B. chlororaphis. Trav. lab. Microbiol. faculté pharmacie Nancy 7:69-71.

Lasseur, Ph. et Dupaix-Lasseur, A. 1936 Influence du pH sur l'aspect des cultures de B. chlororaphis type Ra, B. chlororaphis type Rb_1, B. chlororaphis type Rb_2, B. chlororaphis type S. Trav. lab. Microbiol. faculté pharmacie Nancy 9:35-42.

Lasseur, Ph., Dupaix, A. et Lacaille, R. 1931 Fluorescence des corps microbiens. Trav. lab. Microbiol. faculté pharmacie Nancy 4:85-95.

Lasseur, Ph., Dupaix, A. et Marchal, J. G. 1931 Étude du pouvoir proteolytique de B. caryocyaneus. Beijernick-Dupaix et B. pyocyaneus Gessard dans des milieux de pH croissants. Trav. lab. Microbiol. faculté pharmacie Nancy 4:63-71.

Lasseur, Ph., Dupaix-Lasseur, A. et Marchal, J. G. 1936 Étude sur la production des substances fluorescentes dans les cultures bacteriénnes. Trav. lab. Microbiol. faculté pharmacie Nancy 9:85-103.

Lasseur, Ph., Dupaix-Lasseur, A. et Palgen, W. 1937 Étude comparative de la fluorescence des cultures de B. caryocyaneus Beijernick-Dupaix type Rb et type S. Trav. lab. Microbiol. faculté pharmacie Nancy 10:23-29.

Lasseur, Ph., Dupaix-Lasseur, A. et Marchal, J. G. 1937 Dissociation de B. pyocyaneus Gessard. B. pyocyaneus pyocyanogènes. B. pyocyaneus erythrogènes. Trav. lab. Microbiol. faculté pharmacie Nancy 10:42-46.

Lasseur, Ph., Fribourg, R. et Grojean, M. 1932 Culture de quelques Bactéries chromogènes en milieus synthetiques additionnes d'indicateurs. Trav. lab. Microbiol. facultè pharmacie Nancy 5:125-140.

Lasseur, Ph., Marchal, J. et Dupaix, A. 1930 Modifications temporaires modifications durable observeès chez les Bactéries. Trav. lab. Microbiol. faculté pharmacie Nancy 3:105-158.

Lasseur, Ph., Marchal, J. G., Dupaix, A. et Renaux, M. 1932 Observations sur la chromogènés de B. cyaneo-fluorescens. Zangemeister. Trav. lab. Microbiol. faculté pharmacie Nancy 5:111-123.

Lasseur, Ph. et Marion, C. 1936 Influence du temps sur la transformation des types "R" en "S". Trav. lab. Microbiol. faculté pharmacie Nancy 9:83-84.

Lasseur, Ph. et Marion, C. 1937 Action de substances tensio-actives sur les cultures de B. aurantiacus tingitanus Renlinger et Bailey et de B. caryocyaneus Biejernick-Dupaix. Trav. lab. de Microbiol. faculté pharmacie Nancy 10:30-41.

Lasseur, Ph., Oliver, M., Dupaix, A. et Maguitot, C. 1930 Relation entre le pH des milieux de culture et le pouvoir chromogene de B. caryocyaneus Beijernick-Dupaix. Trav. lab. Microbiol. faculté pharmacie Nancy 3:65-74.

Lasseur, Ph., Thiry, G. et Dupaix, A. 1930 Le fer dans la vie microbienne. Trav. lab. Microbiol. faculté pharmacie Nancy 3:53-66.

Lasseur, Ph., Thiry, G., Dupaix, A. et Oliver, M. 1930 Influence du phosphore et du magnesium sur la fonction fluorescigène du B. caryocyaneus Beijernick-Dupaix. Trav. lab. Microbiol. faculté pharmacie Nancy 3:32-51.

Laxa, Otto. 1901-1902 Ueber die Spaltung des Butterfettes durch Mikroorganismen. Arch. f. Hyg. 41:119-151.

Ledderhose, G. 1888 Ueber den blauen Eiter. Deutsche Z. f. Chir. 28:201-230.

Lehmann, K. B. und Neumann, R. O. 1896 Atlas und Grundriss der Bakteriologie. J. F. Lehmanns, Munchen.

Lepierre, M. C. 1895 Recherches sur la fonction fluorescigène des microbes. Ann. Inst. Pasteur 9:643-663.

Lesage, Adolphe. 1888 Du bacille de la diarrhée verte des enfants du premier age. Arch. de physiol. 20:212-235.

Lewis, I. M. 1929 Bacterial antagonisms with special reference to the effect of Pseudomonas fluorescens on spore forming bacteria of soils. J. Bact. 17:89-101.

Löhnis, F. and Kuntze, W. 1907-1908 Beiträge zur Kenntnis des Mikroflora des Stalldüngers. Centr. f. Bakt., Abt. 2, 20:676-687.

Löhnis, F. 1930 Handbuck Der Milchwirtschaft. II. Mykologie (Mikrobiologie) der milch. 1:162-163, 174-197, Julius Springer, Wien.

Long, H. F. and Hammer, B. W. 1936 Classification of the organisms important in dairy products. I. Streptococcus liquefaciens. Iowa Agr. Exp. Sta. Res. Bul. 206.

Long, H. F. and Hammer, B. W. 1937 Methods for the detection of lipolysis by microorganisms. Iowa State College J. Sci. 12:343-351.

Luxwalda, Wissi Beene. 1911 Wachstum und Wirkung einiger Milchbakterien bei verschiedenen Temperaturen. Centr. f. Bakt., Abt. 2, 31:129-175.

McCombie, Hamilton and Scarborough, Harold A. 1923 The chemical constitution of bacterial pigments. Part I. The isolation of pyocyanine and the preparation of its salts. J. Chem. Soc. 123:3279-3285.

Mammelle, A. 1918 Observations sur un nouvel enchantillon du Bacillus pyocyaneus erythrogène. Compt. rend. Soc. biol. 81:1137.

Marchal, J. G. 1937 Sur une nouvelle Bacterie chromogène des eux. Bacillus roseus fluorescens. N. Sp. J. G. Marchal 1937. Trav. lab. Microbiol. faculté pharmacie Nancy 10:90-99.

Martin, V. D., Naylor, N. M. and Hixon, R. M. 1939 Action of beta-amylase from soybeans on various substances. Cereal Chem. 16:565-573.

Maschmann, Ernst. 1937 Über Bakterienproteasen I. Biochem. Z. 294:1-33.

Meader, P. D., Robinson, G. H. and Leonard, V. 1925 Pyorubin, a red water-soluble pigment characteristic of B. pyocyaneus. Am. J. Hyg. 5:682-708.

Migula, W. 1900 System der Bakterien. Gustav Fisher, Jena.

Migula, W., Engler, A. und Prantl, K. 1895 Die natürlichen Pflazenfamilien, 1, 1a. Engelmann, W. Leipzig.

Müller, M. 1903 Ueber das Wachstum und die Lebenstätigkeit von Bakterien, sowie den oblauf fermentativer Prozesse bei niederer Temperature unter spezieller Beruchsichtigung des Fleisches als Nahrungsmittel. Arch. f. Hyg. 47:127-191.

Mundhenk, R. L. 1922 Bovine mammitis. North Am. Vet. 3: 101-102, 108.

Newman, R. W. 1930 Pseudomonas as a cause of bitter milk. Calif. Dept. Agr. Monthly Bul. 19, p. 640.

Nogier, T., Dufourt, A. et Dujol 1913 Contribution à l'étude des pigments du Bacillus pyocyaneus. J. physiol. path. gèn. 15:633-635.

Oker-Blom, Max. 1913 Über die Wirkungsart des ultravioletten Lichtes auf Bakterien. Z. f. Hyg. u. Infektionskrank. 74:242-247.

Olitsky, L. 1891 Über die antagonistichen Wirkung des Bacillus fluorescens liquefaciens und seine hygienische Bedeutung. Inaug. Diss. Berne, cited by Buchanan, R. E. and Fulmer, Ellis I., Physiology and Biochemistry of Bacteria 3:4, 1930. The Williams and Wilkins Co., Baltimore.

Orla-Jensen, S. 1902 Studien über das Ranzigwerden der Butter. Centr. f. Bakt., Abt. 2, 8:11-16, 42-46, 74-80, 107-114, 140-144, 171-174, 211-216, 248-252, 278-281, 309-312, 342-346, 367-369, 406-409.

Orla-Jensen, S., Otte, N. E. and Snog-Kjaer, Agnete. 1936 Der Vitaminbedarf verschiedener Bakterien ausserhalb der Gruppe der Milchsäurebakterien der Milch. Centr. f. Bakt., Abt. 2, 94:447-452.

Orla-Jensen, S. 1931 Dairy Bacteriology, 2nd Ed., p. 50. Translated by Arup, P. S.: P. Blakiston's Son and Co., Philadelphia.

Pickens, E. M., Welsh, M. F. and Poelma, L. J. 1926 Pyocyaneus bacillosis and mastitis due to Ps. aeruginosa. Cornell Vet. 16:186-202.

Pribram, Ernst and Pulay, Erwin. 1915 Beiträge zur Systematik der Mikroorganismen. I. Die Gruppe des Bacterium fluorescens. Centr. f. Bakt., Abt. 1, 76:321-329.

Pulvertaft, R. J. V. 1934 Bacterial fluorescence with ultraviolet light. J. Path. Bact. 38:355-362.

Quastel, J. J. 1924 On the fermentation of the unsaturated dicarboxylic acids. I. Fumaric acid. Biochem. J. 18: 365-380.

Rahn, Otto. 1906 Ueber den Einfluss der Stoffwechselprokdukte auf das Wachstum der Bakterien. Centr. f. Bakt., Abt. 2, 16:417-429, 609-617.

Ravenel, Mazÿck P. 1896 Notes on the bacteriological examination of the soil of Philadelphia. Memoirs Natl. Acad. Sci. 8:3-41.

Reinmann, R. Von. 1900 Untersuchungen über die Ursachen des Ranzigwerdens der Butter. Centr. f. Bakt., Abt. 2, 6: 131-139, 166-176, 209-214.

Robinson, G. L. 1932 The growth of B. pyocyaneus in synthetic media. Brit. J. Exp. Path. 13:310-317.

Rocha, A., Lepierre, C. and Fonseca, A. 1900 Un casa de fièvre infectieuse simulant la peste pneumonique produite par un bacilli fluorescent nouveau. Compt. rend. Soc. biol. 52:226-227.

Ropetti, Leonilde. 1938 L'analyse spectrale appliquée a' l'étude des fluorescences microbiennes. Ann. des fermentations 4:4247-4249.

Rumment, Julius. 1931 The effect of water on the keeping quality of butter. Ninth Int. Dairy Congress, Denmark. Report of the Delegation of the United States to the Secretary of State, p. 62.

Růžička, Stanislav. 1898 Vergleichende Studien über den Bacillus pyocyaneus und den Bacillus fluorescens liquefaciens. Arch. f. Hyg. 34:149-177.

Sandiford, B. R. 1937 Observations on Pseudomonas pyocyanea. J. Path. and Bact. 44:567-572.

Schmidt-Nielsen, Sigval. 1902 Über einige psychrophile Mikroorganismen und ihr. Vorkommen. Centr. f. Bakt., Abt. 2, 9:145-147.

Schreiber, Karl. 1901 Fettzersetzung durch Mikroorganismen. Arch. f. Hyg. 41:328-341.

Schroeter, J. 1872 Ueber einige durch Bacterien gebildete pigmente. Beitr. Biol. Pflanz 1[2]:109-126.

Sears, H. J. and Gourley, M. F. 1928 Studies in the carbohydrate metabolism of Ps. aeruginosa (B. pyocyaneus). J. Bact. 15:357-366.

Shaffer, Philip A. and Somogyi, Michael. 1933 Copper-iodometric reagents for sugar determination. J. Biol. Chem. 100:695-713.

Sherwood, Noble, P., Johnson, T. L. and Radotincky, Ida. 1926 Studies on Bacillus pyocyaneus. Univ. Kansas Sci. Bul. 16:91-99.

Shutt, D. B. 1928-1929 Contaminated water as a source of surface flavor in pasteurized creamery butter. Sci. Agr. 9:316-320.

Smith, Clayton O. and Fawcett, Howard S. 1930 A comparative study of the citrus blast bacterium and some other allied organisms. J. Agr. Res. 41:233-246.

Söhngen, N. L. 1910 Vetsplitsing door bakteriën. Versl. K. Akad. Wetensch., Amsterdam 19:689-704.

Söhngen, N. L. 1913 Benzin, Petroleum, Paraffinöl und Paraffin als Kohlenstoff-und Energieque für Mikroben. Centr. f. Bakt., Abt. 2, 37: 595-609.

Stark, C. N. and Scheib, B. J. 1936 A study of fat splitting and casein digesting bacteria isolated from butter. J. Dairy Sci. 19:191-213.

Storck, Wennemar. 1936 Beitrag zur Kenntnis der Alkalibildner in Rohmilch. Centr. f. Bakt., Abt. 2, 94: 295-330.

Sullivan, M. X. 1905 Synthetic culture media and the biochemistry of bacterial pigments. J. Med. Res. 14: 109-160.

Supniewski, J. 1923 Recherches sur la transformation des combinaisons carbonies par le Bacille pyocyanique. Compt. rend. Soc. biol. 89:1377-1379.

Supniewski, J. 1924 Untersuchungen über den stoffwechsel der Stickstoffverbindungen in den Kulturen von Bacillus pyocyaneus. Biochem. Z. 154:98-103.

Tanner, F. W. 1918 A study of green fluorescent bacteria from water. J. Bact. 3:63-101.

Thumm, K. 1895 Beiträge zur Biologie der fluorescirenden Bakterien. Arbeiten aus dem Bakt. Inst. der Technischen Hochschule zu Carlsruhe 1:291-377. (summary by Smith, E.F., Bacteria in Relation to Plant Diseases 1:238, 1905. The Carnegie Institute of Washington, Washington, D. C.)

Turfitt, G. E. 1936 Bacteriology and biochemical relationships in the Pyocyaneus-Fluorescens Group. I. The chromogenic function in relation to classification. Biochem. J. 33:1323-1328.

Turfitt, G. E. 1937 Bacteriological and biochemical relationships in the pyocyaneus-Fluorescens group. II. Investigations on the green fluorescent pigment. Biochem. J. 34:212-218.

Uschinsky, N. 1893 Ueber eine eiweissfreie Nährlösung für pathogene Bakterien nebst einigen Bemerkungen über Tetanusfigt. Centr. f. Bakt., Abt. 1, 14:316-319.

Van Slyke, D. D. 1929 Manometric determination of primary amino nitrogen and its applications to blood analysis. J. Biol. Chem. 83:425-427.

Van Slyke, D.D. and Cullem, G. F. 1916 The determination of urea by the urease method. J. Biol. Chem. 24:117-122.

Vickery, J. R. 1936 Action of microorganisms on fat. I. Hydrolysis of beef fat by some bacteria and yeasts tolerating low temperatures. J. Council Sci. Ind. Res. 9:107.

Virtanen, Atturi I. 1931 Butter defects caused by water bacteria. Ninth Int. Dairy Congress, Denmark. Report of the Delegation of the United States to the Secretary of State. p. 42.

Virtanen, Atturi I. and Suolahti, Osmo. 1937 Die Sekretion der Proteinasen bei den gelatine-verflüssigenden Bakterien. Enzymologia 2:89-91.

Virtanen, Atturi I. and Suolahti, Osmo. 1937 Zur Frage der Proteinasesekretion durch gelatine-verflüssigende Bakterien. Enzymologia 3:62-64.

Von Sommaruga, E. 1894 Ueber Stoffwechselprodukte von Mikroorganismen. III Mittheilung. Z. f. Hyg. Infectionshrank 18 :441-456.

Waksman, S. A. and Tomanitz, S. 1925 Contribution to the chemistry of decomposition of proteins and amino acids by various groups of microorganisms. J. Agr. Res. 30:263-281.

Waksman, S. A. 1932 Principles of Soil Microbiology, 2nd Ed., pp. 148, 164, 406, 425. The Williams and Wilkins Co., Baltimore.

Wells, H. Gideon and Carper, Harry J. 1912 The lipase of tuberculosis and other bacteria. Studies on the biochemistry of chemotherapy of tuberculosis III. J. Infect. Dis. 11:388-396.

Widmann, Ernst. 1929 Quantitative Umwandlung of Methylglyoxal in reine d (-) Milchsäure durch das Bacterium fluorescens. Biochem. Z. 216:475-478.

Wolff, A. 1907-1908 Zur Kenntnis der veränderung in der Bakterienflora in der frischens Milch wahrend des sogenannten Incubations-stadiums. Centr. f. Bakt., Abt. 2, 20:545-563, 651-675, 737-780.

Wood, G. E. C. 1889 Enzyme action in lower organisms. Proc. Roy. Soc. Edinburgh 17:27-54.

Wright, J. H. 1895 Report on the results of an examination of the water supply of Philadelphia. Memoirs Nat. Acad. Sci. 7:422-474.

Zimmerman, O. E. R. 1890 Die Bakterien unserer Trink-und Nutzwasser.