

ANAEROBIC SPORULATING THERMOPHILES  
SOME OBSERVATIONS ON A NEW GROUP OF BACTERIA

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Received for publication June 25, 1924

HISTORICAL RÉSUMÉ

In the literature relating to the bacteria growing at high temperatures there are but three references, so far as we are aware, to thermophilic anaerobes. The earliest reference to bacteria of this type is found in the work of Oprescu (1898) who described three species of obligate anaerobes, although there may be a reasonable doubt whether these species were really anaerobic. Somewhat later Benignetti (1905) isolated a single species of a thermophilic bacillus from water, which could be cultivated anaerobically. And finally, in 1906, Bardou (1906) reported the isolation from sewage of four strains of facultative anaerobes that grew equally well at 18° and 60°C. These organisms were characteristically putrefactive at either temperature. In contrast to the organisms that we will describe, it should be noted that the strains isolated by Bardou had the faculty of growing in the presence of atmospheric oxygen.

SOURCE OF THE CULTURES AND METHODS EMPLOYED

The organisms to be described in this paper were isolated from well-rotted horse manure. The method of isolation was the same in all cases. A suspension of the feces was made in sterile water and deep infusion agar shake tubes inoculated with from 1 to 3 drops of suitable dilutions of this suspension, following the technique of Veillon-Zuber (1898). These tubes were incubated from two to five days at 55°C. and the single isolated colonies

cut out, sterilized by the application of tincture of iodine and transferred to the cooked meat medium of Holman (1919) which had previously been boiled for fifteen minutes in order to expel the dissolved oxygen. At this point melted vaseline to a depth of 1.5 to 2 cm. was poured over the meat, as it would seem that anaerobiosis at thermophilic temperatures is somewhat aided by this means. In the majority of cases good growth is observed in the meat tubes after forty-eight hours' incubation although this is confined to the immediate vicinity of the meat particles and the supernatant broth remains clear. More profuse growth in the meat tubes was obtained by the addition of about 5 cc. of sterile aqueous infusion of horse manure to the medium.

From the meat tubes, which were used as stock cultures, transfers were made, using a Pasteur pipette, to the various media. In all cases the growth of the organisms was tested aerobically and anaerobically on infusion agar slants. The anaerobic tests were made in a McIntosh and Fildes (1916) jar. The organisms were also planted on the usual anaerobic media, i.e., egg cube, alkaline egg, litmus milk, brain, and lactose, sucrose, glucose, maltose, starch, glycerol, inulin, and mannitol broth. Only a meager growth was observed in these media and there was no visible reaction.

Throughout all our work veal infusion media were used for both aerobic and anaerobic cultivation. In the cases where sugar free meat was used it was freed from muscle sugar by inoculation with *B. coli-communior*, incubated eighteen hours at 37°C., tubed and sterilized.

The solid media in which a carbohydrate was included were prepared from 1.5 per cent veal infusion agar by adding 1 per cent of the carbohydrate and 1 per cent Andrade indicator.

The test for nitrate reduction was performed by incubating veal infusion nitrate broth for forty-eight hours and then testing for the presence of nitrite by the addition of a'pha-naphthylamin and sulphanilic acid.

Hydrogen sulphide production was observed by the formation of lead sulphide in veal infusion agar shake tubes to which 0.1 per cent basic lead acetate had been added.

The production of indol was tested for by the methods of Ehrlich and Salkowsky and the vanillin test.

The thermal resistance of the organisms described herein was determined by submitting the broth from a meat culture which had been incubated for forty-eight hours at 60°C, to heating for ten minutes at 60°, 70°, 80°, 90°, 100°, 110° and 120°C. The cultures were immersed and heated in a DeKhotinsky oil bath. After the period of heating subcultures were made in veal infusion agar shake tubes and incubated at 55°C. for five days.

#### MORPHOLOGICAL AND CULTURAL OBSERVATIONS

In the description of the following organisms the generic name "Clostridium" has been adopted as suggested by the Society of American Bacteriologists for all anaerobic spore-forming bacilli. The selection of a term to indicate the species has been dictated by the effort to incorporate the thermophilic character and the most outstanding feature of the organism in a single word.

During this investigation many thermophilic cultures have been isolated which were facultative anaerobes. A report on the cultural characteristics of these organisms will be made in a subsequent paper. The detailed descriptions given in this paper apply only to organisms conclusively demonstrated to be obligate anaerobic thermophiles. A few of the cultures show slight growth at 37°C. as well as at 55°C. but in general their characteristic reactions are best observed at the higher temperature.

#### *Clostridium thermoputrificum*

This organism is an obligate anaerobic thermophile isolated from fecal material derived from the horse. It undoubtedly represents a group of organisms having considerable putrefactive power and capable of exhibiting its reactions at 37°C. as well as at 55°C.

*Morphology.* Uniform short rods with homogeneous protoplasm and rounded ends. The average dimensions of the rods taken from a forty-eight hour chopped meat culture, incubated at 55°C. are 5 by 2.5 microns.

*Staining properties.* The organisms are gram-positive in forty-eight hour meat cultures. They lose their ability to retain the gentian violet after five days incubation.

*Motility.* Non-motile in meat cultures examined every two hours from four to forty-eight hours incubation. No flagella demonstrable.

*Spore-formation.* Spores are observed in forty-eight hour meat cultures. They are terminal and oval, and bulge the rod slightly.

*Agar shake colonies.* Single colonies in 2 per cent veal infusion agar are small, discus shaped, with well defined margins as seen under the low power.

*Cooked meat medium.* After two days incubation at from 37° to 55°C. gas bubbles are noted throughout the medium. At 37°C. the vaseline cap is raised about 1 cm. at the end of four days incubation. Upon penetration of the vaseline cap a strong putrefactive odor is noted, and at the end of four days there is a definite proteolysis as indicated by the digestion of the meat. There is no change in the color of the medium.

*Sugar-free cooked meat* shows reddening and a hazy diffuse growth throughout the medium.

In both media the broth above the meat remains clear except for a narrow zone of turbidity immediately above the meat particles.

*Fermentation reaction.* Lactose, glucose, starch. Growth at from 37° to 55°C. but no acid or gas produced.

Maltose, glycerol, sucrose, mannitol and inulin. Growth, with a marked production of gas, but no acid indicated at 37° to 55°C. with Andrade indicator.

*Lead acetate infusion agar.* Hydrogen sulphide produced as evidence by a black coloration of the medium at 37° to 55°C.

This culture does not reduce nitrates; it does not have any action on gelatin, nor does it produce indol.

#### *Clostridium thermoaerogenes*

This organism is an obligate anaerobe growing only at 45° to 55°C. It was isolated from horse manure. A number of cultures were obtained which gave identical reactions as indicated below. None of the cultures exhibited any putrefactive action. Gas, however, was produced in certain of the carbohydrate media, but no acid was formed.

*Morphology.* A uniform medium-sized rod with granular protoplasm and rounded ends. The average dimensions of the rods in a forty-eight hour meat culture incubated at 55°C. are 7 by 1.8 microns.

*Staining properties.* Gram-negative in forty-eight hour meat cultures.

*Motility.* Non-motile in meat cultures examined every two hours after four to forty-eight hours. Incubation at 55°C. No flagella demonstrable.

*Spore-formation.* Spores observed in forty-eight hour meat cultures incubated at 60°C. They are terminal, oval, and bulge the rod slightly.

*Agar shake colonies.* Single colonies in 2 per cent veal infusion agar are round and have well defined margins under the low power.

*Cooked meat medium.* After two days incubation at 55°C. gas bubbles are noted throughout the medium. There is no change in the color of the meat or sign of digestion.

*Sugar-free cooked meat* shows a reddening and a hazy diffuse growth throughout the meat particles. A narrow zone of broth above the meat is turbid, above this the broth is clear.

*Fermentation reactions.* Lactose, glycerol, sucrose, mannitol and glucose show growth but no acid or gas is formed.

Maltose and inulin: Gas is produced, but no acid is indicated by the Andrade. Starch: Gas and acid produced.

*Nitrate reduction.* Nitrates are reduced to nitrites after forty-eight hours incubation at 55°C. These cultures show no reaction on lead acetate or gelatin and do not produce indol.

One culture was isolated which was identical with *C. thermoaerogenes* except that it produced acid in inulin.

Three cultures were isolated which were morphologically similar and exhibited the same reactions at 55°C. as the above organism but also had the ability to grow at 37°C. without any alteration of the media.

#### *Clostridium thermoacidophilus*

This organism is an obligate anaerobic thermophile isolated from horse manure. A number of cultures were isolated which gave acid on some carbohydrates and acid and gas on others. None of the cultures exhibited any digestion of the meat.

*Morphology.* A long rod with granular protoplasm and rounded ends. The average dimensions of the rods in a forty-eight hour meat culture incubated at 55°C. are 10.8 by 1.2 microns.

*Staining properties.* Gram-positive in a forty-eight hour meat culture. The organisms lose their ability to retain the gentian violet after five days incubation.

*Motility.* Non-motile in meat cultures examined every two hours after four to forty-eight hours incubation at 55°C. No flagella were demonstrable.

*Spore formation.* Spores observed in forty-eight hour meat cultures incubated at 60°C. They are terminal, oval, and bulge the rod slightly.

*Agar shake colonies.* In 2 per cent veal infusion agar shake tubes single colonies are like a burr; no definite margin could be seen under low power.

*Cooked meat medium.* After two days incubation at 55°C. gas bubbles are noted throughout the medium. There is no change in color and no digestion is noticed in the meat.

*Sugar-free meat medium* shows a reddening and a hazy diffuse growth throughout the meat particles. The broth is clear except for a small zone just adjacent to the meat.

*Fermentation reactions.* At 55°C. Lactose, maltose and glucose show growth but no acid or gas is observed.

No gas is produced in glycerol, starch and sucrose, but acid is observed. Gas and acid are seen in mannitol and inulin.

*Nitrate reduction.* Nitrates are reduced to nitrites in forty-eight hours. These cultures show no reaction on lead acetate or gelatin, and do not produce indol.

One culture was isolated which was morphologically similar to the above strain and exhibited the same carbohydrate reactions at 55°C., but did not reduce nitrates.

Two cultures were isolated which were similar to *C. thermoacidophilus* but grew at 37°C. with no apparent reactions although one of them has the power to liquefy gelatin at 55°C.

#### *Clostridium thermochainus*

This organism is an obligate anaerobic thermophile isolated from horse feces. Three cultures were isolated which produced acid from starch and sucrose. None of the cultures exhibited any visible action on meat.

*Morphology.* A uniform medium rod with granular protoplasm and slightly rounded ends. The rods may appear singly or in chains of from 3 to 6 organisms. The average dimensions of the single rods in a forty-eight hour meat culture incubated at 55°C. are 7 by 1.8 microns.

*Staining properties.* Gram-positive in a forty-eight hour meat culture. After five days incubation the organisms lose their ability to retain the gentian violet.

*Motility.* The cultures are non-motile in meat cultures examined every two hours after four to forty-eight hours incubation at 55°C. No flagella are demonstrable.

*Spore formation.* Spores observed in forty-eight hour meat cultures incubated at 60°C. They are terminal, oval and bulge the rod slightly.

*Agar shake colonies.* In 2 per cent veal infusion agar single colonies are round with well-defined margins under low power magnification.

*Cooked meat media.* After two days incubation at 55°C. gas bubbles are noted throughout the medium. There is no change in color or digestion noticed in the meat.

*Sugar-free meat medium* shows a reddening and a hazy diffuse growth throughout the meat particles. The broth is clear above the meat.

*Fermentation reactions at 55°C.* Maltose, glycerol, lactose, mannitol, inulin and glucose show growth but no acid with Andrade indicator. No gas bubbles are seen in the medium.

Starch and sucrose show slight acid production with Andrade indicator, but no gas is observed.

*Nitrates* are reduced to nitrites after two days incubation at 55°C. These cultures show no reaction on gelatin or on lead acetate, and do not produce indol.

TABLE 1  
*Carbohydrate reactions*

CULTURES	INCUBATION TEMPERATURE		MALTOSE		GLYCEROL		STARCH		SUCROSE		LACTOSE		MANNITOL		INULIN		GLUCOSE	
	°c.		Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
<i>C. thermoputrificum</i> .....	55		+		+				+				+		+			
	37		+		+				+				+		+			
<i>C. thermoaerogenes</i> .....	55		+				+	+									+	
	37																	
<i>C. thermoacidophilus</i> .....	55				+		+		+				+	+	+	+		
	37																	
<i>C. thermochainus</i> .....	55						+		+									
	37																	

It will be seen that, with the exception of *C. thermoputrificum*, all the species are obligate thermophiles, and that, with this exception, their fermentative activity is observed at 55°C. only.

TABLE 2  
*Morphology, size, colony formation and thermal resistance*

CULTURES	MORPHOLOGY	SIZE IN MICRONS	COLONY FORMATION IN 2 PER CENT AGAR SHAKE TUBES	THERMAL RESISTANCE
<i>C. thermoputrificum</i>	Short rod, Gram-positive, with terminal spore	5 x 2.4	Small disc-shaped	Thermal death point between 110° and 120°C. ten minutes
<i>C. thermoaerogenes</i>	Medium, Gram-negative, granular, with terminal spore	7 x 1.8	Round, sharply outlined	Thermal death point between 110° and 120°C. for ten minutes
<i>C. thermoacidophilus</i>	Long rod, Gram-positive, granular, with terminal spore	10.8 x 1.2	Large, fuzzy, burr-like	Thermal death point between 110° and 120°C. for ten minutes
<i>C. thermochainus</i>	Medium, Gram-positive, granular, chains, terminal spore	7 x 1.8	Round, sharply outlined	Thermal death point between 110° and 120°C. for ten minutes

## DISCUSSION

The organisms described by Oprescu, Benignetti, and Bardou as thermophilic anaerobes have all been observed to have the faculty of growing in the presence of atmospheric oxygen. In contrast to this the species described in this paper grow only under conditions of strict anaerobiosis. This fact is demonstrated by the observation of a clear zone, in which no colonies are seen, to a depth of 2 to 3 cm. in the infusion agar shake tubes.

It is true that certain cultures were seen to grow under anaerobic conditions at 37°C. but exhibited no reaction in any of the media at this temperature. An exception must be made in the case of *C. thermoputrificum*, however, as this organism exhibits all its cultural reactions at 37° as well as at 55°C.



A series of cultures incubated at 65°C. for five days showed no growth, demonstrating that the limiting temperature for growth is between 60° and 65°C.

In the table of carbohydrate reaction it is seen that in two species gas is produced from maltose, glycerol, sucrose, mannitol, and inulin, and that no acid could be detected with Andrade indicator. A possible explanation for this phenomenon is that the little acid which was produced may have been neutralized by the buffer in the medium in those cases where gas but no acid was observed. It is suggested also that a possible explanation may lie in the fact that the unattacked carbohydrate present stimulated the activity of the organisms to the point where protein fermentation took place.

With the exception of *C. thermoputrificum*, the reactions of all the cultures were meager, although perfectly definite. In some of the cultures where gas production was noted there were only a few gas bubbles produced in the media.

Aerobic and anaerobic control tubes were run with each series of media at 37° and 55°C.

No growth, aerobically or anaerobically, was observed in any of the cultures at room temperature.

In the determination of the heat resistance of the spores it was found that some viable spores were found after heating at 110°C. for ten minutes, but no growth could be seen in subcultures in tubes heated at 120°C. for ten minutes even after five days incubation.

The pathogenicity of all the cultures was tested by subcutaneous and intraperitoneal inoculation in guinea pigs, using 0.5 per cent of the body weight of inoculum. No pathogenic action was observed in any of the cultures.

#### CONCLUSIONS

1. A group of obligate anaerobic thermophiles has been described for the first time.
2. The species described belong to the genus *Clostridium* by virtue of their anaerobic spore-forming character.

3. One culture has been isolated which has an optimum temperature range of from 37° to 55°C. and which is actively proteolytic throughout this range.

4. The limiting temperature of growth for these cultures is between 60° and 65°C.

5. The thermal death point of these cultures lies between 110° and 120°C.

6. None of the cultures are pathogenic for guinea-pigs.

#### REFERENCES

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