STUDIES IN THE CULTURAL AND SEROLOGICAL CHARACTERISTICS OF SOME AEROBIC SPORE-PRODUCING BACTERIA

by

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STUDIES IN THE CULTURAL AND SEROLOGICAL CHARACTERISTICS OF SOME AEROBIC SPORE-PRODUCING BACTERIA.

Introduction and Review of Literature

The lack of an adequate classification is largely responsible for the erratic information concerning aerobic spore-producing bacteria[3]. In 1903 Ford attempted to classify these bacteria by using their reactions in a few carbohydrates as a basis. In the majority of investigations (Gottheil 1901, Meyer 1903, Chester[1] 1903, Weide 1904) emphasis was placed on reactions in gelatin, litmus milk, dextrose, saccharose and dextrose-litmus agar. Differentiation by microchemical reactions, size, shape, and location of spores, as well as methods of sporulation, have all been found impracticable.

As bacteria may be classified from a morphological, cultural, or serological viewpoint it is evident that the respective classifications may oppose one another. The aerobic spore-bearing bacteria have been classified from a cultural basis[8] and from a combined morphological and cultural basis. This latter classification, that of Ford,[6] is the one in general acceptance at the present time. It is here given in brief:

Group I. Subtilis Group

Small sluggishly motile; 0.5 by 2.5 microns; no threads on glucose agar; growth on solid media; scums on fluid media.

Bacillus Subtilis Cohn

Group II. Mesentericus Group

Actively motile; 0.5 by 4 microns; often long threads on glucose agar; growth on hard media as soft mass.

Bacillus Mesentericus --Ruber

" " --Vulgatus

" " --Fuscus

" " --Niger

Group III. Cohaerens-Simplex Group

Motile; o.375 by 0.75 to 3 microns; growth on hard media as a soft mass; growth in fluids turbid.

Bacillus Cohaerens

" Simplex

Group IV. Mycoides Group

Large with square ends; 0.5 by 3 to 6 microns; growth on hard media, dry; In fluid media, firm with tenacious scum.

Bacillus Mycoides

Group V. Cereus Group

Large with round ends; 0.75 by 2.75 to 4 microns; growth on hard media as soft mass; in fluid media as thick friable scum.

Bacillus Cereus

" Albolactus

Group VI. Megatherium Group

Very large; 0.75 to 1.25 by 3 to 9 microns; growth on solid media as thick mass; in fluid media there is turbidity.

Bacillus Megatherium

" Petasites

Group VII. Round Terminal-Spored Group

Small; 0.5 to 0.75 by 1.5 to 3 microns; Spores terminal, round and thicker than organisms.

Bacillus Fusiformis

Group VIII. Cylindrical Terminal-Spored Group

Small; 0.375 to 0.5 by 2.5 to 4 microns; spores terminal, cylindrical.

Bacillus Terminalis

Group Ix. Central-Spored Group

Long; 0.375 to 0.5 by 1 to 4 microns; spores in middle of rod which becomes spindle shaped.

Bacillus Centrosporous

An objection to this method of classification is that such a grouping entirely disregards many close physiological relationships. Ford[6] regards Bacillus Centrosporous as a new specie[8] only because of it singular method of spore formation. In a comparison of the sugar fermentations of this organism with that of Bacillus Fusiformis there is but one irregularity, the production of acidity in dextrin by the former. These bacteria are notably inactive in the other sugars and other media.

Part I

Cultural Characteristics of the Organisms.

Scope

The morphology of the organisms was determined from methylene blue and carbol fuchsin smears. The bacterial cultures were observed on plain, dextrose litmus and lead acetate agars, gelatin and the potato. Reactions to Gram's stain, carbohydrates (fifteen), litmus milk, plain and nitrate broths were ascertained and investigation made as to motility, ability to produce indol in Dunham's peptone solution and the production of such enzymes as diastase, rennin and protease.

It is the purpose of this work:

1. To make cultural and serological studies of aerobic spore-

producing bacteria.

2. To compare the results so obtained with those reported by Ford,

3. To attempt if possible to improve upon the present classification.

Cultures of the following eighteen spore-producing bacteria are the subjects for investigation in this work:

B. Subtilis	B. Petasites
B. Calvarens	B. Coreus
B. Terminalis	B. Mesentericus-Ruber
B. Niger Lactis	B. Mesentericus-Fuscus
B. Albolactus	B. Mesentericus-Niger
B. Tumescens	B. Mesentericus-Vulgatus
B. Anthracis	B. Mycoides
B. Megatherium	B. Fusiformis
B. Centrosporous	B. Simplex

Technique

The usual determinative media and methods were used supplemented by newer ones which have appeared from time to time in the literature.

Spore Stain--Chester[1] (1903). The bacterial film is prepared by adding culture of organism to 10% formalin on a glass slide. The film is dried in air and fresh fuchsin (1-10 dilution) added. After being thus exposed for five minutes the film is washed in water and examined.

Rennet Production--Cohn[2] (1922). A milk culture of the organism is incubated for forty-eight hours (37 C) when about 5cc.

of whey appears on the surface. A tube of unsterilized fresh milk is then warmed in a water-bath to 37 C, incubated with 5cc. of the whey and the time necessary for the coagulation of the milk noted.

Diastatic Action--Starch is dissolved in hydrochloric acid by heat- a mixture of the two. After cooling, alcohol is added and the soluble starch which is precipitated, may be obtained by filtering. The starch is now dissolved in cold water, and sufficient sodium hydroxide added to neutralize the hydrochloric acid. Soluble starch agar (5%) may now be made by adding 5cc. of the starch solution to 95cc. of agar. Agar plates are poured and inoculated with the straight wire by making a short streak of perhaps 3 cms. in length across the center of the plate. After an incubation period of forty-eight hours the plates are flooded with iodine solution. If the organism is not a producer of diastase, there is no change in the color of the medium immediately surrounding the line of bacterial growth. Around this halo the medium is dark blue, the color which is characteristic of starch and iodine contact. If diastase is formed it hydrolyzes the starch so that on the the addition of the iodine no starch is present and therefore no blue color is produced.

Carbohydrates--The sugars were added to meat infusion broth and incubated seventy-two hours.

Reaction of Media--All media were adjusted to a reaction of Ph 7 by the colorimetric method.

Preparation of Media--The preparation of cultural media was in accordance with methods given in Standard Methods of

Water Analysis (1923) by the American Public Health Association.

As a precaution two tubes or plates of media were always used, the second serving as a check for the first.

DETAILED CHARACTERISTICS OF ORGANISM

Each of the organisms was incapable of producing gas in the carbohydrates. The three sugars nutrose, dulcite and rhamnose gave uniformly negative results. Indol production occurred in five of the strains only after eighteen days incubation.

The history and morphology of the bacteria as presented below correspond largely to that given by Ford.[5] Detailed information in light of my laboratory findings follows.

BACILLUS SUBTILIS.

Bacillus subtilis, one of the most common organisms found in milk, soil, water and dust, exists under several names: Vibrio Subtilis Ehrenberg (1838), B. Subtilis Cohn (1872), and the generally accepted B. subtilis (Ehrenberg) Cohn.

Morphology- One of the smallest of the eighteen cultures. The spores which occur in the middle or near one end are of such a size as to cause a bulging of the bacterial walls.

Gram stain- Gram positive.

Agar plates- Growth is slow, spreading; individual colonies low.

Dextrose litmus agar- Medium is acidified. The growth is an elevated non-spreading creamy yellow.

Gelatin plate- Medium is liquefied. Colonies are round and of a spreading tendency.

Gelatin stab- Cup shaped liquefaction. Growth slow.

Broth- Sediment appears in twenty-four hours, and pellicle in forty-eight hours. The liquid remains clear throughout incubation.

Litmus milk- Alkaline. Milk changes color on seventy-two hours incubation becoming a grey brown. In ninety-six hours a yellowish brown precipitate is formed.

Potato- Growth heavy and warty. In seventy-two hours a pink pigment makes its appearance.

Diastatic action- positive.

Proteolysis- Negative.

Rennet- No production

Outstanding characteristice- Growth on potato; one of four to produce acidity in arabinose.

BACILLUS CALVARENS.

The origin of this organism is unknown to the author. There is a possibility that it is identical with B. Cohoereus.

Gram stain- Positive.

Agar plate- Growth slow and spreading; colonies are low and small.

Dextrose litmus plate- Medium acidified. growth in places is of a decidedly greenish hue.

Gelatin plate- Medium liquefied.

Gelatin stab- Medium liquefied with heavy growth along stab.

Broth- Pellicle is formed in twenty-four hours; no sediment or turbidity present after 108 hours of incubation.

Litmus milk- No change in forty-eight hours; turns brown in seventy-two hours with peptonization.

Diastatic action- Positive.

Potato- Growth becomes a greenish yellow in twenty-four hours.

Proteolysis- Negative.

Bennet- No production.

Outstanding Characteristics- Potato growth; one of four to produce acidity in arabinose.

BACILLUS TERMINALIS.

This organism was isolated by Flugge (1894) but received the name it now bears by Migula. Chester has applied another name to this organism, that of B. lacteus.

Gram stain- Negative.

Agar plate- Growth slow and delicate; colonies are low and small.

Dextrose litmus agar; Medium acidified; low dull greenish looking colonies; many colonies appear to be formed of concentric rings with brown centers.

Gelatin plate- Slight liquefaction; colonies present an outstanding center with surrounding concentric circles.

Gelatin stab- Liquefaction with formation of a pellicle.

Broth- In twenty-four hours a pellicle is formed; liquid becomes turbid and sediment is present.

Litmus milk- No change for seventy-two hours after which time milk becomes a red later changing to brown; slow peptonization.

Potato- Growth is dark brown in twenty-four hours which in six days becomes a peculiar greenish brown.

Diastatic Action- Negative

Rennet- No production.

Outstanding characteristics- Growth on dextrose litmus agar; one of four to produce acid in arabinose.

BACILLUS NIGER LACTIS.

This organism was first named Bacillus Lactis-niger by Gorin (1894). Since then Migula has given it its present name.

Gram stain- Positive.

Agar plate- Individual colonies large, creamy in color; growth is elevated and spotted in appearance.

Dextrose litmus agar- Medium is acidified; colonies are greyish white.

Gelatin plate- Medium is liquefied.

Gelatin stab- Liquefaction begins within twenty-four hours and extends throughout medium until there is complete liquefaction.

Broth- Pellicle is formed and liquid becomes turbid within twenty-four hours; in forty-eight hours sediment appears.

Litmus milk- No change in twenty-four hours; becomes acid in forty-eight hours with light brown color.

Potato- Part of potato becomes very black in forty-eight hours, the color spreading throughout the medium with time.

Diastatic Action- Negative.

Proteolysis- Negative.

Rennet- The milk becomes coagulated in five hours; the coagulation is not very intense.

Outstanding characteristics- Growth on potato; one of three organisms to produce acidity in lactose; indol is produced after a long period of incubation (eighteen days).

BACILLUS ALBOLACTUS.

This organism was first named Bacillus lactis albus by its isolator, Loeffler, who found it in soured boiled milk, and it has since come to be known as Bacillus albolactus Migual (1900). It is common in boiled milk and is often responsible for the fermentation of this food.

Gram stain- Positive.

Agar plate- Growth is slimy and spotted in appearance, it is elevated, spreading and smooth; individual colonies are large with outstanding centers.

Dextrose litmus agar- Medium acidified; smooth spreading glossy white growth of a greenish hue here and there.

Gelatin plate- Medium liquefied.

Gelatin stab- Funnel-shaped; liquefaction begins within twenty-four hours and extends until there is complete liquefaction of medium.

Broth- No pellicle is formed; there is sediment in twenty-four hours and tubidity in forty-eight hours.

Litmus milk- Medium acidified in twenty-four hours; coagulation within forty-eight hours; at this time medium is a light brown which in seventy-two hours becomes a "cream in coffee" color. The coagulum

at this period is exceedingly hard and becomes white in color at the end of ninety-six hours of incubation, at which time peptonization sets in.

Potato- A growth appears which in six days becomes a yellowish brown.

Diastatic Action- Negative.

Proteolysis- Negative.

Rennet- Milk is coagulated one hour after the addition of its whey.

Outstanding characteristics- One of three organisms to produce acidity in lactose; indol is produced in eighteen days incubation.

BACILLUS TUMESCENS.

Zopf was the first to describe this organism (1885).

Gram stain- Positive.

Agar plate- Growth is very slow. Individual colonies are low and round.

Dextrose litmus agar- Medium acidified. Colonies are greenish white.

Gelatin plate- liquefaction.

Gelatin stab- Medium presents a funnel shaped liquefaction.

Broth- No pellicle is formed; no turbidity appears; sediment appears in twenty-four hours.

Litmus milk- No change in comparison with control in ninety-six hours of incubation.

Potato- Growth grey in twenty-four hours becoming a white or light cream colored in seventy-two hours.

Diastatic action- Positive.

Proteolysis- Negative.

Rennet- Milk is coagulated in five minutes; the coagulation is exceedingly intense.

Outstanding Characteristics- Production of rennet. This organism and Bacillus Petasites are the only ones that produce acidity in galactose.

BACILLUS ANTHRACIS.

The anthrax bacillus is probably the most pathogenic of the aerobic spore producing bacteria. It is of hygienic and commercial importance because it is often present among the bristles of cheap tooth brushes and shaving daubers.[7,9] Its chances for setting up infection through the use of these brushes are readily seen. The organism is the cause of "anthrax" among sheep, cattle and other herbivorous animals often occurring among the herds in epidemic form. The disease is often transmitted to man thru the skin, digestive tract or lungs, due to contact with the sick animals or the handling of their hides.

Gram stain-Positive.

Agar plate- White spreading growth with spotted appearances.

Dextrose litmus agar- Medium acidified. Colonies of a greenish hue.

Gelatin plate- Liquefaction.

Gelatin stab- Liquefaction within twenty-four hours. A growth

appears which has been adequately called an "inverted fir tree." This growth is yellowish in color and is characteristic of the organism.

Broth- No pellicle is formed. The medium becomes turbid in twenty-four hours and sediment appears in forty-eight hours.

Litmus milk- No change in forty-eight hours; in seventy-two hours medium becomes a greyish red.

Potato- No change for seventy-two hours, after which time it becomes slightly discolored.

Diastatic action- Negative.

Proteolysis- Negative.

Rennet- No rennet is produced.

Outstanding characteristics- Gelatin stab.

BACILLUS MEGATHERIUM.

Bacillus megatherium aside from being one of the most common of the spore-bearing bacteria, is possibly the largest of this group of organisms. It was found and named by De Bary (1884, 1887).

Gram-stain- Positive.

Agar plate- Growth spreading, thick, white or cream colored. Individual colonies are very small.

Dextrose litmus agar- Medium first acidified and later becomes alkaline. Colonies are elevated, moist and creamy white in color. The color later acquires a darker tinge.

Gelatin plate- Colonies are round. Liquefaction.

Gelatin stab- Funnel shaped liquefaction.

Broth- No pellicle is formed; sediment and turbidity both appear in twenty-four hours.

Litmus milk- No change in forty-eight hours; in seventy-two hours medium is a reddish color.

Potato- Growth is of a cream color in twenty-four hours. In seventy-two hours this color diffuses throughout the potato.

Diastatic action- Positive.

Proteolysis- Negative.

Rennet- Milk is coagulated within one hour.

Outstanding characteristics- one of three organisms to produce acidity in raffinoise.

BACILLUS CENTROSPOROUS.

This organism is peculiar in that it has been obtained only once. It was found in the normal dejecta of a child by W. W. Ford.

Gram stain- Negative.

Agar plate- Growth white and smooth; colonies are low and small.

Dextrose litmus agar- Medium is alkaline, becoming bluer in color. The colonies are dull and of a greenish blue with their centers brown.

Gelatin plate- Growth exceedingly slow. Medium is liquefied.

Gelatin stab- no change after six days and then liquefaction in form of a funnel or a sphere.

Broth- No pellicle in ninety-six hours; turbidity appears in twenty-four hours and sediment in forty-eight hours.

Litmus milk- Medium becomes a very intense blue in ninety-six hours. No peptonization.

Potato- Growth white, and moist in appearance.

Diastatic action- Negative.

Proteolysis- Positive. In one of the trials there were excellent results before the addition of the acetic acid.

Rennet- No rennet production.

Outstanding characteristics- Growth in litmus milk; dextrin is only sugar (used) in which acidity is produced; growth on dextrose litmus agar.

BACILLUS PETASITES

This organism was originally described by Ghotteil (1901).

Gram stain-Positive.

Agar plate- Colonies dull, yellowish, round. Growth spreading.

Dextrose litmus agar- Medium acidified; growth thick, yellowish,

Gelatin plate- Medium liquefied.

Gelatin stab- Funnel shaped liquefaction.

Broth- No pellicle is formed. Sediment appears in twenty-four hours, disappears in forty-eight hours to reappear in ninety-six hours. At this time the broth becomes turbid.

Litmus milk- No change in twenty-four hours. In forty-eight hours peptonization sets in and medium becomes a reddish color.

Potato, Growth yellow, becoming darker yellow with age.

Diastatic action- Negative.

Proteolysis- Negative.

Rennet- Rennet is produced, the fresh milk being coagulated in five minutes.

Outstanding characteristics- Production of rennet. One of two organisms that produce acidity in galactose.

BACILLUS CEREUS.

Bacillus cereus is undoubtedly the most widely distributed aerobic spor-producing organism. It was isolated by the Franklands in 1887. It is also the most common laboratory contamination. Many investgators have found organisms that are more or less identified with it. One of these organisms, B. cereus fluorescens is closely related to B. Cereus but differs in one characteristic in that it produces a greenish fluoresence.

Gram stain- Positive.

Agar plate- Smooth, dull cream colored, spotted growth. Colonies are round and of medium size.

Dextrose litmus agar- Medium acidified. Colonies are elevated and dull white in color. Growth spreads rather evenly from line of inoculation.

Gelatin plate- Medium liquefied. Colonies have outstanding centers and thin margins.

Gelatin stab. Liquefaction in twenty-four to forty-eight hours, this extends throughout medium until there is complete liquefaction.

Broth- No pellicle formed; precipitate and turbidity appear in twenty-four hours. The precipitate becomes heavy and is of white color in forty-eight hours.

Litmus milk- Immediate peptonization. In forty-eight hours the medium is divided into three zones by its different colors. The

upppermost zone is white curd, the middle zone is a brownish red while the lowest zone is light blue. In seventy-two hours the zone appearance is lost and the entire medium is light brown. In one hundred and eight hours this color remains but there is also present a white coagulum and a bluish ring.

Potato- A common whitish growth.

Diastiatic action- Negative.

Proteolysis- Negative.

Rennet- Milk coagulates in one hour.

Outstanding characteristics- Effect on litmus milk; production of indol in eighteen days.

BACILLUS MESENTERICUS - RUBER

This organism was isolated in 1888 by Globig. It is known also under the name Bacillus Globigii.

Gram Stain - Negative.

Agar Plate- Spreading growth with a spotted appearance. Colonies are small and elevated.

Dextrose litmus agar- Medium acidified; growth dull and greenish white in color.

Gelatin plate- Liquefaction.

Gelatin stab.- Rapid and extensive liquefaction.

Broth- No pellicle; turbidity and sediment in twenty-four hours.

Litmus milk- Medium takes on a red color in twenty-four hours. In forty-eight hours it has a four zone appearance much like the organism

above. The zones from the top to the bottom are white (curd), light brown, red and pale blue. In seventy-two hours the red zone disappears and after ninety-six hours the culture resembles a one-hundred-and-eight-hour culture of Bacillus Cereus.

Potato- Growth with no usual features.

Diastatic action- Negative.

Proteolysis- Negative.

Rennet- Rennet is produced. Fresh milk coagulates in three hours.

Outstanding Characteristics- Effect on litmus milk.

BACILLUS MESENTERICUS - FUSCUS

In 1886 Flugge satisfied himself that this organism was distinct from Bacillus mesentericus-vulgatus. It is one of the less common of the aerobic spore-bearing bacteria.

Gram Stain- Positive.

Agar plate- Slightly spreading cream colored growth. Individual colonies small.

Dextrose litmus agar- Medium acidified; colonies are low and of a greenish hue.

Gelatin plate- Liquefaction.

Gelatin stab- Growth along line of stab; medium is liquefied.

Broth- Pellicle is formed in twenty-four hours; sediment appears in forty-eight hours; turbidity does not appear.

Litmus milk- No change in forty-eight hours; peptonization begins in seventy-two hours with a red color.

Potato- A pellicle-like growth forms in twenty-four hours. The

potato becomes creamy white in forty-eight hours. Growth is wrinkled and yellow in seventy-two hours, changing to a brown.

Diastatic action- Positive.

Proteolysis- Negative.

Rennet- No rennet production.

OUTSTANDING CHARACTERISTICS- Growth on potato. One of these bacteria to produce acidity in raffinoise.

BACILLUS MESENTERICUS - NIGER.

This organism though described by Biel (1896) was given its present name by Lunt (1896). It is also know as Bacillus aterrimus.

Gram stain- Positive.

Agar plate- Growth smooth, spreading and elevated; dull cream in color.

Dextrose litmus agar- Medium acidified. Where growth is heavy the color is failry green while in other places it is lead colored.

Gelatin plate- Medium liquefied.

Gelatin stab- Extensive liquefaction with pellicle production.

Broth- Pellicle is formed in forty-eight hours. Sediment and turbidity appear in twenty-four hours.

Litmus milk- No change in forty-eight hours; in seventy-two hours medium begins to peptonize and takes on a red coloration.

Potato- Medium becomes slightly discolored until it reaches a brown color in seventy-two hours.

Diastatic action- Positive.

Proteolysis- Negative.

Rennet- Slight production of rennet. The fresh milk coagulates in five hours.

Outstanding characteristics- One of five organisms to produce indol.

BACILLUS MESENTERICUS - VULGATUS

This organism was first described by Flugge in 1886. Some investigators that have worked on it are Trevisan (1889), Eisenberg (1891) and Miguła (1900). Each of the latter workers retained its first name of B. vulgatus. It is commonly known as the "potato bacillus."

Gram stain- Positive.

Agar Plate - Growth slow, spreading, individual colonies are small, low and round.

Dextrose litmus agar- Medium acidified. Colonies are greenish white.

Gelatin plate- Liquefaction.

Gelatin stab- No change in twenty-four hours. Liquefaction with pellicle formation within seven days.

Broth- Heavy pellicle with turbidity in twenty-four hours. A precipitate appears in ninety-six hours.

Litmus milk- No acid production. Peptonization occurs in ninety-six hours with a color which changes from red to a brown.

Potato- Intwenty-four hours there is a dry snow-like growth which in forty-eight hours becomes creamy white and very much wrinkled. In seventy-two hours the growth turns to a yellow and in ninety-six turns to a brown.

Diastatic action - Negative.

Proteolysis- Negative.

Rennet- Rennet is produced. Fresh milk curdles in three and one half hours.

Outstanding characteristics- Growth on potato

BACILLUS MYCOIDES

Though this organism has many names that of Bacillus Mycoides is the most common. This namewas given it by its isolator, Flugge (1886).

Gram stain- Positive.

Agar Plate- The growth is an arborescent myceleoid affair of yellowish color. The peripheries are rhizoid in shape. The photograph of this organism is illustrative.

Dextrose litmus agar- Medium is liquefied; growth as above.

Gelatin plate- Liquefaction; growth is much similar to above.

Gelatin stab- Rapid and extensive liquefaction with pellicle.

Broth- Pellicle is formed which is soon precipitated; medium remains clear.

Litmus milk- No change in ninety-six hours. There is peptonization with a heavy light blue precipitate.

Potato- There is a very slight discoloration in twenty-four hours which becomes reddish brown inside of six days.

Diastatic action- Negative.

Proteolysis- Negative.

Rennet- Rennet production is strong. The whey coagulates fresh milk in five minutes.

Outstanding characteristics- Growth on ager plate; rennet production.

BACILLUS FUSIFORMIS

This organism is one of the many that were first described by Fottheil (1901). Its morphology and cultural characteristics are identical with those of Bacillus lactimorbi.

Gram stain- Negative.

Agar plate- Growth low and spreading, white in color.

Dextrose litmus agar- Medium is an intense blue. Growth is spreading and of a greenish hue.

Gelatin plate- Slow liquefaction; colonies small and round.

Gelatin stab- Slow, funnel-shaped liquefaction; growth long; stab tip is black. Increase in the peptone content of the gelatin does not favor the production of the black color nor is this color produced by the other organisms.

Broth- No pellicle is formed. There is sediment and turbidity in twenty-four hours.

Litmus milk- No change in twenty-four hours; medium becomes an intense blue in forty-eight hours, which in seventy-two hours changes to a reddish color and later to a brown. There is no coagulation nor precipitate.

Potato- Growth is greyish brown in twenty-four hours and becomes a dark brown (almost black) in forty-eight hours. In ninety-six hours this color is changed to a very dark brown having a greenish tinge.

Diastatic action- Negative.

Proteolysis- Positive.

Rennet- No rennet production.

Outstanding characteristics- Growth on potato and in Litmus milk; indol production in eighteen days. There is no acid production in any of the sugars.

BACILLUS SIMPLEX

There is also an organism that was described by Gottheil (1901). The organisms are large in comparison with the other aerobic spore-bearing organisms.

Gram stain- Positive.

Agar-plate- Individual colonies are very small; growth is low, spreading and white in color.

Gelatin plate- Round colonies with sharp edges; liquefaction.

Broth- Pellicle and sediment appear in forty-eight hours; turbidity in twenty-four hours.

Litmus milk- Medium becomes a brownish white color with slight coagulation in twenty-four hours. In forty-eight hours the color is that of "cream in coffee". This color is very pronounced and becomes more intense in ninety-six hours.

Potato- Growth is white and becomes discolored in seventy-two hours. In one hundred and forty-four hours the growth is wrinkled and brown in color.

Diastiatic action- Positive.

Proteolysis- Negative.

Rennet- Rennet is produced. Time required for the coagulation of the fresh milk is ten minutes.

Outstanding characteristics- Effect on litmus milk; growth on dextrose litmus agar.

In Table 1 I have attempted to summarize the cultural characteristics and staining reactions of the organisms.

Table 1.

Tabulated Characteristics of Organisms.

	dextrose	Saccharose	salacin	dextrin	maltose	inulin	xylose	arabinose	mannite	lactose	raffinose	galactose	gram stain	indol	nitrate broth	lead acetate	gelatin stab	motility
B. Subtilis	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+	+	+	+
B. Calvarens	+	+	+	−	−	−	+	+	+	−	−	−	+	−	−	+	+	+
B. Terminalis	+	+	+	−	−	−	+	+	+	−	−	−	−	−	−	+	+	
B. Niger lactis	+	+	+	+	+	−	−	−	−	+	−	−	+	+	+	+	+	
B. Albolactus	+	+	+	+	+	−	−	−	−	+	−	−	+	+	+	+	+	
B. Tumescens.	+	+	−	+	+	+	−	−	−	+	−	+	+	−	+	−	+	+
B. Anthracis	+	+	+	+	+	−	−	−	−	−	−	−	+	−	+	+	+	−
B. Megatherium	+	+	−	+	+	−	+	−	+	−	+	+	−	−	−	−	+	+
B. Centrosporous	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	+
B. Petasites	+	+	−	+	+	+	+	+	+	−	+	+	+	−	−	−	+	+
B. Cereus	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	+	+
B. Mes.-Ruber	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+
B. Mes.-Fuscus	+	+	+	−	−	−	−	−	+	−	+	−	+	−	−	−	+	+
B. Mes.-Niger	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+
B. Mes. Vulgatus.	+	+	−	−	−	+	−	−	+	−	−	−	+	−	−	−	+	+
B. Mycoides	+	−	+	+	+	−	−	−	−	−	−	−	+	−	+	−	+	+
B. Fusiformis	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+
B. Simplex	+	+	+	−	+	+	−	−	+	−	−	−	+	−	−	+	+	+

Discussion.

Ford[5] considers Bacillus Megatherium and Bacillus Tumesceus as identical but a study of the carbohydrate reactions presents numerous differences in fermentation power. Bacillus Tumesceus produces acidity in lactose, inulin and galactose whereas Bacillus Megatherium does not. This latter organism produces acidity in mannite, xylose and rafinose, in all of which Bacillus Tumescens is inactive.

Ford and Lawrence[5] take exception with Chester's consideration of Bacilli Subtilis and Mesentericus-Vulgatus as being the same species. The findings in this work bear out the contentions of Ford and Lawrence. Bacillus Subtilis is a more active bacterium, producting acidity in maltose, dextrin, salacin, xylose, arabinose and all sugars in which Bacillus Mesentericus-vulgatus produces acidity.

So slightly do the organisms differ among themselves that the classification of the aerobic spore-producing bacteria by Ford[6] is hardly consistent. A classification is devised herein which is essentially an identification guide. The reaction to Gram's stain is first used to differentiate the organisms and the two groups are then subdivided with reactions in dextrose and dextrin as the primary basis. It is to be noted that all Gram-positive bacteria are also dextrose-positive.

This method of identification by sugar fermentation does not distinguish between Bacillus Niger Lactis and Bacillus Albolactus. This distinction may be readily determined from the observation of

a potato culture of the organisms. The potato in the case of the former organism becomes jet black within forty-eight hours but remains unchanged by the latter organism save for the presence of an ordinary white bacterial growth.

Bacilli Anthrax, Cereus and Mesentericus Niger make up another group of spore producing bacteria that are not differentiated by the carbohydrate media. The identity of an organism as Bacillus anthracis is determined by the presence of the "inverted fir tree", growth in a gelatin stab culture. A potato tube may be used to distinguish between Bacilli Cereus and Mesentericus-Niger. In this medium Bacillus Cereus produces an ordinary white growth while Bacillus Mesentericus-Niger discolors the medium, producing a dark brown or black growth. Lead acetate agar may also be used with respectively similar results.

A further classification is attempted by denoting the bacteria as Gram-positive or Gram-negative and dividing them by reactions in lead acetate agar and their ability to produce rennet. Nitrate broth reactions are also used. Wherever there are more than one bacterium occurring in the same position under nitrate broth additional media are listed in which the bacteria may be readily differentiated.

With slight modification this identification gives a consistent grouping of the bacteria. It is given below.

Classification of Aerobic Spore-Producing Rod-Like Bacterial Forms.

These bacteria grow well at 20° C. The presence of oxygen is required.

Identification Chart 1.

Gram Positive Spore Producers Uniformly Dextrose Positive

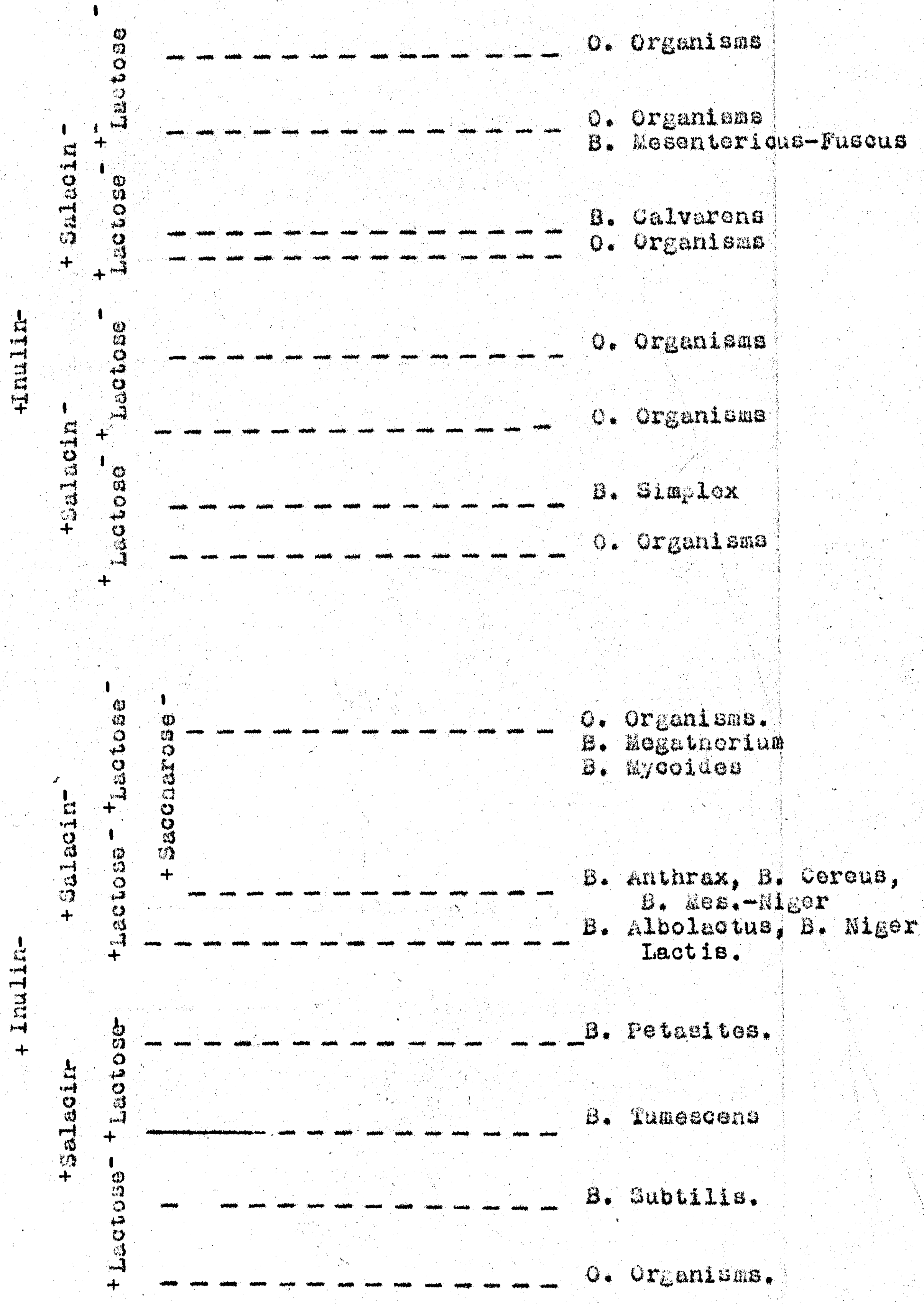


Table 2. (cont.)

Identification Chart 1.

Gram Negative Spore Producers.

+Dextrose-

+Dextrin- | -Dextrin-

B. mesentericus-ruber | B. terminalis | B. centrosporus. | B. fusiformis

Lead Acetate Rennet Identification of Gram Positive Spore Producers.

+Lead Acetate Agar.-

+Rennet-

+Nitrate Broth-

- Inulin — B. Megatherium
+ Inulin — B. Petasites.
+ Potato — B. Mesentericus-Vulgates.
- Litmus Milk — B. Tumescens
+ Agar Plate — B. Mycoides
+ Litmus Milk — B. Cereus

+Nitrate Broth-

-Potato — B. Anthrax
+Potato — B. Subtilis

+Nitrate Broth-

+Rennet-

+Nitrate Broth- — B. Simplex.

Potato (black) — B. Niger Lactis
Potato (brown) — B. Mes.- Niger
+ Litmus Milk — B. Albolactus.

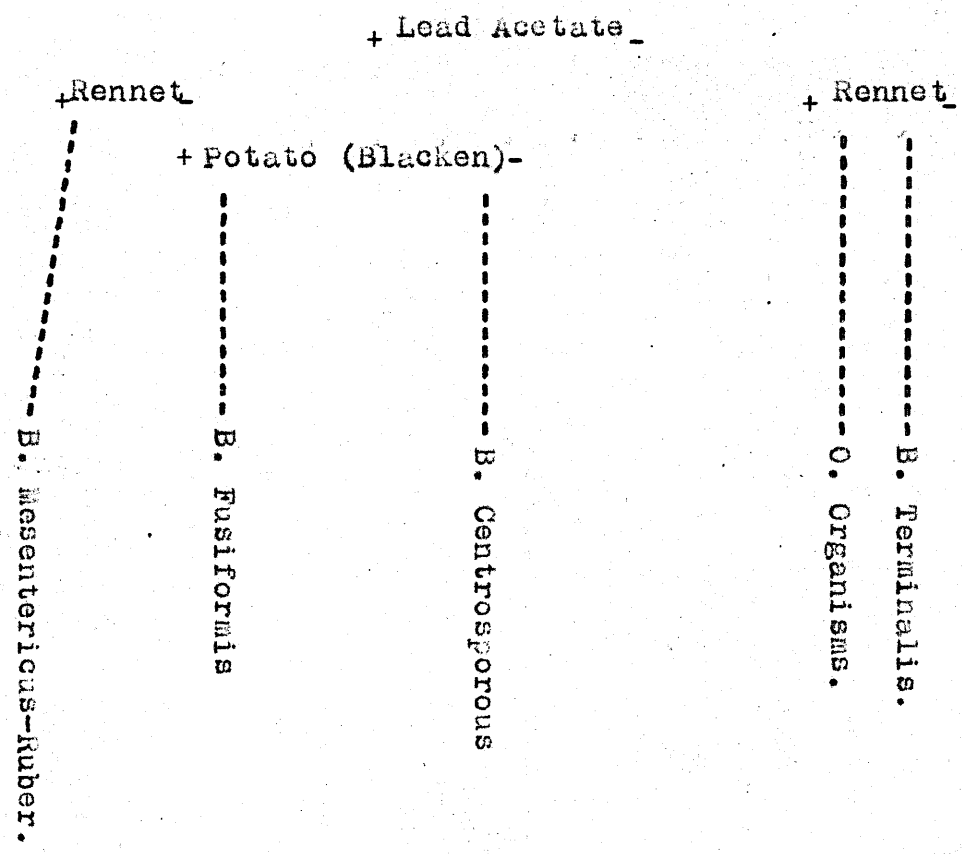
+Nitrate Broth- — B. Mesentericus-Fuscus

+Nitrate Broth- — O. Organisms

Table 3. (cont.)

Identification Chart 2.

Lead Acetate Rennet Identification of Gram Negative Spore Producers.



GROUP I.

Gram-positive; lead acetate agar positive; rennet production-positive.

Bacillus albolactus.

" mesentericus-niger.

" niger Lactis.

" Simplex.

GROUP II.

Gram positive; lead acetate agar positive; rennet production-negative.

Bacillus anthracis.

" Subtilis.

" Calvarens.

GROUP III.

Gram-positive; lead acetate agar-negative; rennet production positive; nitrate broth positive.

Bacillus Tumescens.

" Mycoides.

" cereus.

GROUP IV.

Gram-positive; lead acetate-agar-negative; rennet production-positive; nitrate broth-negative.

Bacillus Megatherium.

" Petasites.

" Mesentericus-Vulgatus.

" " Fuscus.

GROUP V.

Gram-negative; lead acetate agar-positive; rennet pro-

duction-negative.

Bacillus Fusiformis.

" centrosporous.

GROUP VI.

Gram-negative; lead acetate agar-positive; rennet production-positive.

Bacillus mesentericus-Ruber.

GROUP VII.

Gram-negative; lead acetate agar-negative; rennet production-negative.

Bacillus Terminalis.

A convenient chart of the classification is here given.

TABLE Four

Lead Acetate-Rennet Classification of the Aerobic Spore-Producing Bacteria.

Group	Gram Stain	Lead Acetate Agar	Rennet Production	Nitrate Broth
I	+	+	+	
II	+	+	-	
III	+	-	+	+
IV	+	-	+	-
V	-	+	-	
VI	-	+	+	
VII	-	-	-	

In comparing this original Lead acetate-Rennet classification of the aerobic spore-producing bacteria there is found both

concurrence and disagreement. The former classification does not keep intact the Mesentericus Group of Ford. Bacilli Mesentericus-Vulgatus and Mesentericus-Fuscus fall into Group IV with two other bacteria while Bacilli Mesentericus-Niger and Mesentericus-Ruber occur elsewhere, the latter bacterium being placed in a group to itself. Bacilli Cereus and albolactus, placed in the same group by Ford, are here placed in different groups. The close relationship between the following organisms is recognized in the two classifications by the appearance of the bacteria in the same group: Bacilli Mesentericus-Vulgatus and Mesentericus-Fuscus; Bacilli Megatherium and Petasites.

The author's classification seems more simple than the one by Ford.

Conclusions.

1. My results do not indicate the close similarity between Bacilli Megatherium and Bacillus Petasites as reported by Ford.

2. The Gram's stain and carbohydrate reactions serve as convenient bases for the identification of the organisms.

3. A classification of the aerobic spore-producing bacteria based on reactions to Gram's stain, lead acetate agar, nitrate broth and the production of rennet is simple, useful and consistent.

Agglutinin	Subtilis	Calvarens	Terminalis	Niger Lactis	Albolactus	Tumescens	Anthrax	Megatherium	Centrosporous	Cetasites	Cereus	Mes. Ruber	Mes. Fuscus	Mes. Niger	Mes. Vulgatus	Mycoides	Fusiformis	Simplex
Agglutinogen																		
Subtilis	950	800	200	400	150	200	200	50	200	50	200	200	50	50	50	50	50	50
Calvarens	900	900	200	450	150	50	400	50	50	200	200	200	200	50	50	100	100	50
Terminalis	150	200	800	350	400	200	150	50	250	50	200	150	50	50	50	50	200	50
Niger Lactis	450	150	300	600	300	200	150	200	100	50	100	100	150	150	100	100	50	50
Albolactus	600	450	200	200	450	400	200	50	50	200	150	50	150	50	50	350	50	50
Tumescens	200	850	200	50	200	450	50	50	150	250	200	50	50	50	50	200	50	50
Anthrax	200	450	200	450	400	200	650	150	150	200	200	50	50	200	100	350	50	50
Megatherium	600	300	150	450	400	200	200	450	100	150	50	50	50	50	50	150	150	150
Centrosporous	300	350	400	50	350	50	400	50	500	200	150	150	50	50	50	50	50	50
Cetasites	450	200	250	50	100	50	150	50	50	450	50	150	150	150	50	50	150	50
Cerues	350	200	200	50	150	200	50	50	450	400	250	50	50	50	50	50	50	50
Mes. Ruber	50	300	200	50	250	200	50	50	150	50	150	350	150	150	50	50	50	50
Mes. Fuscus	50	50	300	150	150	150	150	50	100	50	100	150	300	200	50	400	200	50

Mes. Niger	150	200	450	50	450	150	200	200	200	50	50	50	50	500	50	450	200	100
Mes. Vulgatus	50	350	50	150	150	50	50	50	50	50	100	150	200	350	800	350	50	50
Mycoides	450	700	200	200	400	350	200	50	100	100	50	50	50	50	150	560	50	50
Fusimormis	400	400	200	200	150	250	300	150	200	300	150	200	150	200	50	200	250	150
Simplex	450	200	200	50	300	100	150	400	200	150	150	50	50	350	50	350	50	200

PART II.

Serological Reactions of the Organisms.

Scope.

As agglutination reactions[10] have yielded satisfactory results in the identification of members of the Pneumoccus, Meningococcus and Bacillus Thphosus groups and have been found rather unsatisfactory in the identification of membersof the Bacillus Coli, Bacillus Diphtheria and many other groups, it was thought desirable to note whether these reactions would be of value in determining a classification of the aerobic spore-producing bacteria.

The experimental rabbits were first examined for the presence of normal agglutinins, the macroscopic method of performing agglutination experiments being used here and thruout the work.

As the animals presented uniformly negative results, they were injected with suspensions of the desired bacteria as antigens according to the following schedule.

Table 5.

Scheme of Injection.

Number of Injection	Method	Days interval	Dose
1.	Intraperitoneally	-	1.0 CC.
2.	"	6	1.5 CC.
3.	Intravenously	5.	2.0 CC
4.	"	4	2.0CCC

On the ninth day after the last injection the titre of each antiserumwas determined against each bacterial suspension. The chart below, Table 6, represents in a compact form the results of these titrations. All controls thruout these tests showed unquestionable negative results. Additional controls were made by titrating two of

the antisera against formalinized antigens of some non-spore-producing bacteria.

Technique.

Preparation of antigen for Titrations--The bacterial suspensions for use in all the agglutination experiments were made according to a method suggested by Dreyer.[4] The bacteria are grown in 30 cc. of well filtered, sterilized plain broth for twenty-four h hours at 37° C. The culture is well shaken and 0.3 cc. of formalin added. The shaking is repeated on that day and on the following days. Twenty-four hours after the addition of the formalin, the culture in filtered under sterile conditions. Whenever not in immediate use, the suspension is kept in the ice chest. The same suspension of each organism was used throughout every experiment of this work.

Preparation of Antigen for Injection -- Ten c.c. of normal salt solution were added to a twenty-four hour (37° C) agar slant culture of the desired bacteria. With a platinum loop the growth is brought into suspension with the salt solution. The tube is plugged with cotton and allowed to stand for ten minutes, thus permitting the larger clumps of bacteria to sink to the bottom of the slant. Eight or nine c.c of the suspension are drawn off in such a manner that the sediment is not disturbed. The tube is replugged with sterile cotton, its portion above the level of the liquid well exposed to a flame and then placed in a 63°C. water-bath for one hour. The heating is repeated on the next day for the same length of time. The antigen is kept in the ice chest at all future times when not in use.

use.

Period of Incubation--After receiving both antiserum and antigen the tubes are placed in the water-bath for two hours at 37° C, after which the results are read, the tubes removed to the ice chest, allowed to remain there over night (approximately 12 hours) and re-read the following morning.

Discussion.

The titre of the homologous sera was hardly high enough in any case to be regarded as specific. Heterologous spore-producing bacteria were agglutinated to approximately the same extent as the homologous bacteria. Accordingly, these experiments can offer no basis for classification of the bacteria. It is quite possible that this can be obtained thru absorption tests which were not performed in this work.

Only negative results were shown in the titrations of antisera of Bacilli Cereus and Mesentericus-Vulgatus against the non-spore-forming bacteria, Bacilli Coli and Typhosus, Sarcina Lutea and Staphylococcus Albus.

CONCLUSIONS.

1. The agglutination experiments offer no basis of classification for the aerobic spore-producing bacteria.

2. Cross-agglutination is consistently present among the organisms.

3. Immune sera gave negative results with a numberof non-spore producing strains such as Bacillus Coli, Bacillus Typhosus, Sarcina Lutea and Staphylococcus Albus.

GENERAL CONCLUSIONS.

1. Each of the eighteen aerobic spore-producing bacteria studied showed definite physiological differences.

2. There is considerable antigenic similarity among the organisms as evidenced by cross-agglutination reactions.

3. Agglutination tests offer no basis for a classification.

4. A classification based on the reaction to Gram's stain, the blackening of lead acetate, the production of rennet and the reaction to nitrate broth is offered as a means of identifying aerobic spore-producing bacteria.

5. An identification key based on Gram stain reaction and fermentation of dextrose, dextrin and salacin also seems to offer a more satisfactory means of identification than that offered by Ford.

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(10) Zinsser, Hans, Infection and Resistance. (Second Edition.) Chapter IX., pp. 218-247.

Photographs of Bacterial Cultures on Agar.

These photographs are taken from agar plate cultures of the bacteria after an incubation of ninety-six hours at room temperature. Forty-eight hours of this time was caused by the photographer's being unable to photograph the plates sooner. This excess incubation destroyed the delicate growth which is present at the end of forty-eight hours, an ideal time for observing agar plate growth of the Aerobic Spore-Producing Bacteria.

Plate I.

Bacillus Subtilis Bacillus Calvarens.

Bacillus Terminalis Bacillus Niger-Lactis.

Bacillus Albolactus Bacillus Cereus.

Plate II

Bacillus Mycoides Bacillus Potasites.

Bacillus Centrosforous Bacillus Megatherium

Bacillus Anthracis Bacillus Tumescens.

Plate III.

Bacillus Mesentericus-Ruber Bacillus Mesentricus-Fuscus.

Bacillus Mesentericus-Niger Bacillus Mesentericus-Vulgatus.

Bacillus Fusiformis. Bacillus Simplex.

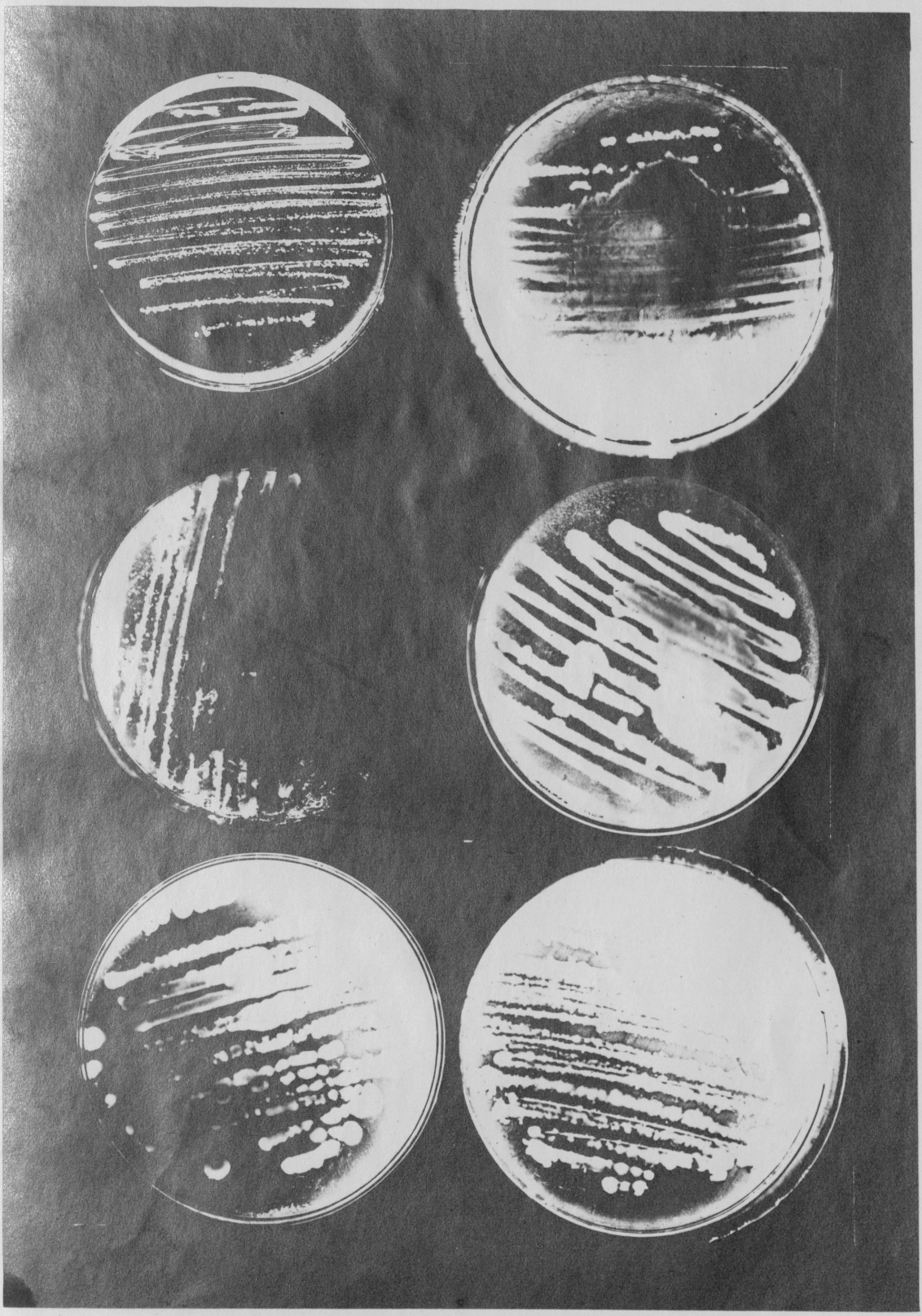

PLATE I

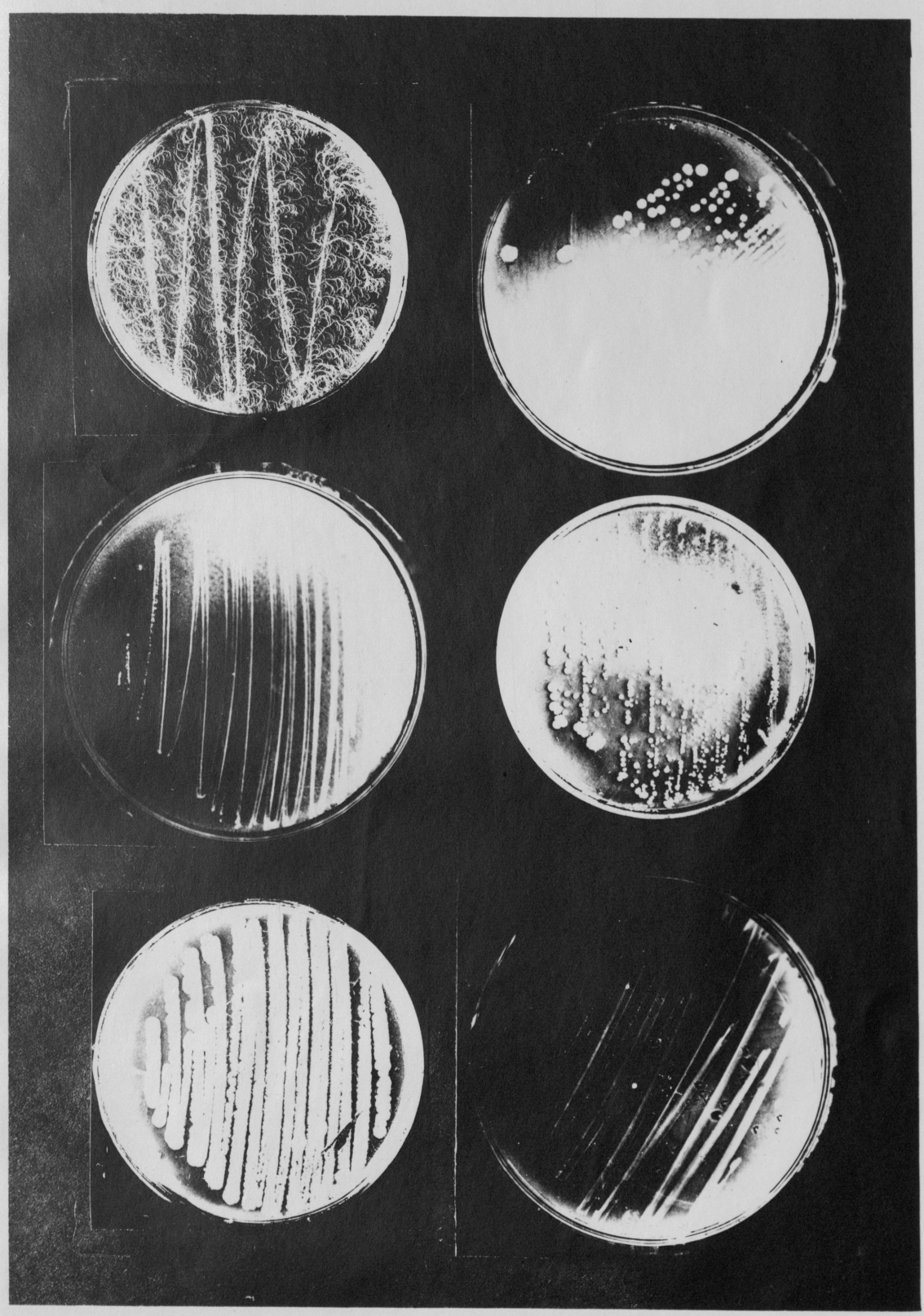

PLATE II

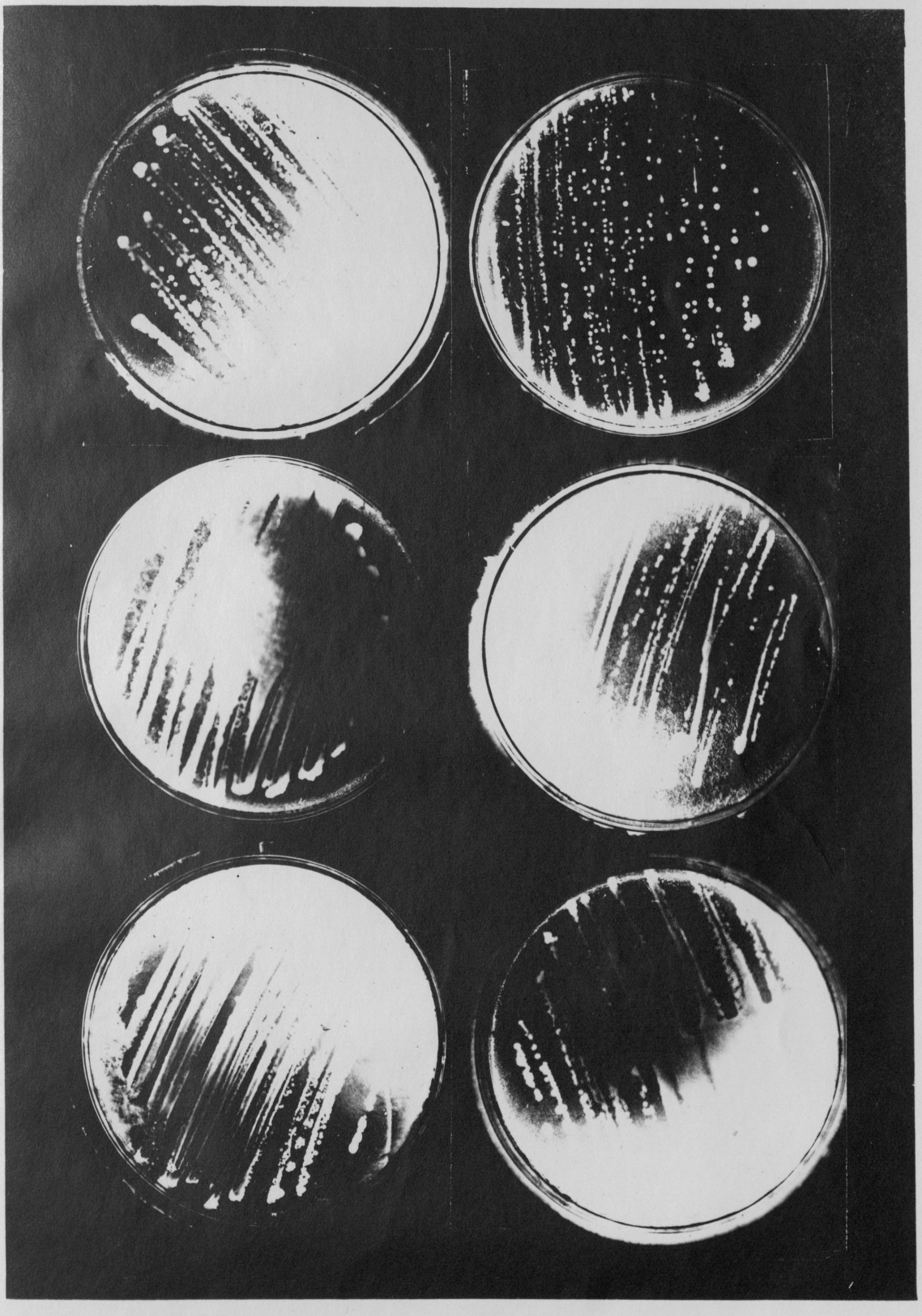

PLATE III