MARINE SPORE FORMING BACTERIA

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By

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Introduction.

This investigation was undertaken with the object of isolating and studying the spore forming bacteria found in the sea, in fish and shell-fish such as herring, clam, lobster, etc. The economic application of the investigation is based on the fact that certain of these organisms are able to resist the heating process to which canned fish are subjected, and when these bring about undesirable changes by their growth, become a source of considerable loss to the fishing industry.

The plan of the work consists in: (1) the isolation of spore formers from the sources mentioned; (2) the selection of those which, because of their high thermal death points and the production of hydrogen sulphide, are likely to be responsible for the spoiling of canned fish; (3) the identification of these forms by a study of their morphological and physiological characters; and (4) an accurate determination of the thermal death points.

Isolation of spore-formers.

The first part of the investigation, namely, the isolation from marine sources of spore-forming organisms, was carried out at the Atlantic Biological Station, St. Andrews, New Brunswick, in July and August, 1921. As organisms in any of

the sea-water animals, and in the sea-water itself, are liable to occur in or upon fish commonly canned, isolations were not confined to the last class only. The author repeatedly isolated similar organisms from as widely different sources as herring, haddock, limpets, clams, and sea-water. Further proof was established when an organism isolated from canned lobster was found to be identical with that isolated from the slime of a haddock.

Plate cultures were made from the contents of the alimentary canal of lobsters, clams, mussels, limpets, flounders, herring, pollock, alewives, and deep water fish caught on the troll-line, such as cod, haddock, and hake. Cultures were also made from sea-water at different stages of the tide, samples of water being taken from the brackish water of the St. Croix below the town of St. Stephens to the more saline waters around the islands in Passamaquoddy Bay, and in the Bay of Fundy.

(a) Technique.

The medium used for all isolation work was standard nutrient agar, made up with sea-water instead of distilled water.

Cultures were always made from fish and shell-fish within a few hours after being caught. Clams and mussels were thoroughly scrubbed under the tap to remove the mud, before being opened. The animal was placed on a sterile surface, opened by instruments previously standing in a jar of 60 per cent. alcohol, and several loopfuls of the intestinal contents transferred with

a flamed platinum needle to sterile water blanks. The instruments were washed, replaced in alcohol, and flamed off, before a second specimen was dissected. Samples of sea-water were obtained in sterile test-tubes plugged with cotton-wool. These tubes of sterile water, with intestinal contents, and sea-water, were placed in a water bath, heated to 80°C., and held at that temperature for ten minutes, in order to destroy vegetative forms.

Agar plate cultures were then made, using 2 c.c. portions of the samples in each petri dish, and incubating at room temperature, or at 37°C. The plates were first incubated at room temperature, because it was thought sea-water organisms might not grow well at higher temperatures. However, it was later found that colonies were as numerous, and growth was more rapid at 37°, so all subsequent cultures were incubated at that temperature.

Stained preparations of all the colonies occurring on the plates were made with Loeffler's alkaline methylene blue, applied to the fixed smear for two or three minutes. This stain is taken up by vegetative cells, and not by spores. When a spore is encased within a vegetative cell, the clear spore and the blue cell are in sharp contrast. Free spores show a narrow rim of blue around the outside of the spore wall. By this method spore-formers occurring on the plates were quickly and efficiently demonstrated. These were transferred to agar slopes, and stored at room temperature.

(b) Results.

At the end of six weeks, a fairly representative collection of the spore-forming bacteria inhabiting the sea-water, fish, crustaceans, and mollusks, in the vicinity of Passomaquoddy Bay, had been made. About twenty obvious duplicates were discarded, and the remaining eighty pure cultures were taken to the laboratory at Macdonald College.

Selection of forms most resistant to heat.

The first method of sterilization of canned fish, adopted in this country in 1839, was three intermittent boilings for periods as long as one and a half hours, with tapping and sealing between each boiling to expel air and produce a vacuum. Now, the prevailing method at fish canneries is a straight three hours boil after packing and sealing, which saves labor, but is not completely efficient, the proof being the large annual losses due to bacteriological spoilage of canned fish. Prominent among the types of spoilage in canned fish, is a blackening of the contents in contact with the cover or sides of the container, due to the action of hydrogen sulphide upon the iron of the can with the production of ferrous sulphide.

Long continued heating tends to disintegrate and darken canned goods, and adds to the expense of canning, so the aim of the canner is to reduce the time of processing as much as possible. Of first economic importance then, was to find out what percentage of the common marine spore-forming organisms would survive three hours boiling, what lengths of boiling they would survive, and whether they were capable of producing

hydrogen sulphide.

(a) Technique.

The eighty organisms were inoculated into beef-broth, and placed in baths of boiling water. Exactly three hours after the water had resumed boiling, the test tubes were removed and placed in the incubator.

In order to test for the production of hydrogen sulphide, the organisms were inoculated into test tubes of Dunham's solution, over which was suspended strips of filter paper soaked in lead acetate solution. Such tubes were incubated for two weeks at 37°C.

(b) Results.

Twelve organisms survived the three hours boiling, and twenty showed production of hydrogen sulphide. These results are concerned with the former group, to which was added five isolations from canned lobster. The canned lobster had been sent from Prince Edward Island, and had been subjected to the regular three hours processing.

Six, out of the twelve heat resisting organisms, showed production of hydrogen sulphide. The twelve were re-tested, along with the five new isolations from canned lobster, on lead carbonate agar, a positive result (a blackening of the medium) being obtained in every case.

Study of morphological and physiological characters for the purpose of classification.

Technique.

For the study of the morphology of the seventeen

organisms, Loeffler's methylene blue was used in all cases except for the determination of flagella. Slides were made from cultures at different periods of their growth in order to follow the development of the spore, and note any irregular forms present in young or old cultures. Drawings were made with the aid of the camera lucida, for the purpose of comparison, accuracy, and record.

Special attention was paid to the method of germination of the spores. For this purpose, an agar culture about three weeks or a month old was used. At this age the culture contained practically nothing but free spores. The spores were smeared with a platinum needle as a thin film over several square centimeters of an ager slant. The sloped tubes were placed in the incubator at 37° . After one and a half to two hours, the first examination was made. The platinum needle was rubbed gently over the surface of the smear, and the adhering material transferred to a drop of water on the slide. The smear was stained in the usual way, and examined. Successive examinations were made every hour until the desired stage was found.

Successful flagella stains were made of each organism using the following method:- To 10 c.c. of a 20 per cent. solution of tannic acid were added 8 c.c. of a cold saturated solution of ferrous sulphate and 1 c.c. of a saturated solution of gentian violet in absolute or 95 per cent. alcohol. Smears were made on the slide and fixed in the usual way, special

care being taken not to overheat. The above mordant was filtered on to the preparations, left for three minutes, washed with water, and stained with anilin gentian violet. The stain was applied for five minutes, during which time the slide was heated several times until the liquid began to vaporize. The mordant, after mixing, was never used for more than two days. This method proved very satisfactory, although some granular precipitate was always present on the slide.

In the study of the physiology of the organism, the standard methods adopted by the American Society of Bacteriologists were followed. Agar and gelatine were adjusted to a P_H of 6.8 to 7.0. All cultures, except gelatine, were incubated at 37°C.

Results.

Spore germination was found to proceed as follows:- After one and a half to two and a half hours, depending on the organism, the spore walls, which, on staining, previously showed only a narrow rim of pale blue, showed now a wider rim of deep blue. The deepening of the blue color of the spore wall sometimes started at one end, sometimes at the side, but usually simultaneously all around the spore. In the second stage, the whole spore began to swell appreciably, accompanied by a faint staining of the central part, either evenly or deeper towards the ends. The whole spore finally stained a deep blue, the spore-wall ruptured, and the tip of the new vegetative cell pushed its way out. In the case of polar

germination, the spore and issuing vegetative cell together had a typical flask shaped appearance. As the contents of the spore passed out into the vegetative cell, the spore case sloughed off in fragments or was left behind entire, retaining the original spore shape or becoming collapsed. The cast spore cases stained very faintly, except in the case of D. The first few cells formed after germination tended to be much larger than those formed later on.

In numbers 48, 62, and 136, the method of germination was not definitely established, for, although germination proceeded as above up to the point where the whole spore stained a deep blue, the next stage seemed to be that of an ordinary vegetative cell, differing only in the fact that it tapered slightly towards one end, and that the ends were more rounded. No sign of cast spore cases was found. Possibly the spore had grown directly into a new vegetative cell without rupturing the spore case.

In general morphological and physiological characteristics, the organisms isolated at St. Andrews corresponded to those described by Russell ('93) at Woods Hole. He mentions the granular appearance of the protoplasm as being very characteristic of marine bacteria. Three out of the twelve heat resisting organisms from St. Andrews exhibited this property conspicuously. He also says the majority of the organism he isolated from the sea-water belonged to the liquefying group, producing a peptonizing enzyme that slowly liquefied gelatine,

and producing ferments that digested the casein in milk, changing the insoluble proteins into soluble peptones. This was found to be true of all the organisms discussed in this paper.

The detailed results of the study of the morphology and physiology of the seventeen organisms are embodied in the key, and in the separate descriptions of each organism which follow. For purposes of identification the references consulted and compared were Russell's two articles, "Untersuchungen uber im Golf von Neapel lebende Bacterien," and "The bacterial flora of the Atlantic Ocean in the vicinity of Woods Hole, Massachusetts"; Migula's "System der Bakterien", Chester's "Manual of Determinative Bacteriology", Matzuschita's "Bacteriologische Diagnostik", and the key of the American Society of Bacteriologists.

Bacterium. Produces endospores. Growth at room temperature. Gram negative. Gelatine liquefied. Chromogenic. Aerobic and facultative aerobic. No gas production. Produces H_2S (Lead carbonate agar). D.

Bacillus.

1. Produces endospores. Aerobic and facultative aerobic, liquify gelatine, non-chromogenic, no gas production, produces H_2S (Lead carbonate agar), non-lactose fermenting. Peritrichous flagella.

A. Rods not swollen at sporulation, germination polar.

B. Dextrose and saccharose + , Glycerine - .

C. Liquefaction of gelatine slow.

D. Gram-positive. Colonies on gelatine circular, zoned, with opaque rims.

E. On agar stroke, growth membraneous. spores formed profusely. U.

EE. On agar stroke, growth viscid or butrycus. 72

DD. Gram-negative. Colonies on gelatine cream, homogeneous in density from centre to border, saucer-shaped liquefaction. 239

BB. Dextrose, saccharose and glycerin + . Gram-negative, germination doubtful.

C. Liquefaction of gelatine slow.

D. Colonies on gelatine with densely opaque

centre, and crinkled ribbon-like, opaque border. 62

DD. Colonies on gelatine with centre filamentous, rim opaque. Sub-surface colonies on agar rhizoid. 136

DDD. Colonies at first a homogeneous, cream-colored hollow of liquefaction, spreading by long painted fingers, or circular and zoned, with cream centre. 48

AA. Rods spindle shape at sporulation, Gram-positive, polar germination.

B. Dextrose, saccharose, and glycerine+ .

C. Liquefaction of gelatine rapid.

D. Gelatine colonies circular, zoned, with opaque border. Gelatine stabs, faint uniform, growth along line of inoculation followed by crateriform liquefaction.

E. Non-chromogenic on potato K, K_2.

EE. Producing red pigment on potato. 246

DD. Gelatine colonies circular, not zoned, but with opaque granular centre. Sub-surface colonies on agar rhizoid. 271

B. Dextrose and saccharose +, glycerin - .

C. Liquefaction of gelatine rapid. Growth alone line of inoculation in stab, slightly villous.

D. Sub-surface colonies on agar crystal-like to

naked eye, under low-power composed of clusters of dense, ovate colonies. 201

CC. Liquefaction slow. Distinctly villous or arborescent growth on gelatine stab.

DD. Sub-surface colonies on agar, rhizoid. Sub-surface colonies on gelatine, stellate. Vegetative cells occur in very long chains. False branching observed. 288

BB. Dextrose and glycerin + , saccharose - .

C. Liquefaction of gelatine rapid.

D. Colonies on gelatine white or cream colored centre, and indistinct border.

E. Sub-surface colonies on agar clouded, granular to naked eye, rhizoid under microscope. Surface colonies have rhizoid projections at the edge. B.

EE. Surface colonies slightly umbonate, usually with curled edges. 145

AAA. Rods clavate at sporulation. Gram-negative. No gas production. Dextrose, saccharose, lactose and glycerin - . Liquefaction of gelatine slow, non chromogenic, hydrogen sulphide production with both lead carbonate agar, and Dunham's solution with lead acetate paper. Peritrichous flagella. 162, 165

Note.- After eight months on an agar slope the spores of the above species isolated at St. Andrews were still viable.

D.

Isolated at Macdonald College, P.Q., November 1921.

Source - canned lobster.

Vegetative cells.

Length 4 - 5μ. Width 1 - 1.4μ. The first rods formed after the germination of the spore tend to be much wider than those produced later on. In young agar cultures (six hours or less) long chains of vegetative cells connected by bridging strands of protoplasm, occur frequently. Older cultures usually contain many shadow forms consisting of enlarged, rounded or irregular, faintly staining vegetative cells. Gram-negative.

Motility.

Non-motile.

Spore formation.

The vegetative cells first assume a granular appearance, then swell slightly towards one end as the spore begins to develop. After 24 hours spore formation has begun, with occasional free spores present; in 48 hours free spores are fairly numerous.

In old cultures chains of vegetative cells often become beaded in appearance, producing groups of round refractile bodies resembling spores. Free spores measure 1.25 - 2μ x 1 - 1.2μ. Germination, equatorial.

Agar stroke.

Thin soft spreading growth, glistening and moist. Color, after 24 hours, light cream, after several days incubation

deepening to Isabellinus dotted with numerous small round yellowish transparent areas, resembling globules of fat. Medium, much browned.

Agar colonies.

Colonies circular, smooth, flat, measuring two to eight m m.in diameter. Edges entire. Color of colonies at first creamish, on further incubation becoming Isabellinus*, and then developing the translucent yellowish spots described in the stroke.

Gelatine stab.

Faint growth along line of inoculation with slow crateriform liquefaction beginning at the surface in three days.

Gelatine colonies.

Colonies small from 1 - 1.5 m m.,in hollows of liquefaction. Distinctly yellowish in color, the central portion being the darkest, followed by a somewhat hyaline zone and opaque border. Under the low power of the microscope the edges are filamentous.

Potato plugs.

Thick yellow shiny growth. Medium much darkened.

Milk.

After two days hard curd formed with extrusion of whey. In litmus milk the surface of the curd assumes a pinkish color. After ten days, complete peptonization.

* Saccardo, P.A. Chromotaxia seu nomenclator colorum. Pata VII. 1894.

Fermentation.

	Dextrose.	Lactose.	Saccharose.	Glycerine.
Gas.	-	-	-	-
Acid.	+	+	+	+

Production of H_2S.

with lead carbonate agar + .

T.D.P. of spores.

1 minute boiling.

Classification.

According to Chester, this organism is a Bacterium of the Anthrax group. It is not in Migula's group of bacteria which produce spores with equatorial germination, neither does it conform to his description of spore forming organisms which produce color. In Matzuschita's ('02) classification, it agrees with Bacillus multipediculus flavus, Zimmermann, except in its ability to grow at blood heat, and upon potato.

162 & 165.

Isolated at St. Andrews, N.B., July, 1921.

source - Mussel.

Vegetative cells.

Length 1.5 - 3μ. Width .6 - .7μ.

Slender rods, usually occurring singly, and as a rule containing deeply staining round granules at the ends or near the centre. Shadow forms larger than the ordinary vegetative cells, crescent, oval or rectangular shaped, and always possessing one or more of the deeply staining granules, are commonly present witnin 36 hours. Gram negative.

Motility.

Very motile.

Flagella.

Peritrichous, ten to twelve per vegetative cell.

Spore formation.

After twenty-four hours only occasional cells forming spores. In 36 nours free spores abundant. Spores are always located at the ends of the rods from which they spring, forming a characteristic drum-stick. The free spores may be round, oval, or hemi-ovoid. Some of the spores retain small tags of protoplasm for a long time in culture, and in this state may have a somewhat triangular appearance. They measure .8 - 1.2μ. The spore wall takes the stain deeply, even in very old cultures.

Agar Stroke.

Raised, glistening contoured growth, at first almost translucent, later growing opaque and deep cream colored.

Agar colonies.

Surface colonies circular or ameboid in form, slightly raised, smooth, glistening, edge entire, or irregular, sending out numerous pseudopodium like processes. Color, brownish cream.

Sub-surface colonies, punctiform, under low power of microscope, ovate in shape.

Gelatine stab.

Growth along line of inoculation echinate. After eight days a slow infundibuliform liquefaction starts. After thirty days, 10 m m. liquefaction in test tube of 15 m m. diameter. The long narrow funnel of liquefaction extending to the bottom of the tube contained a thick spirally twisted membraneous precipitate.

Gelatine colonies.

Surface colonies 5 - 7 m m. in diameter with alternate hyaline and opaque zones. Edge entire. Sub-surface colonies about 4 m m. in diameter. Center faintly cream colored with fuzzy edge.

Potato plugs.

Thick, smooth, shiny growth, somewhat amber in color. Medium grayed.

Milk.

In three days unchanged except slightly more alkaline. In one week coagulated, peptonization commenced, and litmus completely reduced. After twelve days peptonization almost complete, and in litmus milk a greenish scum around the top.

Fermentation.

	Dextrose.	Lactose.	Saccharose.	Glycerine.
Gas.	-	-	-	-
Acid.	-	-	-	-

H_2S Production.

With lead carbonate agar + .

With Dunham's solution and lead acetate paper + .

T.D.P. of spores.

2 minutes boiling.

Classification.

This organism agrees with B. sublanatus, Wright (Chester '01) except in the coagulation of milk and the character of the growth on agar slant.

288.

Isolated at St. Andrews, N.B., August, 1921.

Source - Sea-water.

Vegetative cells.

Length 1.5 - 2.25 μ. Width .6 - .75 μ. Occur in long filaments. False branching observed. In chains of nomogeneous rods, shadow-forms staining faintly in the centre and deeply at the ends, sometimes occur. Gram-positive.

Motility.

Sluggishly motile in beef-broth.

Flagella.

Peritrichous. Up to twelve per vegetative cell.

Spore formation.

Spores formed abundantly in 24 hours at 37° on plain agar. They are slightly ex-central, and are wider than the vegetative cells from which they spring. Size of free spores .7 x 1 μ. Polar germination.

Agar stroke.

Abundant, flat, somewhat glistening, opaque growth. Central portion of stroke punctate. Edge grown out into numerous filaments.

Agar colonies.

Surface colonies show a tendency to spread in rhizoid manner a from small, round, central portion. Surface somewhat punctate, or in non-spreading colonies, slightly wrinkled, edges fuzzy and indefinite. Internal structure,

coarsely granular. Sub-surface colonies irregularly round, with internal structure resembling a tangled mass of roots, and edges marked by numerous irregular rhizoid extensions.

Gelatine Stab.

Villous growth along the lines of inoculation, followed by a slow stratiform liquefaction. After 30 days at room temperature about one-quarter of the tu be of gelatine was liquefied.

Gelatine colonies.

surface colonies have round dense creamish centres about 2 m m. in diameter, surrounded by a clear zone of liquefaction. Sub-surface colonies measure .75 to 1.5 mm. and are composed of oval creamish centres with delicate prickly radiations giving the whole colony a stellate appearance.

Potato plugs.

Thick cream growth with granular appearance. Medium much darkened.

Milk.

After three days milk unchanged. After 10 days there is some peptonization, a slight reduction of the litmus, and a granular curd.

Fermentation. (Sugar-free bouillon + 1% sugar + 1% Andrade's indicator.

	Dextrose.	Lactose.	Saccharose.	Glycerine.
Gas.	-	-	-	-
Acid.	+	-	+	-

Production of H_2S.

With lead carbonate agar +.

With Dunham's solution and lead acetate paper + .

T.D.P. of spores.

10 minutes boiling.

Classification.

This organism corresponds with the description of Bacillus licheniformis Weigmann, as given in Chester ('01), except tnat it is gram-positive, and that growth on potato is not slimy.

B. 145, 271.

Isolated at Macdonald College, January, 1922.

Source - Canned lobster, B.

Isolated at St. Andrews, N.B., July, 1921.

Source - Pollock, 145.

Source - Sea-water 271.

This type occurred very commonly in sea-water, deep-water fish, and shell fish. Three strains from widely different sources were studied, and small differences recorded.

Vegetative cells.

Length 2 - 3 μ. Width 1 - 1.2 μ. Large homogeneous rods with ends rounded or square, usually occurring in long chains, in young cultures often connected by bridging strands of protoplasm. Shadow forms may appear, much larger than the ordinary rods, and taking the stain irregularly or faintly. These often occur in pairs, each cell becoming hemi-ovoid in shape, or singly, becoming rounded, or retaining the rectangular shape. Gram positive.

Motility.

Sluggishly motile, in long chains. Lashing movement.

Flagella.- Peritrichous.

Spore formation.

In strains 145 and 271 the vegetative cells become conspicuously granular, and slightly wider, in the first stage of spore formation. In B, the granulation is not as

conspicuous, and is sometimes absent. In all cases spore formation begins within 24 hours, but free spores are not present in abundance until 48 hours. Spores are in central or sub-central position in the mother cell. When free, they measure 1.25 - 2.25μ x 1 - 1.2μ . Germination polar.

Agar stroke.

Thick, moist, glistening, creamish growth, with irregular edge, along line of inoculation. Smooth or contoured. Consistency at first butyrous, gradually becoming softer. In old cultures the strokes often have a milky looking appearance.

Agar colonies.

Surface colonies B. and 271 irregularly circular, smooth, glistening, raised or convex, with rhizoid projections giving the edge a fuzzy appearance. Sub-surface colonies have clouded or granular appearance to the naked eye. Under the low power of the microscope they resemble a tangled mass of fine roots. The surface colonies of 145 are more regularly round, with slightly umbonate elevation, and either curled or fuzzy edges. Sub-surface colonies, punctate, ovate, or small, round with fuzzy edges and finely granular internal structure.

Gelatine stab.

Rapid infundibuliform liquefaction, complete in eight days with formation of a heavy membranous precipitate.

Gelatine colonies.

The colonies rest in saucer-like excavations caused by liquefaction. They have a distinctly white or creamish centre,

followed by a hyaline zone, and a faintly opaque rim. Internal structure finely granular, edge entire, but not sharply defined.

Potato plugs.

Thick, dull, creamish growth. May be smooth or slightly rugose. Medium grayed.

Milk.

In 24 hours at 37°C. a firm curd formed, peptonization began, and reduction of litmus started. After seven days complete peptonization.

Fermentation.

	Dextrose.	Lactose.	Saccharose.	Glycerine.
Gas.	-	-	-	-
Acid.	+	-	-	+

The production of acid in glycerinewas very weak. 271 produced acid in saccharose bouillon.

Production of H_2S.

With lead carbonate agar + .

T.D.P. of spores.

145 & 271 - 2 minutes boiling.

B. - 3 minutes boiling.

Classification.

This organism corresponds with the diagnosis of Bacillus cereus, Frankland, as given in Matzuschita ('02), but differs from Chester's ('01) description in the absence of outgrowths in gelatine colonies, the opaqueness on agar slant, and the cream color of the potato growth.

K, K_2, 201.

Isolated at Macdonald College, P.Q., November, 1921.

Source - Strains K and K_2 canned lobster.

Isolated at St. Andrews, N.B., August, 1921.

Source - Strain 201 Limpet.

Vegetative cells.

Length 2 - 3.3 μ. Width .7 - .8 μ.

Homogeneous rods with slightly rounded ends. Shadow forms present in old cultures. Gram-positive.

Motility.

Motile.

Flagella.

Peritrichous.

Spore-formation.

Free spores are produced abundantly on agar in 24 hours at 37°C. They are formed centrally or ex-centrally with slight enlargement of the mother cell. Free spores are oval, and measure 1.2 - 1.6 μ x .75 - .8 μ. Polar germination.

Agar stroke.

Pale cream glistening growth, slightly raised and spreading, with irregular edges. The surface is thrown up into distinct ridges, which are usually more or less reticulate. Consistency, butyrous.

Agar colonies.

Surface colonies are smooth and glistening or dull and finely punctate, spreading or circular. The circular colonies

may be faintly zoned, with a less dense central area, and edges appearing entire to the naked eye, or they may be uniformly opaque from centre to border. Occasionally, some colonies have centres finely rugose and more opaque than the borders.

The sub-surface colonies are of two types, first punctiform and crystal-like, under the low power of the microscope composed of clusters of dense ovate colonies, or small, round and faintly opaque, with edges entire. Internal structure, finely granular.

Gelatine stab.

Uniform growth along line of inoculation, followed by rapid saccate liquefaction, and formation of a surface pellicle.

Gelatine Colonies.

Colonies resting in saucer-like hollows of liquefaction, internal structure finely granular.

K. Opaque rim, with remainder of colony hyaline or with central opaque dot, edge entire.

K_2. Surface colonies may have a ring of opaque spots around rim, and faintly opaque, more coarsely granular centre. Sub-surface colonies are distinctly zoned. Edge entire.

201. Surface colonies have opaque edges, followed by a hyaline zone and opaque, more granular centre.

Potato plugs.

Thick creamish growth, surface both verrucose and rugose. Medium darkened to a greyish brown. In 201 the growth

is more rugose and less verrucose than in the other two.

Milk.

K. and K_2. After 24 hours at 37°C. a hard curd formed, and peptonization began. Peptonization complete in one week.

201. After 48 hours at 37°C. a soft curd formed, and peptonization began. Peptonization complete in one week.

Fermentation.

	Dextrose.	Lactose.	Saccharose.	Glycerine.
Gas.	-	-	-	-
Acid.	+	-	+	+

In strains K and K_2 the production of acid in glycerine is very weak. In strain 201 it is absent.

Production of H_2S.

With lead carbonate agar + .

T.D.P. of spores.

seven minutes boiling.

Classification.

This organism exactly corresponds with the short description of B. granulosus, Russell, given by Matzuschita. Russell ('93) in his original description, however, lays stress on the granular condition of the protoplasm, but this condition was not noticeable in the cultures under consideration.

246.

Isolated at Bliss Island in the Bay of Fundy, N.B., August, 1921.

Source - Sea-water.

Vegetative cells.

Length 2 - 3.5 μ . Width .65 - .75 μ . Homogeneous rods with slightly rounded ends, occurring singly or in short chains. Gram-positive.

Motility.

Very motile, tumbling or boring.

Flagella.

Peritrichous.

Spore-formation.

Spores are formed freely within 24 hours. They are cylindrical, central or ex-central, produced with slight enlargement of mother cell. When spore is completely free from adhering tags of protoplasm the ends appear slightly rounded. Free spores measure 1.1 - 1.3 μ x .65 - 8. μ . Germination polar.

Agar stroke.

Flat, dull, rugose, deep cream or ecru growth spreading widely from line of inoculation at base of tube, where water of condensation present. Edges irregular, consistency, slightly viscous or butyrous.

Agar colonies.

Surface colonies smooth and spreading, or small, rugose and irregularly round. Edge slightly undulate or ragged, with finely granular internal structure. Sub-surface colonies

punctiform, appearing as dense irregular masses under the hand lens; or small, circular, nomogeneous and faintly opaque.

Gelatine stab.

Uniform growth along line of inoculation with liquefaction also along entire line. The liquefaction becomes saccate, and is complete in about 10 days.

Gelatine colonies.

Circular colonies set in saucer shaped hollows of liquefaction. They have an opaque dot in centre followed by a hyaline zone and a hazy, faintly opaque rim.

Potato plugs.

Thick, deep cream or ecru, verrucose growth. After twenty-four hours the liquid in the bottom of the tube becomes the color of dilute blood. After forty-eight hours the potato itself assumes a pale blood-red tinge along the margin of advancing growth. The part beneath the older growth becomes grey with a faint pinkish tinge.

Milk.

After three days soft curd formed at bottom, with an amber colored peptonized zone at the surface. Litmus is reduced. Peptonization continues until in two weeks the entire milk tube is converted into an amber fluid.

Fermentation.

	Dextrose.	Lactose.	Saccharose.	Glycerine.
Gas.	-	-	-	-
Acid.	+	-	+	+

Production of H_2S.

with lead carbonate agar + .

T.D.P. of spores.

Seven minutes boiling.

Classification.

This organism agrees with B. vitalis (Chester '01) (= B. mesentericus - ruber, Globig, Matzuschita '02) except in the character of the growth in the gelatine stab, and in the production of H_2S.

C.

Isolated at Macdonald College, January, 1922.

Source - Canned lobster.

Vegetative cells.

Length 2 - 3 μ . Width .6 - .65 μ .

Slender homogeneous rods with rounded ends usually occur singly, sometimes in short chains of three or four. In cultures several days old shadow forms appear, larger than the original cell, and made up of faintly staining protoplasm with sometimes deeply staining particles at the ends, towards the centre, or at the periphery.

Motility.

Moderately motile, showing both tumbling and lashing motion.

Flagella.

Peritrichous.

Spore formation.

Free spores formed abundantly in 24 hours on agar. Spores are formed either centrally or towards one end, without bulging of the rod. They measure 1 - 1.5 μ x .6 - .75 μ . Germination polar.

Agar stroke.

Flat, dull cream-colored growth, covered with irregular network of fine raised lines. In consistency membranous.

Agar colonies.

Surface colonies may be round and smooth with edges entire, or they may be irregularly round, wrinkled, with undulate or lobate margins, showing in some cases a tendency to spread. Sub-surface colonies, small, round, faintly opaque, or irregular, clouded and crystalline in appearance.

Gelatine Stab.

Faint growth along line of inoculation, a rapid, saccate liquefaction beginning at the surface in two days, complete in three weeks.

Gelatine colonies.

Gelatine colonies circular, crateriform, edge entire. They have a white central dot, surrounded by an opaque zone of granular appearance, then a hyaline zone bordered by an opaque rim, consisting of more densely packed bacteria.

Potato plugs.

Thick, dry, rugose, and verrucose growth, showing faint pink tinge. Medium browned.

Milk.

Soft curd formed at the bottom with layer of peptonization at the surface. Complete peptonization in 10 days.

Fermentation.

	Dextrose.	Lactose.	Saccharose.	Glycerine.
Gas.	-	-	-	-
Acid.	+	-	+	-

Production of H_2S.

With lead carbonate agar + .

T.D.P. of spores.

2 hours 10 minutes boiling.

Classification.

This organism does not conform to any spore-forming, motile, gelatine liquefying species described by Migula ('00), Chester ('01), Matzuschita ('02) or by Russell ('91). It is probably a new species.

72.

Isolated at St. Andrews, N.B., July, 1921.

Source - Herring.

Vegetative cells.

Length - 1.5 - 2.5μ. Width .6μ.

Homogeneous rods, either straight or slightly curved, with rounded ends. Shadow forms are abundant in a 24 hour culture, as well as in very old cultures. Gram-positive.

Motility.

Very motile.

Flagella.

Peritrichous.

Spore formation.

Spore formation proceeds slowly, and spores are never produced abundantly. In 24 hours only very occasional rods are forming spores, and no free spores are present. In 48 hours a few free spores are present. In old cultures (three weeks or more) the majority of vegetative cells seem to have lost their protoplasm, and turned into shadow forms, leaving a small percentage of spores. Oval spores are produced centrally or ex-centrally without distortion of mother cell, and measure 1.1 - 1.2μ x .6 - .65μ. Germination, polar.

Agar stroke.

Flat, or slightly raised, glistening, opaque growth, spreading somewhat from line of inoculation towards the bottom of tube, with irregular edges. Surface, either smooth, or

delicately rugose, depending on moisture content of medium. Color, cream. Consistency, butyrous, or, in young cultures, slightly viscid.

Agar colonies.

Surface colonies glistening, opaque, finely granular, with smooth margins and rugose centres, or sometimes spreading by stubby fingers. The edges are definite and entire. Under the low power there are distinct reticulations radiating from centre, and corresponding to the wrinkling on the surface. Sub-surface colonies, small, circular, opaque, finely granular, with edges entire, or punctiform and irregular.

Gelatine Stab.

Uniform growth along line of inoculation, followed by slow crateriform liquefaction, complete in three weeks.

Gelatine colonies.

The colonies on gelatine have a dense central portion, to the naked eye, resembling ragged floating pieces of skin, under the microscope somewhat filamentous.

Potato plugs.

Cream-colored, spreading growth, smooth or rugose. Medium slightly greyed.

Milk.

After three days at 37°C. unchanged. After seven days, a soft curd produced, peptonization started, and litmus completely reduced. After two weeks, peptonization complete.

Fermentation.

	Dextrose.	Lactose.	Saccharose.	Glycerine.
Gas.	-	-	-	-
Acid.	+	-	+	-

Production of H_2S.

With lead carbonate agar + .

T.D.P. of spores.

Four minutes boiling.

Classification.

This organism does not conform to any spore-forming, motile, gelatine-liquefying species described by Migula ('00), Chester ('01), Matzuschita ('02), or by Russell ('91). It is probably a new species.

239.

Isolated at St. Andrews, N.B., August, 1921.

Source - Lobster.

Vegetative cells.

Length 1.5 - 2.2μ . Width .5 - .7μ .

Slender nomogeneous rods, with slightly rounded ends, usually occurring singly. Gram negative.

Motility.

Extremely motile.

Flagella.

Peritrichous.

Spore formation.

Spores may be formed centrally or ex-centrally, without distortion of mother cell. Spore formation starts within 24 hours, but at this time no free spores are present. Free spores are oval in shape and measure 1 - 1.2μ x .6 - .7μ . Germination polar.

Agar stroke.

Smooth, glistening, opaque growth, spreading little from line of inoculation. Cream color, later assuming a pale ecru tinge. During the first four months of culturing, the consistency was always slightly viscid in young cultures. In later culturing it lost this property, and became butyrous.

Agar colonies.

Surface colonies may be smooth, glistening and spreading from a central nucleus by stubby fingers; or circular

with an inconspicuous depressed ring near the margin and a slightly umbonate centre. Sub-surface colonies are punctiform, irregular, or circular, opaque, 1 - 1.5 m m. in diameter. The edges in all cases are definite and entire.

Gelatine Stab.

Slight uniform growth along entire line of inoculation, followed by a slow crateriform liquefaction. At the end of three weeks liquefaction almost complete, with a flocculent precipitate.

Gelatine colonies.

Surface colonies, circular, homogeneous, cream in color, finely granular internal structure, edge entire; set in saucer-shaped hollows of liquefaction.

Sub-surface colonies irregularly circular, dotted in appearance, with filamentous edges.

Potato plugs.

Thick creamy grey growth, finely rugose, fairly flat. Medium, much darkened.

Milk.

After seven days unchanged. Litmus completely reduced.

Fermentation.

	Dextrose.	Lactose.	Saccharose.	Glycerine.
Gas.	-	-	-	-
Acid.	+	-	+	-

Production of H_2S.

With lead carbonate agar + .

With Dunham's solution and lead acetate paper + .

T.D.P. of spores.

Two minutes boiling.

Classification.

This organism does not conform to any spore-forming, motile, gelatine-liquefying species described by Migula ('00), Chester ('01), Matzuschita ('02) or by Russell ('91). It is probably a new species.

62 and 136.

Isolated at St. Andrews, N.B., July, 1921.

Source - 62, Haddock.

136, Cod.

Vegetative cells.

Length 1.5 - 2.8μ . Width .6 - .7μ .

Homogeneous rods with slightly rounded ends. Shadow forms present in old cultures. Gram-negative.

Motility.

Motile.

Flagella.

Peritrichous.

Spore formation.

Spores are formed abundantly within 24 hours, either centrally or towards one end of the vegetative rod, with little or no distortion. They are oval in shape and measure 1 - 1.3μ x .6 - .7μ . Germination, doubtful.

Agar stroke.

Moderate, smooth, glistening, opaque, cream-colored, flat growth, spreading little from line of inoculation except at the base. Occasionally the surface is faintly punctate. Consistency, butyrous.

Agar colonies.

Surface colonies are smooth, glistening, opaque, and usually spreading. The non-spreading colonies are circular,

convex or slightly umbilicate, very occasionally showing a tendency to wrinkle in the centre. Edge entire.

Sub-surface colonies.

62. - They may be of two types, punctiform, dense, cream, and ovate, or circular and faintly opaque, with edges entire.

136. - The majority of the colonies are rhizoid, looking ragged and fuzzy to the naked eye. There occurs also the circular and faintly opaque colony, with entire edge.

Gelatine Stab.

Faint uniform growth along line of inoculation, followed by a crateriform liquefaction, beginning at the surface, and complete in 30 days at room temperature.

Gelatine Colonies.

62. - Colony circular, set in saucer shaped hollow of liquefaction. The centre is round, cream color, finely granular, followed by a less dense area, and then a crinkled, ribbon-like, opaque border. In some colonies the central portion is irregular in shape, and followed by a faintly opaque zone, under the low power of the microscope shown to be composed of irregular floating clumps of bacteria.

136. - Surface colonies have a slightly opaque central zone, filamentous in character, followed by a hyaline zone, and opaque rim.

Sub-surface colonies are 2 to 5 m m. in diameter, and are distinctly zoned, with a creamish centre.

Potato plugs.

Thick, spreading, rugose, creamy-grey growth. Medium darkened.

Milk.

After four days, a soft curd formed, peptonization begun, and litmus reduced. After ten days, completely peptonized.

Fermentation.

	Dextrose.	Lactose.	Saccharose.	Glycerine.
Gas.	-	-	-	-
Acid.	+	-	+	+

Production of H_2S.

With lead carbonate agar, 62 and 136 + .

With Dunham's solution and lead acetate paper, 62 + .

T.D.P. of spores.

Four minutes boiling.

Classification.

This organism does not conform to any spore-forming, motile, gelatine-liquefying species described by Migula ('00), Chester ('01), Matzuschita ('02), or by Russell ('91). It is probably a new species.

48.

Isolated at St. Andrews, N.B., July, 1921.

Source - Jelly-fish.

Vegetative cells.

Length 2 - 3μ . Width .5 - .6μ . Slender, homogeneous rods, with slightly rounded ends. Occasional shadow forms present after 24 hours at 37°.

Motility .

Very motile.

Flagella.

Peritrichous.

Spore-formation.

Spores are formed either centrally or towards one end of the mother cell. They are formed abundantly within 24 hours, and are oval, measuring 1μ x .55 - .6μ. Germination, doubtful.

Agar stroke.

Moderate, smooth, opaque, cream-colored, flat growth, spreading little from line of inoculation except at the base. Consistency, butyrous.

Agar colonies.

Surface colonies are smooth, glistening, opaque, as a rule, widely spreading. The non-spreading colonies are circular, convex, or sometimes slightly umbilicate, with an inconspicuous wrinkling in the centre. Edges are entire or undulate. Color, cream.

Sub-surface colonies punctiform, dense, creamish, and irregular.

Gelatine stab.

Faint uniform growth along line of inoculation, followed by a crateriform liquefaction beginning at the surface, and complete in 30 days.

Gelatine colonies.

The colonies first appear as a homogeneous, cream-colored hollow of liquefaction, which spreads rapidly from this central nucleus by long irregular pointed fingers. Some of the colonies remain circular and increase uniformly in diameter, still retaining the cream-colored centre, and becoming zoned in appearance.

Potato plugs.

Thick, creamish, rugose growth. Medium slightly greyed.

Milk.

After one week at 37°C., no change, except slight reduction of litmus. After two weeks litmus completely reduced, solid curd formed, and no peptonization.

Fermentation.

	Dextrose.	Lactose.	Saccharose.	Glycerine.
Gas.	-	-	-	-
Acid.	+	-	+	+

Production of H_2S.

With lead carbonate agar + .

With Dunnam's solution and lead acetate paper + .

T.D.P. of Spores.

Five minutes boiling.

Classification.

This organism does not conform to any spore-forming, motile, gelatine-liquefying species described by Migula ('00), Chester ('01), Matzuschita ('02), or by Russell ('91). It is probably a new species.

Determination of Thermal Death Points.

Technique.

For the determination of the thermal death point, cultures were used which had been incubated for 36 to 48 hours at 37°, and stored at room temperature from one to two weeks. Young growth on agar slants often tended to be viscid or membranous, but with age became more friable and granular. The latter state was much preferable from the standpoint of obtaining spore suspensions free from large clumps of spores. As will be pointed out later, the difference in age would not affect the thermal death point.

A heavy suspension of spores was made in sterile distilled water, triturating the material with a platinum needle against the side of the tube in order to break up the spore clumps. A direct microscopic examination of this spore suspension showed that even after the most careful trituration with the needle, clumps of spores were still present. The size varied with the consistency of the original inoculum. In organisms like C. where the growth always remained fairly membranous there were several clumps of from ten to fifty spores in every field, frequent clumps of 500, and occasional clumps too numerous to count, probably containing one or two thousand spores. In spore suspensions of organisms showing a soft butyrous growth, numerous clumps containing from nine to fifty spores were present in every field. When a serious difference in the thermal death point of

a single spore and spores in clumps was found, further treatment was given the spore suspension to rid it of clumps. Two methods were followed, and their relative$ efficiency estimated by direct microscopical examination. The first meth od was mentioned by Harriette Unick ('08), namely, centrifuging the spore suspension for several minutes, and pipetting off the supernatant liquid. The second method was filtering the suspension through filter paper. (In this work No. 1 Whatman filter paper, prepared by W. & R. Balston, Ltd., was used). The closeness of the weave of the filter paper would probably affect the results. Organisms showing both membranous and granular growths were chosen for the test. Portions of the same spore suspension were centrifuged for three, five and ten minute periods at about 3600 revolutions per minute, and filtered once and twice through filter paper. A loopful of the suspension after each treatment was transferred to a slide and allowed to evaporate without spreading. The residue was stained, the number of clumps per field, and the average number of spores in the clumps recorded.

The results showed that absolute efficiency was not obtained by either methods. Both, however, greatly reduced the size and number of clumps present in the suspension. Continued centrifuging lowered the total number of spores in the supernatant liquid, tending to throw out the clumps, rather than to break them up. When chains of vegetative cells were present along with the spores, centrifuging gave rather erratic results. These chains tend to wrap around clumps of spores, and owing to

their lower specific gravity, to prevent the clump from being thrown to the bottom of the tube. Filtering twice also had the effect of reducing the total number of spores present, without altering the size of clumps very materially. One filtering showed greater total efficiency in reducing the number and size of clumps than centrifuging for ten minutes. This was more marked in the spore suspensions from a membraneous growth than in that made from a granular or butyrous growth . In the case of the last two, the size of the spore clumps ranged from three to twelve in both centrifuged and filtered suspensions. when growth was membranous the number of spores per clump in centrifuged material varied from three to fifty. In the filtered, the average number per clump was about ten, but one clump of forty was observed, showing the possibility of clumps of that size passing through the filter paper. Centrifuged and filtered material were compared experimentally in several cases, resulting in no marked difference of the thermal death point.

As filtering was shown by the microscopic method to be slightly more efficient, and as it required much less time, this method of treating spore suspensions was adopted.

Two c.c. portions of the filtered spore suspension were transferred with sterile capillary pipettes to long necked, thin walled glass bulbs of about four c.c. capacity. The bulbs were sealed in the blow-flame, and placed in wire baskets. The baskets were inverted into baths of boiling water in order that the bulbs would be kept completely submerged. One half minute after the water resumed boiling, the spores were assumed to be at

the temperature of boiling water, and the time of exposure was counted from that second. The bulbs were removed at successive periods, at first long, and gradually narrowing down to one minute periods, as some idea of the probable thermal death point was obtained. The neck of the bulb was dipped in alcohol, the top snipped off with a pair of pincers, also dipped in alcohol, and the boiled contents transferred with a sterile capillary pipette to duplicate agar slopes. The agar slopes were incubated and examined up to the end of five days, for the presence or absence of growth. (Both bulbs and capillary pipettes were made by the author ,using glass rods, and the blow-flame.)

Results.

The results obtained without filtering the spore suspension were extremely inconsistent, as will be shown by the following list of positive results in two tests.

Time boiled -	½ hour		1 hour.		1½ hrs.	2 hours		2½ hrs.		3 hrs
	1st. test.	2nd. test.	1st. test.	2nd. test.	One test.	1st. test.	2nd. test.	1st. test.	2nd. test.	
Growth present from numbers-	C	C	201	201	C	C	C	C	C	C
	288	288	K_2	K_2	246	K_2	K_2	K	K	
	201	201	C	C	B	201	201	288	K_2	
	K	K	246		K					
	62	72	K		201					
	B	B	288							
	145	145	136							
	239	239								
	K_2	48								
	136	246								

A special test was made of C, allowing spore clumps of large size to be present in the suspension. Growth was obtained after boiling seven hours. No longer period was tried.

The filtered material gave unexpected results. The thermal death point was found to range from one to ten minutes boiling in all organisms except C, in which it was over two hours. A comparison of these results with the above will show the serious effect of clumping in raising the thermal death point of spores. Evidently the central spores in the clumps are greatly protected. As was mentioned previously, all these organisms had survived either three hours boiling in beef broth, or the three hour processing given the canned lobster. Although these results were not obtained four months later, that is, only three organisms survived three hours boiling, two explanations might be given. The first is loss of vitality due to continued culturing on artificial media. The second is much more probable, that is, the organisms freshly isolated from their natural habitat tended to show much tougher and more viscous growth than they did after four months continued cultivation. Their survival at higher temperatures was no doubt due to the fact that clumps could not be as efficiently broken up by the platinum needle.

Another important point is the astonishing effect of an increase of several minutes in the thermal death point of the individual spores in prolonging the time required for their destruction in clumps. Spores with a thermal death point of two

minutes seldom survived over half an hours boiling in clumps, those with a thermal death point of four to five minutes, lived after one hour's boiling in clumps, while spores with a thermal death point of seven to ten minutes were capable of growth after two to two and a half hours boiling in clumps.

If the time required for the destruction of spores in clumps was increased proportionately in organisms like C, in which the thermal death point of individual spores was over two hours, then sterilization by boiling temperature would not be practicable. Probably, tests made of the death-point of spore-bearing bacteria in the presence of clumps accounts for the high thermal death points given in articles like W.W. Ford's "Studies on aerobic spore-bearing, non-pathogenic bacteria".

In repeating the determination of the thermal death point several times, absolutely consistent results were not obtained. Occasionally, variations of one to two minutes occurred. This inconsistency may have been due to a difference in concentration of spores (Chick '08) or by an individual variation of the spores of one organism in their power to resist heat.

Experiments were tried with cultures of different ages, to test the effect upon the thermal death point. The experiments were too few to be conclusive, but no constant difference was found between cultures forty-eight hours, two weeks, or two months old. The indications were that spores lose their vitality very slowly.

Conclusions.

The conclusions drawn from this study of marine spore forming bacteria are:-

1. Spore forming bacteria are found commonly in sea-water, and in the alimentary tract of fish, crustaceans and mollusks.

2. The majority are capable of producing hydrogen sulphide, and consequently, if they are not killed by sterilization, may produce the blackening of canned fish.

3. The three hours boiling given to canned fish is far more than is necessary to kill the isolated spores of the organisms commonly found in the sea.

4. The growth of freshly isolated cultures is far more viscous or membraneous than that of the later cultures, transferred twenty to thirty times, therefore the tendency to clumping or massing of the spores is probably quite marked in the natural habitat.

5. The thermal death point of spores in clumps is extremely high in comparison with that of the individual spore, and may often exceed three hours boiling. Consequently, one of the principal factors in the bacteriological spoiling of canned fish is the inefficient method of attempting sterilization by boiling.

Acknowledgments.

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Explanation of plates.

N.B. The number or letter beneath each group of drawings is the same as that under which the organism illustrated in the figure is described in the text.

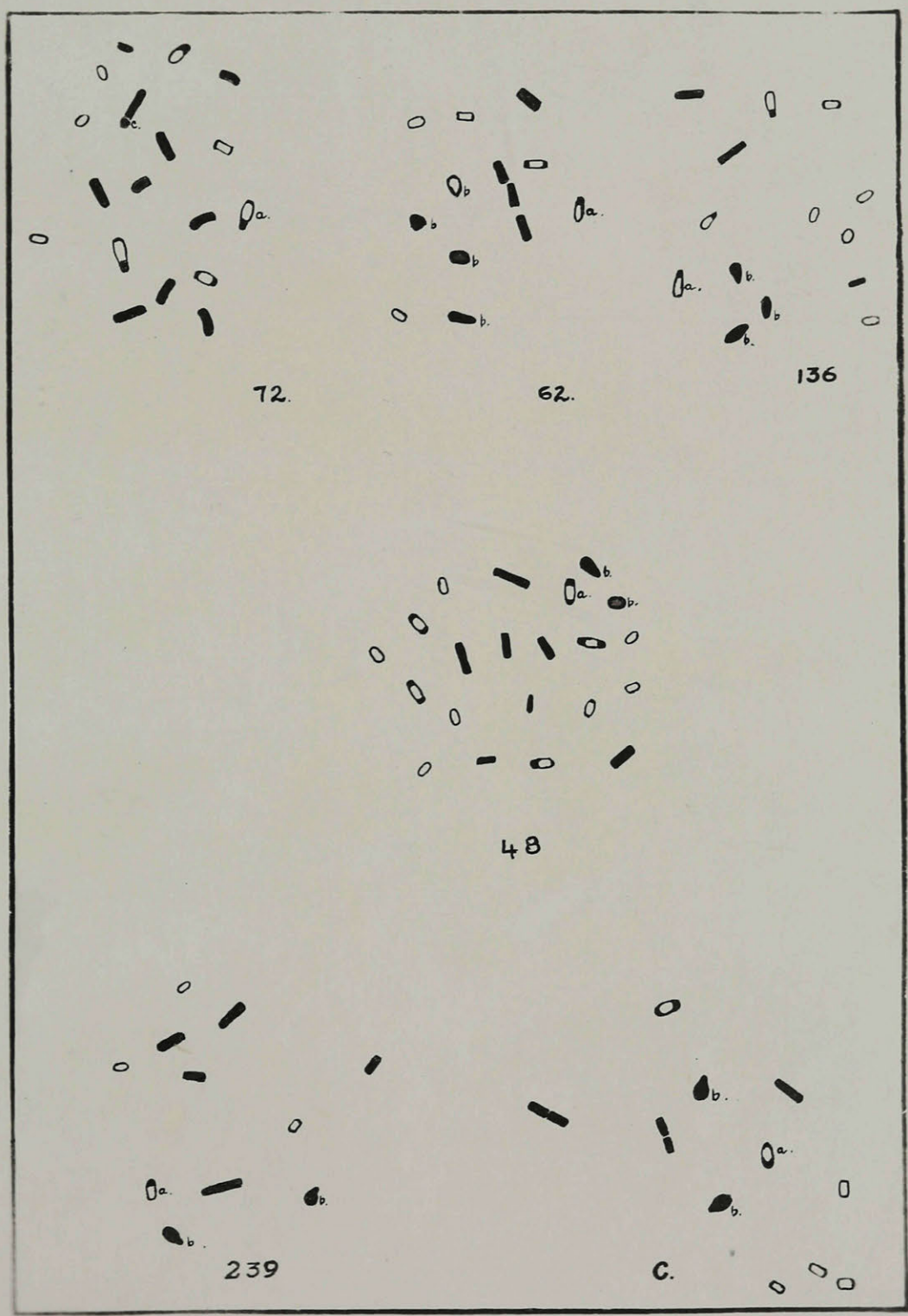
Reference lettering -

a. Spore in formation.

b. Spore germinating.

c. Cast spore case.

All figures magnified 1700 diameters.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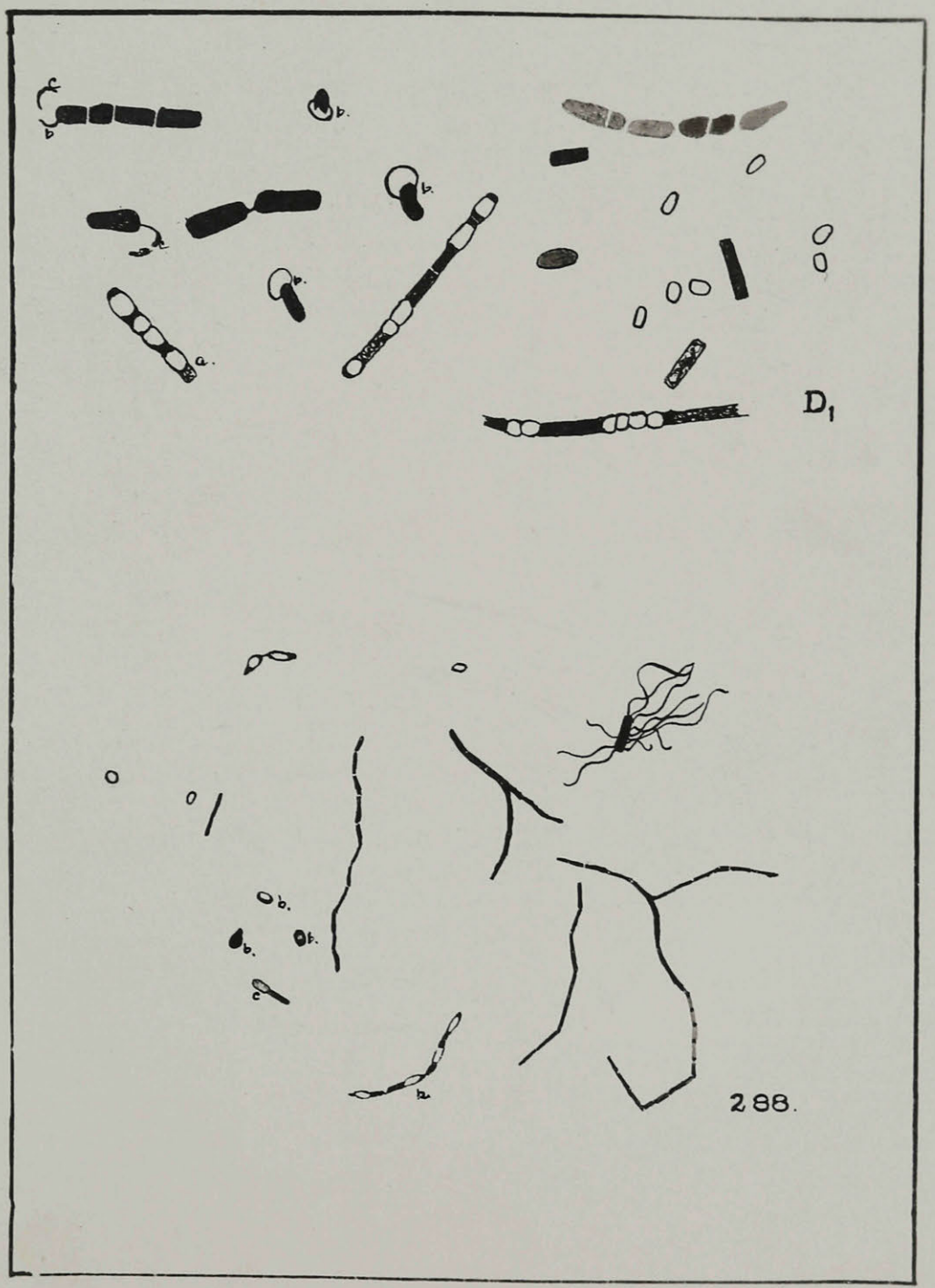
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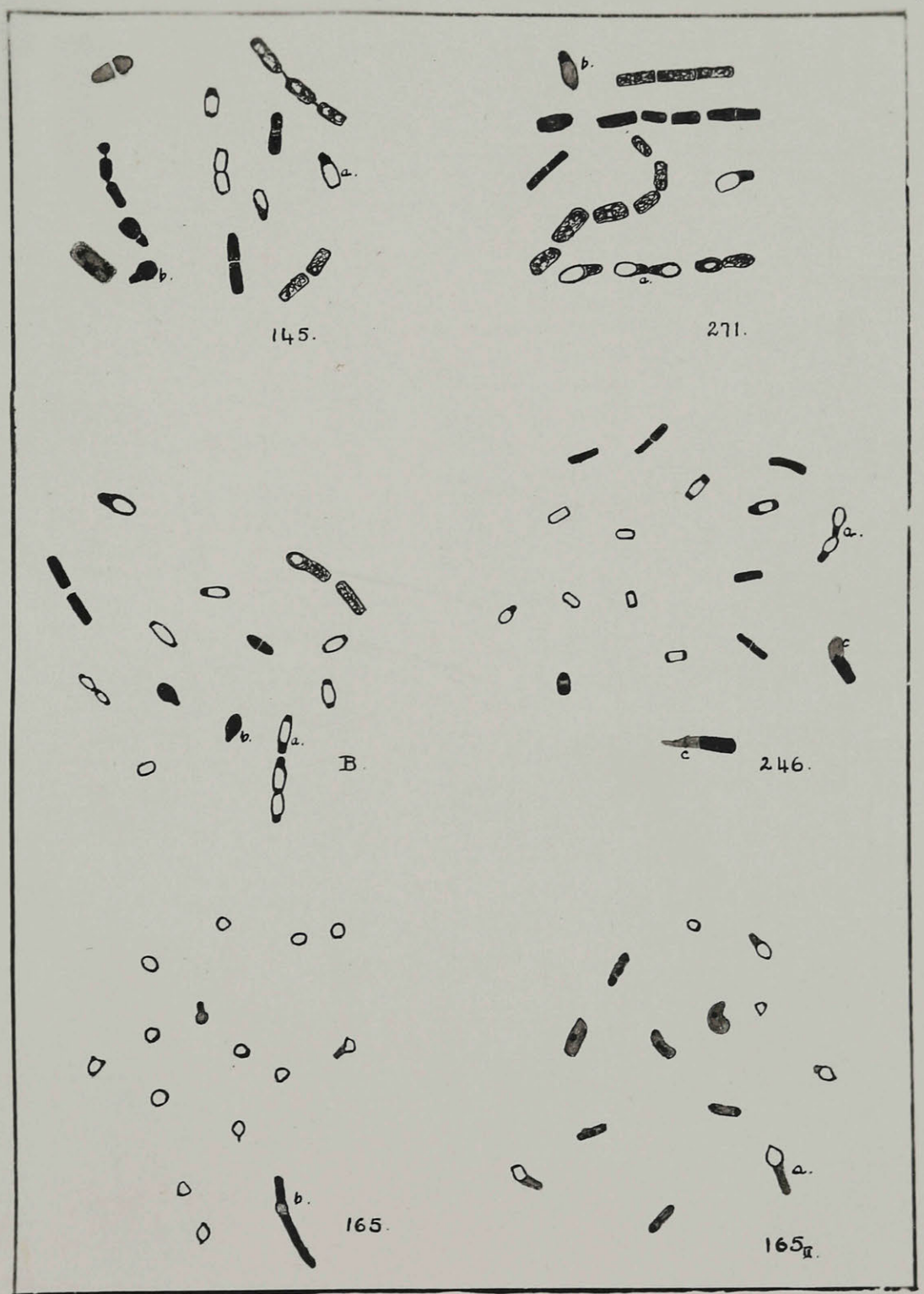
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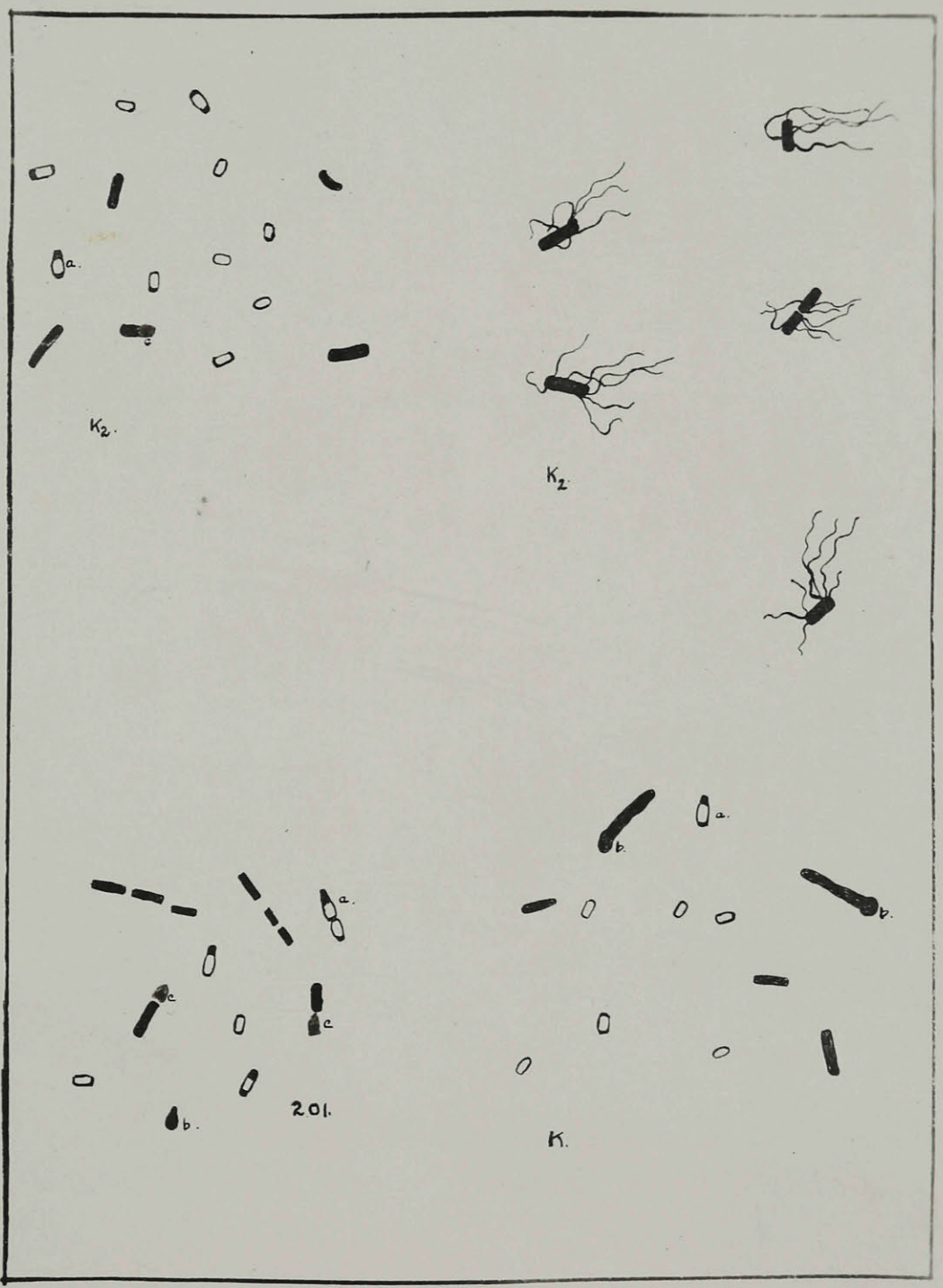
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