

A CULTURAL, MORPHOLOGICAL, BIOCHEMICAL AND NUTRITIONAL
STUDY OF THE GENUS MICROBACTERIUM ORLA-JENSEN

By

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CHAPTER I

HISTORICAL

The genus Microbacterium was first proposed by Orla-Jensen in 1919. His cultures were obtained from such diverse materials as pasteurized milk, cheese, butter, rotten beets and calf feces. The species of this genus were described as gram positive, non-motile, non-sporeforming rods considerably smaller than the true lactic acid bacteria. Physiologically these organisms were characterized by their ability to grow upon the surface of agar slants, production of catalase and their pronounced thermal resistance. The genus embodied four species, viz., M. lacticum, M. flavum, M. liquefaciens and M. mesentericum.

M. lacticum was described as a small rod 0.3 by 1.0 μ . At times it appeared "coccus-like." This organism was able to survive heating to 80 C for 15 min. M. flavum formed "clumsy rods" 0.5 by 1.0 to 2.0 μ although it could attain a length of 10.0 μ . It was nearly as heat resistant as M. lacticum but its most distinctive characteristic was the production of a yellow pigment when cultivated on a solid medium. When stained with methylene blue it presented a granulated appearance. M. liquefaciens was morphologically similar to M. lacticum. It differed, however, in being able to liquefy gelatin. M. mesentericum was characterized by the formation of a mesentery-like growth on solid media. It was killed by heating to 70 C momentarily.

Orla-Jensen (1919) considered these organisms closely allied from a physiological standpoint to the Tetracocci.¹ The organisms of this genus

¹"Tetracoccus" according to Orla-Jensen refers to acid-producing forms of Micrococcus and Sarcina. Much of the acid they produce is acetic acid, and many liquefy gelatin. They generally produce yellow, orange, or pink pigments.

presented a physiological gradient with the saccharolytic acid-former (M. lacticum) at one extreme and the proteolytic gelatin liquefyer (M. liquefaciens) at the other extreme.

The taxonomic position of this genus was considered briefly by Orla-Jensen (1921). He stated:

Microbacterium is to be understood as merely a provisional collective name for gram positive rods of size a little smaller than ordinary bacteria. In biological respects some of these rods (Bacillus acidophilus) are closely related to the true lactic acid bacteria, whereas others approach the Tetracocci or the aerobic bacilli.

No further detailed investigations were made on this genus for 14 years.

Robertson (1927) encountered M. lacticum in his study of thermophilic organisms isolated from heated milk. He reported that this bacterium could survive in milk held at 62.8 C for 4 to 5 days. It was found on all plates of milk samples held at pasteurizing temperatures and plated hourly over a 7 hour holding period.

Robertson's data agreed well with those given by Orla-Jensen (1919) for M. lacticum. A curious feature of Robertson's work was the suggestion that M. lacticum and Lactobacillus thermophilus were identical. The description of L. thermophilus by Ayers and Johnson (1924) leaves little doubt that these organisms are distinctly different. L. thermophilus is a thermophile with an optimum temperature of 55-63 C. In addition, it is catalase-negative, destroyed easily by heating to 80 C and morphologically distinct. It measures 0.5 to 1.0 by 2.5 to 6.0 μ and hence is a much larger organism than M. lacticum.

A valuable contribution to present knowledge of the genus was made by Wittern (1933). Her work confirmed and extended that of Orla-Jensen (1919). A major portion of her investigation dealt with the taxonomic

position of M. mesentericum. After a detailed morphological and physiological study she allocated this organism to the genus Mycobacterium.² She concluded that morphologically M. mesentericum resembled a mycobacterium on the basis of the appearance of liquid cultures, consistency of colonies on solid media, the formation of coccus-like elements, and the thread-like structure of the cells. Physiologically the ability of M. mesentericum to use paraffin as a carbon source, and its sensitivity to methylene blue supported this point of view.

Jensen (1932) made a morphological study of the genus. His observations led him to the conclusion that M. mesentericum was a proactinomyces and hence should be Proactinomyces mesentericus. He described the organism as a non-acid-fast rod capable of developing an extensive mycelia composed of branching hyphae of somewhat variable thickness. This species was considered closely related to Mycobacterium coeliacum and Mycobacterium equi. Further recommendations by Jensen were that M. lacticum and M. liquefaciens be designated as Corynebacterium lacticum and Corynebacterium liquefaciens. C. lacticum was considered to be similar to C. pseudodiphthericum except for the lower optimum temperature and acid production of the former. C. liquefaciens was considered to be closely related to C. fimi. Jensen concluded:

The genus Microbacterium cannot possibly stand as defined by Orla-Jensen, since of its four species, one is a true Proactinomyces, one - flavum - an organism on the borderline between Mycobacterium and Corynebacterium, and lacticum and liquefaciens doubtless corynebacteria. As pointed out. . . their faculty of producing lactic acid does not make them a separate genus.

²This organism is classified as Nocardia mesenterica in the 6th edition of Bergey's Manual (1948).

Dill (1933) isolated organisms from the intestinal tract of man and identified several of them as M. lacticum.

Jensen (1934) suggested that M. flavum was actually a mycobacterium and therefore should be designated as Mycobacterium flavum.

Orla-Jensen, Otte and Snog-Kjaer (1936) reported that M. lacticum did not require "lactoflavin" or "milk-bios" for growth.

Hansen (1938) found that hydrocyanic acid or moniodoacetate inhibited the respiration of M. lacticum and suggested that the microbacteria differed from the true lactic acid bacteria in catalase and hemin content.

In 1940 Orla-Jensen and Foulberg reported M. lacticum had an optimum of pH 7.5 in milk and pH 7.0 in broth.

Speck (1943) found that 70 to 95 per cent of the acid formed by M. lacticum was lactic. He also presented data which indicated that this species produced small amounts (0.01 per cent per 20 ml medium) of carbon dioxide from glucose. This worker took issue with the taxonomic suggestions of Jensen (1932, 1934). He felt that to place the microbacteria into the genera Mycobacterium or Corynebacterium on a morphological basis would be a serious error. He concluded that the microbacteria were closely related to both the propionibacteria and the lactobacilli. The microbacteria were said to be physiologically related to the propionibacteria except for their more aerobic nature and lactic acid production, and to the lactobacilli except for their catalase production and aerobic growth. Speck felt that any system of classification should indicate a close relationship between the genera Microbacterium, Propionibacterium, and Lactobacillus.³

³This suggestion has been followed in the 6th edition of Bergey's Manual (1948).

Orla-Jensen (1943), in an attempt to show the taxonomic relationships of the microbacteria to other groups, was prompted to diagram their very close relationship to the corynebacteria and actinomycetes. He was, however, well aware of the implications involved when he concluded:

Der Vollständigkeit halber habe ich - mehr oder weniger berechtigungsweise - in unserem System auch die Mikrobakterien und die Propionibakterien angebracht. Ob es richtig ist, Corynebakterien und Actinomyceten von den Mikrobakterien und von Em. bifidum entspringen zu lassen, muss die Zukunft lehren.⁴

⁴"For completeness sake I have more or less qualified the Microbacterium and Propionibacterium in our system. Whether or not it is correct to diagram Corynebacterium and Actinomycetes extending from the microbacteria and Em. bifidum the future will decide."

CHAPTER II

METHODS OF CULTURE ISOLATION

Orla-Jensen (1919) isolated microbacteria from raw and pasteurized milk, cheese, cow feces and rotten beets. Robertson's (1927) cultures of M. lacticum were isolated from pasteurized milk. Orla-Jensen (1931) stated that:

. . . . bacteria found in incompletely sterilized evaporated milk seem to be not so often hay and potato bacilli as a weakly peptonizing, non-acid forming, non-sporing rod bacterium. This organism is presumably related to the group of microbacteria which are able to stand comparatively high temperatures.

Düll (1933) isolated M. lacticum from the intestines of adults. The majority of Wittern's (1933) cultures were also obtained from heat-treated milk. Speck (1943) found microbacteria in raw and pasteurized milk, milk stone and cheese. From these investigations it would seem probable that the microbacteria are most often to be found in milk as contaminants from milking and pasteurizing equipment. Since they are probably present in the intestinal tract of cows, they are occasionally introduced into milk from cow feces.

The heat resistance of the microbacteria is generally used as a basis for their isolation. Orla-Jensen (1919) stated that colonies of M. lacticum could be obtained in pure culture by plating out high temperature pasteurized milk. Robertson (1927) found M. lacticum regularly in milk held for extended periods at pasteurizing temperatures. In 1933, Wittern described in detail the methods used in isolating microbacteria. She heated samples of milk at 80 C for 5 min. and streaked decimal dilutions on China blue-lactose agar. Colonies of M. lacticum grew on this medium after 3 to 4 days at 30 C.

Since M. flavum was unable to resist 80 C for 5 min., isolations were made on milk samples heated at 60 C for 5 min. or 75 C for 1 min. The China blue in the medium served as an indicator of the small amount of acid produced by the microbacteria. Colonies outlined by yellow (acid) zones were examined microscopically. Small, thin, gram positive rods were concluded to be members of the genus Microbacterium. These methods were not always successful and Wittern suggested alternative enrichment procedures. These were as follows: For isolating M. lacticum a milk sample was heated at 80 C for 5 min. and placed into an equal amount of peptone-levulose broth plus calcium carbonate. Levulose is easily attacked by members of the genus Microbacterium and hence is the preferred carbon source. The mixture of heated milk sample and broth was allowed to stand 5 to 8 days at room temperature. The resulting sediment was streaked on plates of China blue-lactose agar and incubated at 30 C. For isolating M. flavum a milk sample was heated at 65 C for 5 min. and placed into an equal amount of peptone-levulose broth containing calcium carbonate and 5 per cent sodium chloride. M. flavum is capable of growing in salt concentrations up to 10 per cent hence 5 per cent salt would exert an inhibitory effect on other types of organisms which survived the heat treatment. The mixture was allowed to stand at room temperature for 5 to 8 days and the resulting sediment streaked on China blue-lactose agar. Typical colonies of M. flavum developed after 3 days' incubation at 30 C.

Speck (1943) obtained microbacteria from milk, milk stone and cheese. Milk samples were heated at 61.1 C for 30 min. or 71 C for 16 sec. and decimal dilutions plated on tryptone-glucose-meat-extract-skinmilk agar. The plates were incubated at 30 C. Cheese samples (1.0 g) were suspended in 2.0 ml of sterile water containing 0.1 g

sodium citrate. After complete trituration, 8.0 ml of water were added and the mixture was heated at 61.1 C for 2 min. Isolations were then made as for milk samples.

The University of Maryland cultures were isolated in the following manner: Samples of raw and pasteurized milk were obtained from the University dairy. Five ml portions were pipetted into sterile test tubes and heated at 61 C for 30 min. or 73 C for 16 sec. The samples were cooled immediately in an ice bath and decimal dilutions made. The dilutions were plated on three different media. Medium #1 was of the following composition:

| | |
|--------------------------------------|----------|
| Proteose-peptone..... | 0.5 g |
| Beef-extract..... | 0.3 g |
| Glucose..... | 0.1 g |
| Potassium phosphate (dibasic)..... | 0.4 g |
| Potassium phosphate (monobasic)..... | 0.1 g |
| Agar..... | 1.5 g |
| Water to..... | 100.0 ml |

pH 7.0

Medium #2 was dehydrated tryptone-glucose-meat-extract-skinmilk agar.

Medium #3 had the following composition:

| | |
|--------------------------------------|----------|
| Peptone..... | 0.5 g |
| Beef extract..... | 0.3 g |
| Levulose..... | 0.1 g |
| Potassium phosphate (dibasic)..... | 0.4 g |
| Potassium phosphate (monobasic)..... | 0.1 g |
| Water to..... | 100.0 ml |

pH 7.0

After 3 days' incubation at 30 C colonies 1.0 mm or less in diameter were examined microscopically. If small, short, gram positive rods were present, the colony was transferred to litmus milk and incubated at 30 C for 3 days. At this time one drop of the litmus milk culture was placed on an agar slant of medium #1, #2, or #3, and again incubated at 30 C. When good growth appeared on the slant and micro-

scopic examination showed that the culture was pure, it was placed in the refrigerator for future study. Forty cultures were isolated in the above manner. Five representatives of this collection were included in the more complete studies described in later sections. Other cultures used in this study were obtained from the sources indicated in table I.

TABLE I
Source and designation of cultures

| Culture Designation | Code Number | Source of Cultures |
|-----------------------------------|-------------|--|
| M. lacticum | 3 | Prof. S. Orla-Jensen, Biochemical-Technical Institute, Copenhagen, Denmark |
| M. lacticum | 6 | |
| M. flavum | 8 | |
| M. flavum | 9 | |
| M. lacticum | 8180 | American Type Culture Collection, Georgetown University, Washington, D. C. |
| M. lacticum | 8181 | |
| M. lacticum | 513 | Dr. Johanna Westerdijk, Centraalbureau voor Schimmelcultures, Baarn, Netherlands |
| M. lacticum | 516 | |
| M. flavum | 531 | |
| M. flavum | 534 | |
| M. lacticum | 3-2 | Dr. M. L. Speck, National Dairy Research Laboratories, Inc., Baltimore, Maryland |
| M. lacticum | 30-3 | |
| M. flavum | 342-51 | |
| M. lacticum | 3PMb-9 | Original isolations, University of Maryland, College Park, Maryland |
| M. lacticum | 1PM3 | |
| M. lacticum | 3RM2 | |
| M. species | 3RM5 | |
| M. species | 3RMb-1 | |
| Bacillus acidophilus ¹ | 738 | Mr. M. Rogosa, Bureau of Dairy Industry, Department of Agriculture, Washington, D. C. |
| Bacillus acidophilus | 750 | |

¹Orla-Jensen (1919) believed that this species was related to M. lacticum.

CHAPTER III

CULTURAL CHARACTERISTICS OF THE GENUS MICROBACTERIUM

Orla-Jensen (1919) did not characterize the colonial morphology of the genus Microbacterium in his original description of these organisms. Robertson (1927) described the agar colonies of M. lacticum as "dull, grey or white, opaque, raised centers (when not crowded), round, glistening, slow in developing." Wittern (1933) described the colonies of M. lacticum on meat-extract peptone agar as "punctiform, glassy, translucent, non-fluorescent, round, convex, smooth, finely amorphous, 1.0 to 1.5 mm in diameter." Colonies of M. flavum on dextrose agar were described as "1.0 mm in diameter, citron yellow, round, even and finely amorphous." Dill (1933) described M. lacticum colonies as white, yellow or greenish-white in color and ranging from 2.0 to 10.0 mm in diameter. Jensen (1934) stated that colonies of M. flavum were ". . . narrow, convex, smooth, glistening, opaque, light yellow." Speck (1943) stated that colonies of microbacteria found on tryptone-glucose-meat-extract-skim milk agar were:

. . . very small and smooth, the surface colonies round, the sub-surface ones lens-shaped. After 7 days' incubation at 30 C the colonies averaged about 0.7 mm in diameter Frequently the colonies would be hardly more than visible after 3 days at 30 C on this medium.

Observations on the colonial morphology of the microbacteria used in this study were made on streak plate cultures grown on tryptone-meat-extract-glucose-skim milk agar and incubated 3 days at 30 C.

The surface colonies of M. lacticum were 0.25 to 0.50 mm in diameter. They were punctiform, glistening, smooth, convex, pearl white or grey, translucent, and finely amorphous. They may be described as "dew-drop"

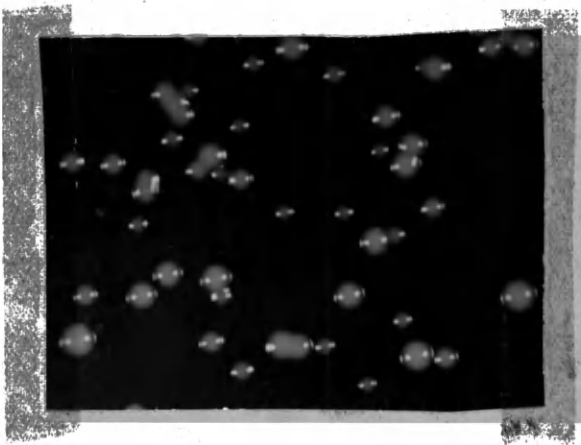
colonies. Cultures OJ3, OJ6, SE, 30-5, 513, 516, 8180, 750, 1PM3, SRM2, 738 and 3PMb-9 were of this type. Cultures 342-S1 and SRM5 were also of this type but were cream or slightly yellow in color. Culture 3RMb-1 was slightly different in that the colony edge curled upward and the center appeared slightly depressed. Pictures of typical colonies are shown in figures 1 and 1 a.

The surface colonies of M. flavum averaged 2.0 mm in diameter. They were glistening, smooth, convex, and cream or canary yellow. They were also generally round and possessed an even edge. Cultures 531, 534, OJ8, and OJ9 were of this type. The pigment varied from the color of rich cream (OJ8) to a very bright canary yellow (534). These colonies are shown in figure 2.

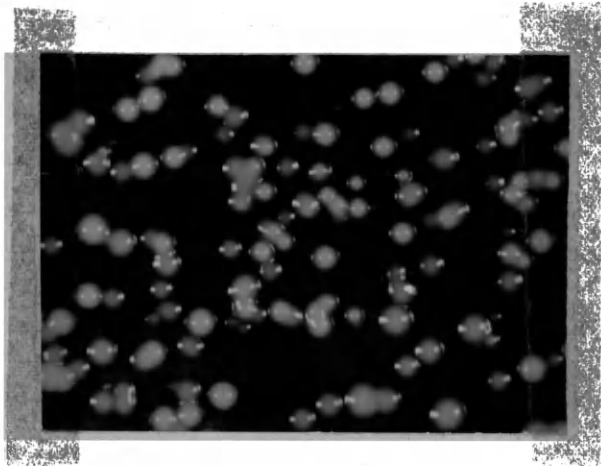
The chief difference between the colonies of M. lacticum and M. flavum appeared to be one of color. Generally the colony diameter of M. lacticum averaged 0.5 mm, whereas that of M. flavum averaged 2.0 mm. This was not, however, a very distinguishing characteristic.

When these organisms were grown under anaerobic conditions, (see Chapter V for method used), they appeared much the same as under aerobic conditions. The colonies took longer to develop (5 to 7 days at 30 C) and were generally smaller. Pigmentation was minimal.

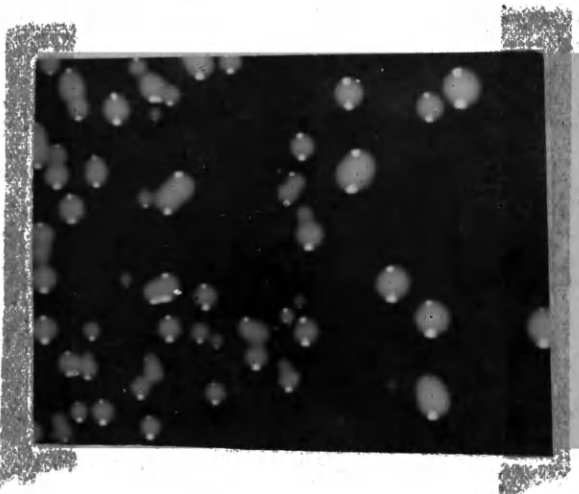
Sub-surface colonies of microbacteria were usually lens-shaped and smaller than the surface colonies. Several of the cultures isolated at this laboratory also formed round sub-surface colonies. "Lumpy" and "diffuse" sub-surface forms were also seen. A given culture would generally form all of the above mentioned sub-surface types. In addition, the organisms present in one colony form were morphologically identical to those of another colony form. The location of the colony within the



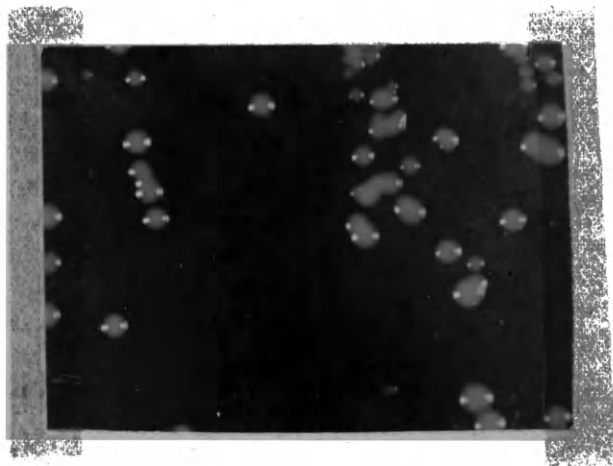
OJ3



S13

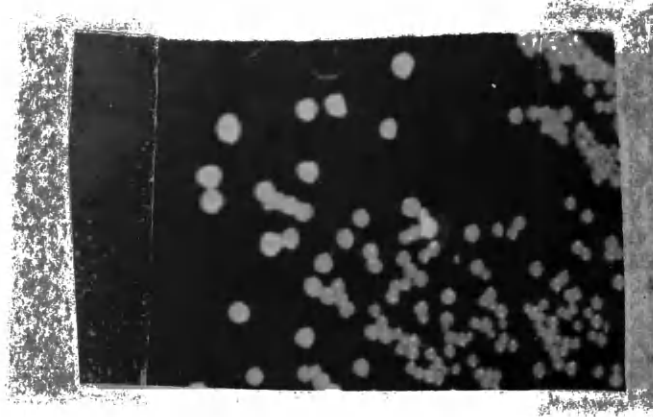


OJ6

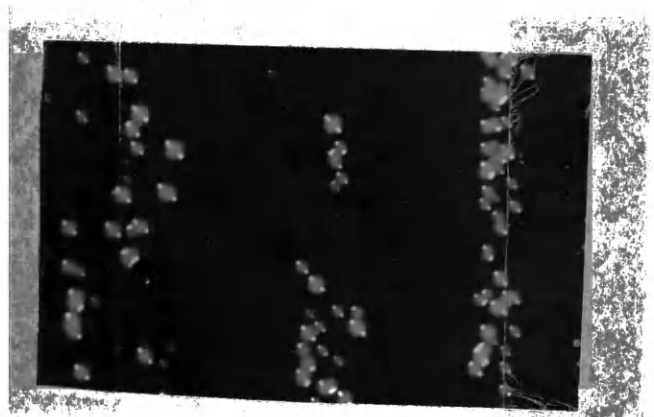


S180

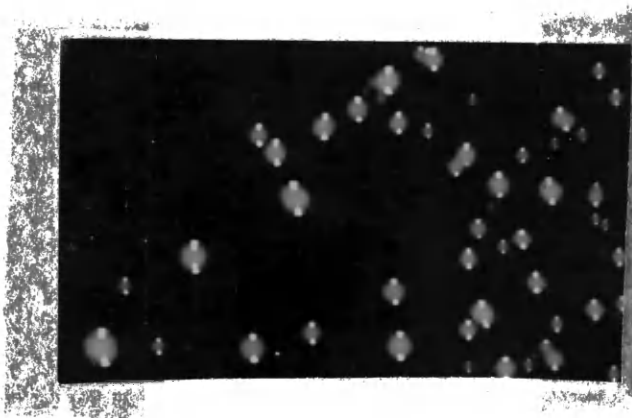
Fig. 1 Surface colonies of Microbacterium lacticum (X 500)



SRMb-1



SRM2

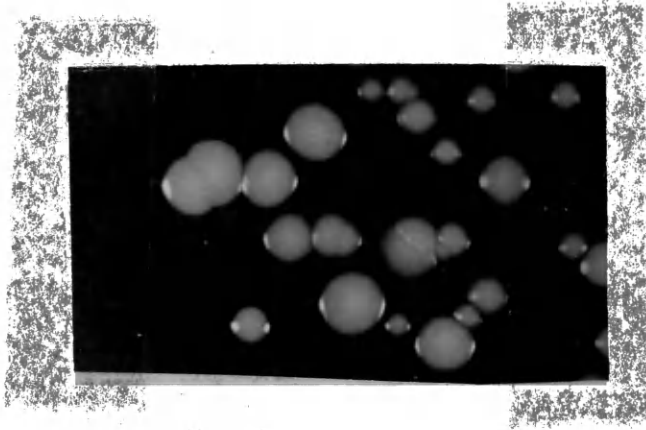


S-2

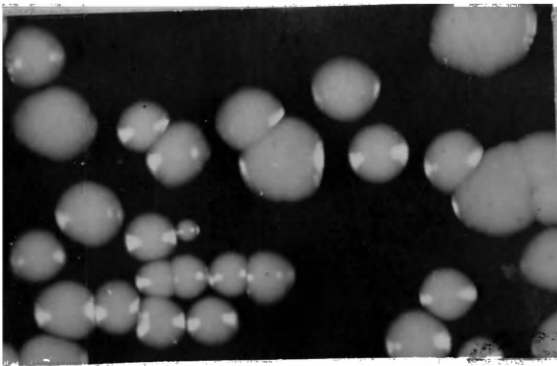


750

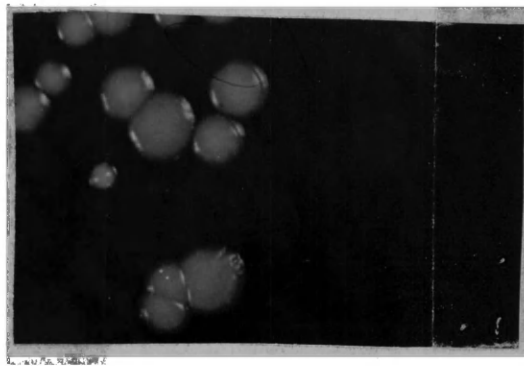
Fig. 1 a Surface colonies of Microbacterium lacticum (X $5\frac{1}{2}$)



531



0J8



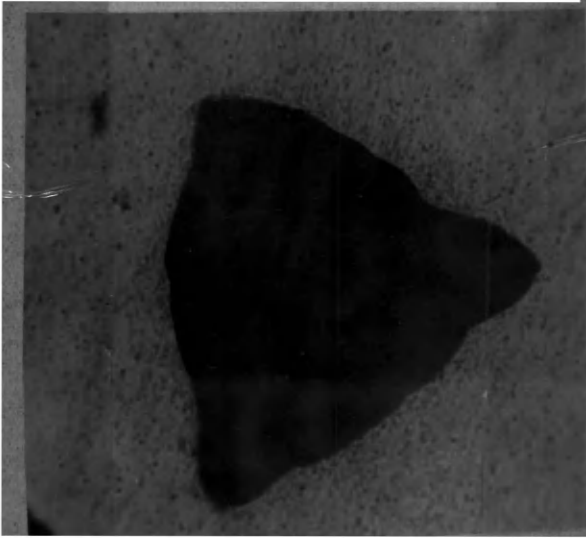
0J9

Fig. 2 Surface colonies of Microbacterium flavum (X $5\frac{1}{2}$)

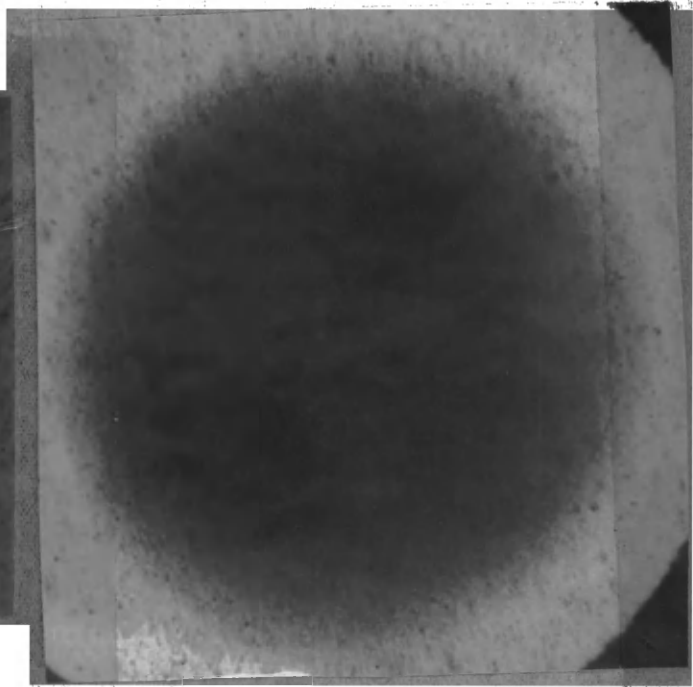
agar had an effect on the colony morphology. The sub-surface colonies between the glass-agar interface were generally "diffuse." Those within the agar were round, "lumpy," or lens-shaped. These colony forms are shown in figure 3.

Yeast-extract-proteose-peptone-glucose broth cultures of the organisms generally showed varying degrees of turbidity from faint to heavy. Most of the cultures formed a stringy, viscid, sediment after 4 days at 30 C. (See table II).

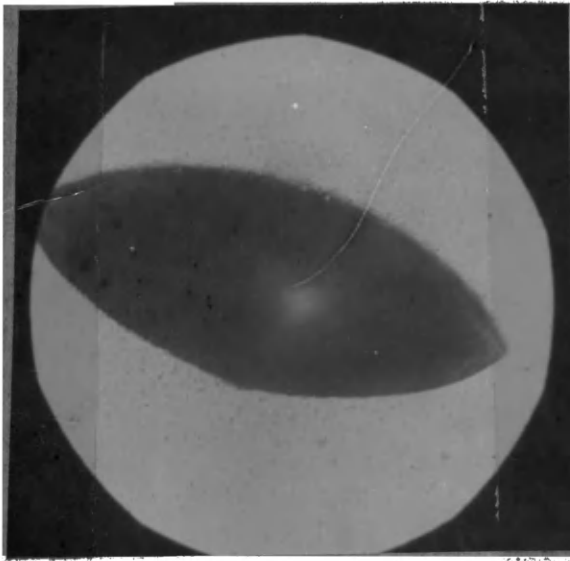
Agar slant cultures of the above medium showed good surface growth after 2 days at 30 C. In the case of M. lacticum, the growth was greenish-white, white or grey. Many cultures showed a translucent film of growth over the surface of the slant. M. flavum gave quite heavy growth which was usually cream or canary yellow.



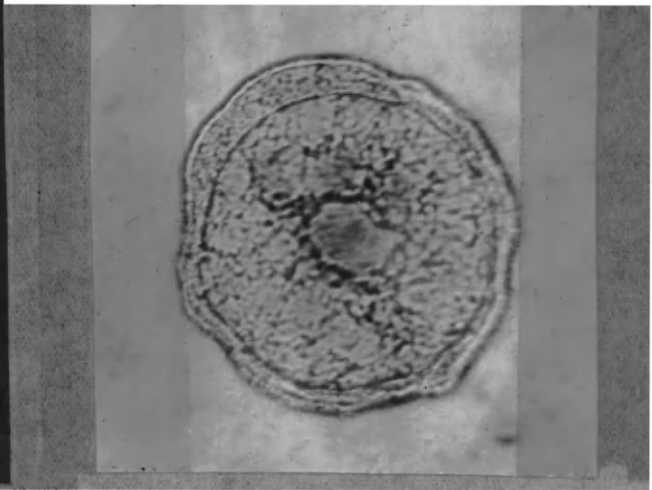
LIMPY



ROUND



LENS



DIFFUSE

Fig. 3 Sub-surface colony forms of microbacteria (X 30)

TABLE II

Description of broth cultures of microbacteria grown 3 days at 30 C.¹

| Culture No. | Description |
|-------------|---|
| OJ3 | slight turbidity, viscous sediment |
| OJ6 | slight turbidity, viscous sediment |
| 516 | even turbidity, viscous sediment |
| 8180 | slight turbidity, viscous sediment |
| 8181 | slight turbidity, viscous sediment |
| 30-5 | pellicle, even turbidity, no sediment |
| S2 | even turbidity, viscous sediment |
| 1FM3 | even turbidity, viscous sediment |
| 3RM2 | even turbidity, viscous sediment |
| OJ8 | slight turbidity, very heavy viscous sediment |
| OJ9 | pellicle, slight turbidity, very heavy sediment |
| 531 | even turbidity, viscous sediment |
| 534 | no turbidity, slight powdery sediment |
| 342-S1 | slight turbidity, viscous sediment |
| 738 | even turbidity, powdery sediment |
| 750 | even turbidity, viscous sediment |
| 3RM5 | slight turbidity, viscous sediment |
| 3RMb-1 | slight turbidity, viscous sediment |
| 3FMB-9 | even turbidity, viscous sediment |

¹Grown in Yeast-extract-protocose-peptone-glucose broth.

CHAPTER IV

MORPHOLOGICAL CHARACTERISTICS OF THE GENUS MICROBACTERIUM

Oria-Jensen (1919) described the microbacteria as "Gram-positive, rod-shaped lactic acid bacteria considerably smaller than the rod forms hitherto described" M. lacticum was found to occur mostly in the form of single rods 0.3 by 1.0 μ . At times it was also found to exhibit a coccus-like appearance. When stained with methylene blue the cells were highly granulated and resembled extremely small streptococci. M. flavum was found to form rods 0.5 to 2.0 μ . At times this species was found to form cells 10.0 μ long. They were distinctly granulated when stained with methylene blue.

Robertson (1927) stated that M. lacticum was a rod 0.3 to 1.2 μ . It was gram positive and occurred singly, in pairs or small irregular clumps of 4 to 16 individual cells. It was non-motile and non-spore-forming.

Wittern (1933) described M. lacticum as

. . . . ein kleines, dünnes Stübchen, ohne Sporen, die Grösse beträgt 0.3 x 1.0 μ . Als auffallend und typisch kaum war die geringe Dicke bezeichnen auch war stets ein schwaches Lichtbrechungsvermögen vorhanden. Die Länge des Bakteriums variiert von 1-2 μ . Eine typisch Lage war nicht zu erkennen, oft lagen sie in Häufchen aber nie in Ketten.¹

¹" a thin, small rod, without spores, the largest measuring 0.3 by 1.0 μ . A slight swelling, hardly typical, was observed in some instances. The length of these organisms varied from 1 to 2 μ . A typical arrangement was not observed, they often occur in clumps but never in chains."

She described M. flavum as

ein plumpes, Stäbchen von der Grösse 0.5 x 1-2 μ , kann oft bis zu 10 μ lang werden. Sehr oft lagen die Stäbchen zu vieren mit der Langsseite nebeneinander, oft mals auch in Häufchen zusammen, jedoch nie in Ketten.²

Dhill (1933) found most of his strains to be small rods measuring 0.2 to 0.5 by 1.0 to 6.0 μ .

Jensen (1934) described M. flavum as ". . . rod-shaped or bluntly cuneate, 0.6 to 0.8 by 1.5 to 3.5 μ , multiplying by purely snapping growth." Jensen also found this species to ". . . exhibit a very typical angular arrangement in stained preparations." Old cultures were found to contain shorter cells up to 1.0 to 1.2 μ thick. Milk cultures were definitely club-shaped. Jensen concluded that M. flavum was the ". . . least acid-fast of all mycobacteria."

Speck (1943) found that Newman-Lampert stains of microbacteria grown in milk ". . . may often be misleading as to their actual morphology." Using this method he stated that the microbacteria resembled small cocci in groups because of their irregular staining. Agar slant cultures stained evenly and were found to exhibit characteristic angular and pallisade arrangements. Speck's cultures measured 0.4 to 0.5 by 0.6 to 1.0 μ .

The morphology of the cultures used in this study agreed well with the descriptions of the preceding investigators. Small, thin cells exhibiting angular and pallisade arrangements, especially in preparations from solid media, were frequently seen. These cells measured 0.5 to 0.7 by 1.0 to 1.7 μ . This type of cell also showed a slight swelling at one end thus giving it a club-shaped appearance.

². . . a plump rod 0.5 by 2 μ , may be 10 μ long in some instances. The rods often lie in fours with their sides parallel to one another, they often occur in clumps but never in chains.

Microbacterium organisms which were quite plump (cocco-bacillary) were also seen. They resembled micrococci superficially but were very similar to photographs in Orla-Jensen's (1919) monograph. These cells measured 0.7 to 1.4 μ in diameter. The most rod-shaped cells measured 1.0 to 1.4 μ . As pointed out by Wittern (1933), many of the cells lay side by side.

All of the cultures used in this investigation were found to be gram positive, non-motile, and non-sporforming. They showed granulation when stained with Loeffler's methylene blue and were non-acid-fast when stained with Ziehl-Neelsen's carbol fuchsin and decolorized with acid alcohol. A description of the shape, arrangements and tinctorial characteristics of the cultures used in this study is presented in table III.

On the basis of cell morphology the cultures have been arbitrarily divided into 4 types: Type 1: Short, thin rods, which were regarded as the classical type-morphology of M. lacticum. Occasionally club-shaped cells were observed. Pictures of this type are shown in figure 4. This type has been described by Orla-Jensen (1919), Robertson (1927), Wittern (1933), Dill (1933), and Speck (1943). Cultures 3RM2, 1PM3, S2, 30-3, 3PMb-9, S180 and S181 are of this type. Angular and palisade arrangements were frequently noted in preparations made from cultures grown on solid media. They were quite small (0.5 to 0.7 by 1.4 to 2.1 μ) and showed granulation when stained with Loeffler's methylene blue. The granulation was more evident in cultures grown in milk and stained with the Newman-Lampert stain. Culture OJ3 was very similar to the organisms in this group except for its tendency to form definite club-shaped rods. Type 2: Cocco-bacillary and wedge-shaped rods as described by Orla-Jensen (1919) and Jensen (1934). The organisms superficially resembled

TABLE III

Morphology and tinctorial reactions of members of the genus Microbacterium

| Culture | Gram Stain | Morphology | Size (μ) | Acid Fast Stain | Granules |
|---------|------------|---|--------------------------|-----------------|----------|
| OJ3 | + | Thin, long & short rods, some club-shaped | 0.4 to 2.1 | - | + |
| OJ6 | + | Blunt, cocco-bacillary, wedge-shaped | - | - | + |
| 513 | + | Long, filamentous thread-like | 0.4 to 14.0 | - | + |
| 516 | + | Blunt, cocco-bacillary, wedge-shaped | - | - | + |
| 8180 | + | Short, thin rods | - | - | + |
| 8181 | + | Short, thin rods | - | - | + |
| 30-3 | + | Short, thin rods | - | - | + |
| S-2 | + | Short, thin rods | - | - | + |
| 19W3 | + | Short, thin rods | - | - | + |
| 3RM2 | + | Short, thin rods | 0.7 to 2.1 | - | + |
| OJ8 | + | Rods, thick and some coccus-like | 0.9 to 2.8 | - | + |
| OJ9 | + | Large rods | - | - | + |
| 631 | + | Large rods | - | - | + |
| 634 | + | Blunt, cocco-bacillary, wedge-shaped | - | - | + |
| 342-21 | + | Blunt, coccus-like, wedge-shaped | - | - | - |
| 738 | + | Short, thin rods | - | - | + |
| 750 | + | Blunt, cocco-bacillary, wedge-shaped | - | - | - |
| 3RM5 | + | Blunt, cocco-bacillary, wedge-shaped | - | - | - |
| 3RMb-1 | + | Blunt, cocco-bacillary, wedge-shaped | 0.7 to 0.8 1.0 to 1.4 | - | - |
| 3RMb-9 | + | Short, thin rods | - | - | + |

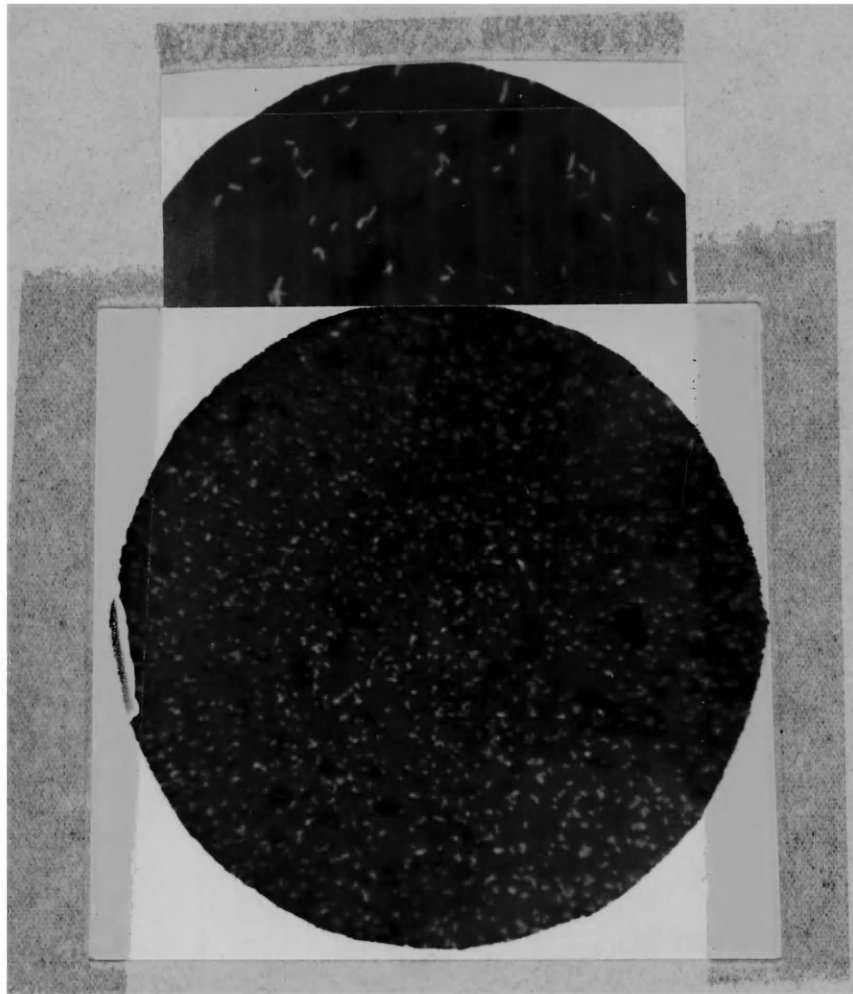


Fig. 4 Negative preparations of type 1 organisms. (Y 970)

micrococci. In most instances the major axis of the cell was only slightly longer than the minor axis. They had diameters ranging from 0.7 to 1.4 μ . Granulation was apparent in preparations made from milk cultures. Cultures SRMb-1, 516, 534, 750, 342-51, SRM5 and OJ6 were of this type. Pictures are shown in figure 5. Type 3: Rods of ordinary bacterial dimensions. Cells of this type have been described by Orla-Jensen (1919) and Wittern (1933). M. flavum was representative of this morphological type. The above mentioned investigators stated that M. flavum could grow to a length of 10 μ . Cells of M. flavum which were this length were not encountered. It was difficult to distinguish between the smaller cells of this type and those of type 2. Cultures OJ8, OJ9, and 531 were representative of type 3. They were 0.7 to 0.9 by 1.4 to 2.6 μ . Pictures of this type are shown in figure 6.

Type 4: Long, thin filamentous cells. This type has been described by Wittern (1933) as representative of M. mesentericum. Culture 513 (labeled M. lacticum) was of this type. It had an unusually long, thread-like appearance and differed markedly from all other cultures used in this study. (Figure 7)

When the microbacteria were grown under anaerobic conditions, their morphology was not significantly altered. The rod-shaped appearance of some of them was more difficult to detect and, in general, all were more coccus-like.

From what has been previously reported and on the basis of the observations made in this study, the genus Microbacterium may be described as follows: gram positive, granulated, non-motile, non-sporeforming, non-acid-fast organisms occurring as thin rods (0.5 to 0.7 by 1.4 to 2.1 μ), cocco-bacillary rods (0.7 to 1.4 μ diameter), or rods of ordinary bacterial

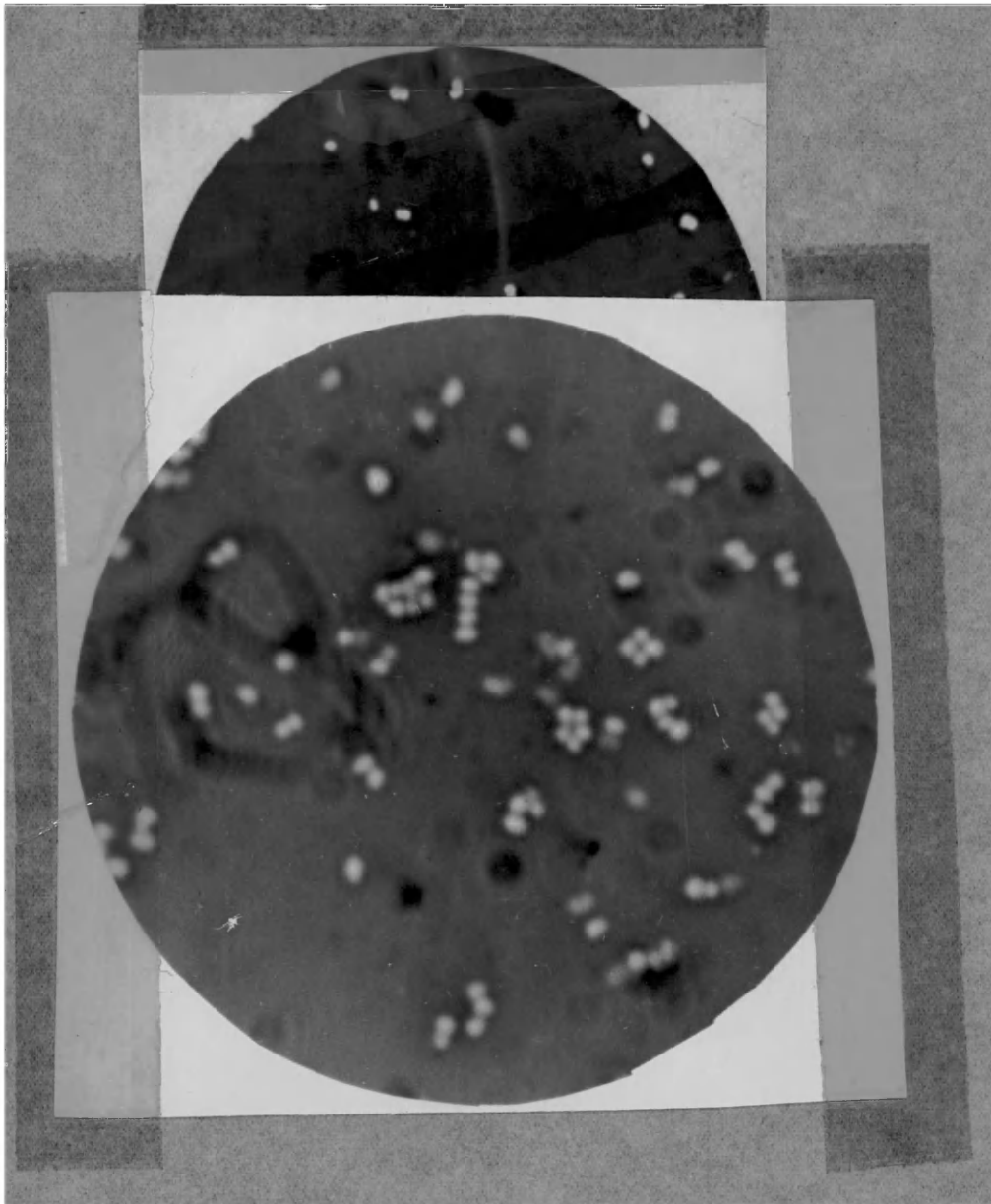


Fig. 5 Negative preparations of type 2 organisms. (X 970)

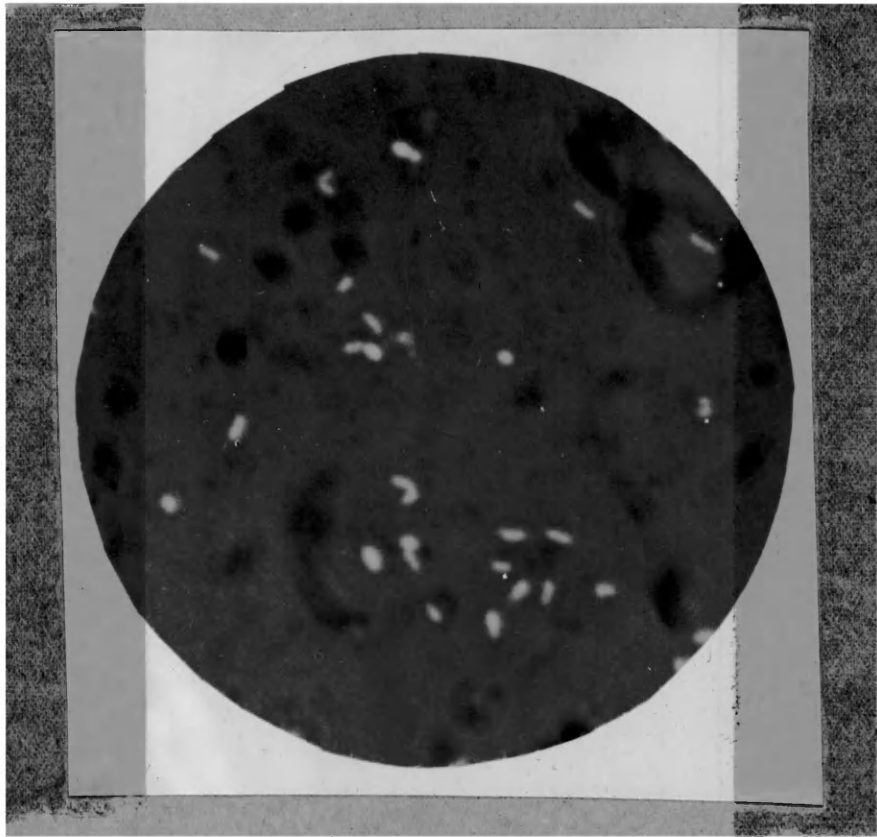


Fig. 6 Negative preparation of type 3 organisms. (X 970)

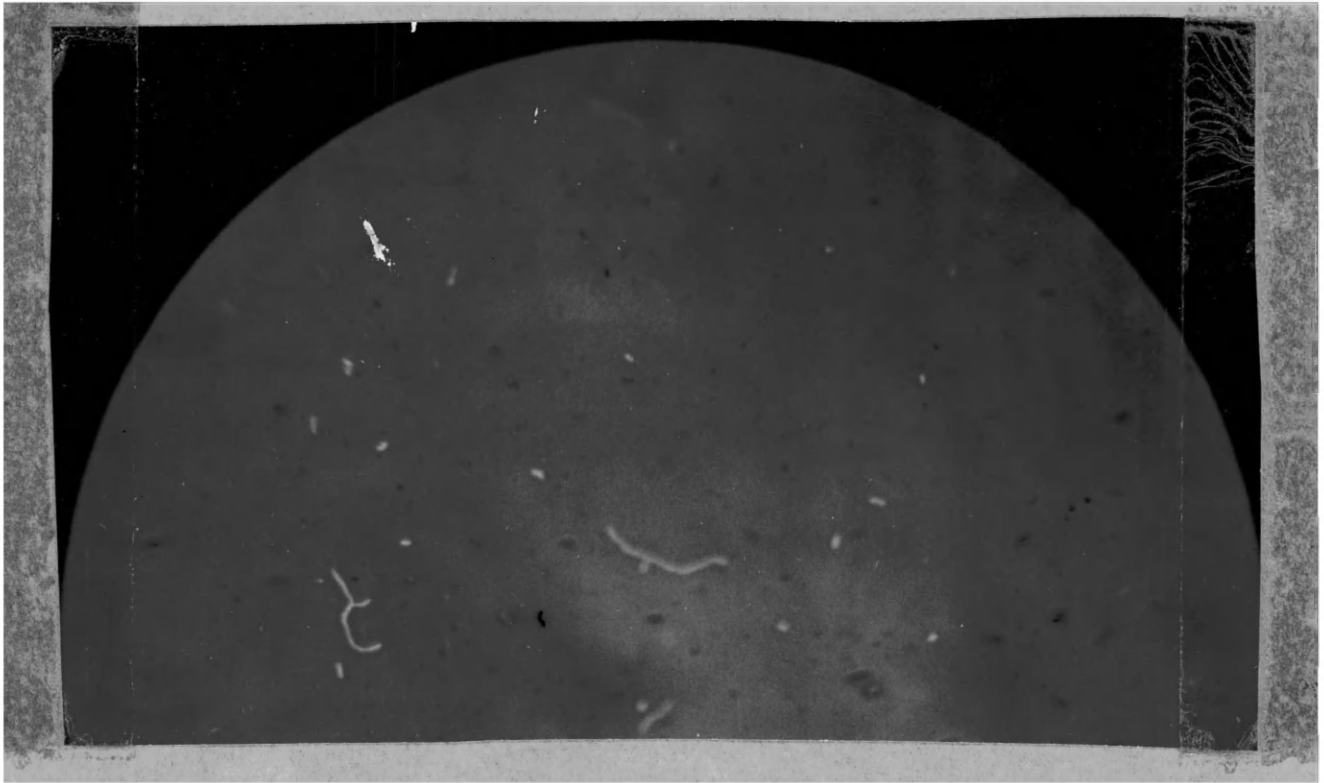


Fig. 7 Negative preparation of type 4 organisms. (X 970)

dimensions (0.7 to 0.9 by 1.4 to 2.6 μ). They may or may not show clubbing and exhibit characteristic angular and pallasade or side-by-side arrangements when stained from preparations grown on solid media.

CHAPTER V

PHYSIOLOGICAL REACTIONS OF THE GENUS MICROBACTERIUM

The physiological reactions of this genus as reported by Orla-Jensen (1919), Robertson (1927), Wittern (1933), Orla-Jensen (1943) and Speck (1943) are summarized in table IV. Orla-Jensen (1919) found that the microbacteria were weak acid-formers and generally failed to curdle milk. These organisms generally produced d-lactic acid from sugars. Yeast extract was found to be a poor nitrogen source, whereas casein peptone was satisfactory. All of the organisms included in this genus produced catalase and most of them were able to reduce nitrate to nitrite. M. lacticum and M. flavum were reported to be exceptionally thermotolerant. M. lacticum was able to survive 80 C for 15 min. and M. flavum was able to survive 75 C for 15 min.

Robertson (1927) found that M. lacticum

. . . may or may not ferment the pentoses and trisaccharides (raffinose); usually ferments the hexoses, disaccharides, glucosides, complex carbohydrates, and hexose and pentose alcohols, and generally fails to utilize the organic acids and amino acids. Hippuric acid is available.

He also found that litmus milk was made acid and that nitrate was generally not reduced. The latter finding was in direct opposition to the results of Orla-Jensen (1919).

Wittern (1933) also found casein peptone to be the best nitrogen source for the microbacteria when potassium nitrate, ammonium chloride, urea, asparagine, glyceroll, Witte's peptone, Liebig's meat extract and casein peptone were compared. She also reported that the microbacteria did not produce lipase, hemolysin or reductase and that they were non-pathogenic for mice.

TABLE IV

Physiological characteristics of the genus Microbacterium as described by some previous investigators

| Investigator | General Description of Species |
|-----------------------|--|
| Orla-Jensen (1919) | Weak acid formers; generally failed to curdle milk. Catalase positive. Most species able to reduce nitrate to nitrite. <u>M. lacticum</u> found to ferment most carbohydrates including starch. <u>M. flavum</u> generally not physiologically as active as <u>M. lacticum</u> . Both species exceptionally thermotolerant. |
| Robertson (1927) | <u>M. lacticum</u> ". . . . may or may not ferment the pentoses and trisaccharides (raffinose); usually ferments the hexoses, disaccharides, glucosides, complex carbohydrates, and hexose and pentose alcohols, and generally fails to utilize the organic acids and amino acids. Hippuric acid is available." Litmus milk was made acid and nitrate not generally reduced. Exceptionally thermotolerant. |
| Wittern (1933) | Found essentially the same fermentative reactions for <u>M. lacticum</u> and <u>M. flavum</u> as did Orla-Jensen (1919). The species of this genus were non-pathogenic for mice; did not produce hemolysin, reductase or lipase. |
| Hansen (1938) | <u>M. lacticum</u> and <u>M. flavum</u> were inhibited by potassium cyanide and monoacetate and therefore differ from lacto-bacilli in their hemin content. |
| Orla-Jensen (1945) | "The microbacteria do not attack the pentoses, raffinose, nor inulin. Mannitol is the only higher alcohol occasionally fermented. <u>M. lacticum</u> attacked starch, glucosides (arbutin) and hippurate. <u>M. flavum</u> attacked only mannitol, levulose, glucose and mannose. The final pH in levulose broth was 3.7." |
| Speck (1945) | Small amounts of carbon dioxide were produced from glucose by <u>M. lacticum</u> . Approximately 75 per cent of the acid produced in skim milk by <u>M. lacticum</u> was found to be lactic acid. |

Jensen (1934) stated that M. flavum hydrolyzed starch "slightly", reduced nitrate to nitrite, did not utilize paraffin as a sole carbon source, and produced acid from dextrose, levulose, galactose, and glycerol.

Hansen (1938) reported that the respiration of M. lacticum and M. flavum was inhibited by potassium cyanide or moniodoacetate and concluded that these organisms differed from the true lactic acid bacteria in their hemin content.

Orla-Jensen (1943) recharacterized the genus as follows:

... dass die Mikrobakterien weder Pentosen, Raffinose noch Inulin vergären können. Von den höheren Alkoholen wird Mannit nie und da vergoren. Mm. lacticum greift meistens die von uns geprüften Disaccharide, Stärke und Glucose (auch Arbutin) an und hydrolysiert Hippurate. Mm. flavum dagegen vergärt ausser Mannit eigentlich nur Xylulose, Glukose und Mannose. Es zeihlt Lävulose vor, und hat darin ein pH von 3.7 erzeugt.¹

Speck (1943) found that most of his cultures produced acid from starch. He further stated that this species was generally unable to reduce nitrate. Small amounts of carbon dioxide were produced by his cultures when grown in glucose broth. Approximately 75 per cent of the acid produced in milk by the microbacteria was found to be lactic acid.

1. . . . the microbacteria do not attack pentoses, raffinose nor inulin. Mannitol is the only higher alcohol occasionally fermented. M. lacticum attacked most of the disaccharides, starch, glucosides (including arbutin), and hydrolyses hippurate. M. flavum attacked only mannitol, levulose, glucose and mannose. The final pH of levulose broth was 3.7."

The workers who have investigated this genus are in agreement that the microbacteria do not produce indole, hydrogen sulfide, nor do they liquefy gelatin. They also agree that these bacteria are endowed with unusual thermotolerant ability. Catalase production and weak acid formation are also generally reported.

Most of the investigations summarized in table IV were limited in their scope since they did not combine a representative culture collection with extensive physiological determinations. Thirty-eight physiological reactions were determined for each of the cultures used in this study with the purpose of correlating them, if possible, into a useable scheme for identifying members of the genus.

Methods and Media:

The inoculum for the physiological determinations consisted of 4 day cultures (30 C) grown in a medium of the following composition:

| | |
|--------------------------------------|----------|
| Yeast extract..... | 1.0 g |
| Proteose-peptone..... | 0.5 g |
| Potassium phosphate (monobasic)..... | 0.2 g |
| Potassium phosphate (dibasic)..... | 0.2 g |
| Water to..... | 100.0 ml |

pH 6.6

Carbohydrate, alcohol and glucoside fermentation was studied by adding 1 per cent of the substrate to a previously sterilized and cooled cystine agar base of the following composition:

| | |
|----------------------|-----------|
| Trypticase..... | 20.0 g |
| Sodium chloride..... | 5.0 g |
| Agar..... | 3.5 g |
| Cystine..... | 0.5 g |
| Sodium sulfate..... | 0.5 g |
| Phenol red..... | 0.017 g |
| Water to..... | 1000.0 ml |

pH 7.3

The test substrate was sterilized separately by Seitz filtration and added aseptically to the cystine agar base to give a final concentration

of 1 per cent. The cystine agar base was sterilized by autoclaving 15 min. at 121 C. Four ml amounts were placed in sterile 16 by 128 mm test tubes and incubated 24 hours at 30 C to detect contamination. At the end of this time the tubes were inoculated with the cultures to be tested. Controls consisting of uninoculated media and inoculated media without substrate were used. Readings were made after 4 and 8 days incubation at 30 C.

Gelatin liquefaction was determined using a medium of the following composition:

| | |
|-------------------|----------|
| Gelatin..... | 10.0 g |
| Peptone..... | 0.5 g |
| Beef-extract..... | 0.3 g |
| Water to..... | 100.0 ml |

pH 7.0

Readings were made after 5, 8 and 14 days' incubation at 30 C. The tubes were placed in the refrigerator along with uninoculated controls and after 20 min., they were observed for evidence of liquefaction.

Hydrogen sulfide production was determined using lead acetate agar. Readings were made after 4 and 5 days' incubation at 30 C.

Methyl red and Voges-Proskauer reactions as well as the final pH determination were made using M.R.-V.P. medium. At the end of 8 days' incubation at 30 C, one drop of the culture was added to one drop of 0.05 per cent aqueous-alcoholic methyl red and the reaction recorded. The Voges-Proskauer test was made by adding 2 ml of 40 per cent potassium hydroxide containing 0.3 per cent creatine to 2 ml of the 8 day culture. Readings were made 30 min. after the addition of this reagent. The final pH was determined using a Beckman pH meter (Model G).

Indole production was determined by growing the cultures in a 1 per cent solution of trypticase for 8 days at 30 C. At the end of this time,

a few drops of Kovac's reagent were added and the color observed.

Catalase production was determined by adding 1.0 ml of 3.0 per cent hydrogen peroxide to a 4 day slant culture of the test organism. Evolution of gas was taken as evidence of a positive catalase test.

Nitrate reduction was determined in a medium of the following composition:

| | |
|------------------------|----------|
| Yeast extract..... | 1.0 g |
| Proteose-peptone..... | 0.5 g |
| Glucose..... | 0.1 g |
| Potassium nitrate..... | 0.1 g |
| Water to..... | 100.0 ml |

pH 7.0

After 8 days' incubation at 30 C the presence of nitrate in the medium was tested by addition of 0.5 ml each of sulfanilic acid and β -naphthylamine as described in leaflet V of the "Manual of Methods for Pure Culture Study" (1944).

Litmus milk was prepared by adding litmus powder to fresh skim milk until the desired shade of lavender was obtained. This medium was sterilized in the autoclave 10 min at 121 C. The tubes were immediately cooled in cold water. Readings were made after 4, 8 and 15 days' incubation at 30 C.

Acid production in skim milk was determined after 7 days' incubation at 30 C. Twenty ml of skim milk culture were titrated with 0.1 N sodium hydroxide to the phenolphthalein (1.0 per cent aqueous-alcoholic) endpoint. The acidity was calculated as lactic acid from the formula

$$\text{per cent lactic acid} = \frac{\text{ml} \times \text{normality} \times 0.09 \times 100}{\text{grams sample}}$$

Growth in 10 per cent salt broth was determined in a medium of the following composition:

Yeast-extract.....1.0 g
 Proteose-peptone.....0.5 g
 Glucose.....0.1 g
 Sodium chloride.....10.0 g
 Water to.....100.0 ml

pH 7.0

The cultures were observed for growth after 4 and 8 days' incubation at 30 C.

Hydrolysis of sodium hippurate was determined in a medium of the following composition:

Yeast extract.....1.0 g
 Proteose peptone.....0.5 g
 Sodium hippurate.....1.0 g
 Water to.....100.0 ml

pH 7.0

The sodium hippurate was sterilized by Seitz filtration and added aseptically to the above medium to give a final concentration of 1 per cent. After 4 days' incubation at 30 C, 0.6 ml of a 12 per cent solution of ferric chloride in 2 per cent hydrochloric acid was added to 4.0 ml of the culture and the resulting mixture observed for the presence of a ferric benzoate precipitate. Uninoculated controls were compared with the inoculated cultures.

Anaerobic growth was observed on cultures made by streaking plates of tryptone-glucose-meat-extract-skim milk agar and incubated in Spaulding anaerobic jars containing a previously heated and cooled palladinized asbestos as a catalyst. The air was evacuated by means of a vacuum pump and the enclosed system flushed out with carbon dioxide. This gas was then evacuated from the jar and a mixture of 90 per cent hydrogen and 10 per cent carbon dioxide introduced. The jars were incubated at 30 C for 10 days. 100 mm of vacuum were maintained in the jars during the incubation period to prevent the expanding gas from loosening the lid.

The heat-resistance of the cultures was determined in a medium of the following composition:

| | |
|--------------------------------------|----------|
| Yeast-extract..... | 1.0 g |
| Proteose-peptone..... | 0.5 g |
| Glucose..... | 0.1 g |
| Potassium phosphate (monobasic)..... | 0.2 g |
| Potassium phosphate (dibasic)..... | 0.2 g |
| Water to..... | 100.0 ml |

pH 6.6

Five ml amounts of this medium were placed in 50 ml screw-top vials of uniform diameter and sterilized 15 min at 121 C. One drop of a 4 day culture grown in the same medium was added to a vial and then heated to 85 C for $2\frac{1}{2}$ min. Two minutes were necessary to heat the medium to the desired temperature. Immediately after heating, the vials were cooled in an ice bath to 30 C. After cooling they were incubated 7 days at 30 C and the presence or absence of growth was recorded.

The results of these determinations are summarized in table V.

The cultures used in this study showed several physiological characteristics in common (see table V). None of the cultures studied were able to produce indole, hydrogen sulfide or liquefy gelatin. In addition, none of the cultures fermented rhamnose, inulin, adonitol or sorbitol. Glucose, galactose, fructose and mannose were fermented and catalase was produced by all cultures studied.

Physiological Characteristics of *Microbacterium lacticum*:

Consideration of the fermentation reactions of *M. lacticum* reveals that they failed to ferment the pentoses, arabinose, and xylose. Culture 513 was found to be the exception. In addition to fermenting arabinose and xylose this culture was morphologically distinct in that it produced filamentous thread-like cells instead of the typical small, thin type of rod. The hexoses, dextrose, galactose, levulose and mannose were fermented

with the production of acid by all the M. lacticum strains tested. Acid production from galactose was weak. Rhamnose was not fermented. With the exception of melibiose, most of the disaccharides were fermented. Culture 8181 was unable to ferment any of the disaccharides. The majority of strains of M. lacticum were unable to ferment the trisaccharides, melizitose and raffinose. The polysaccharides, inulin, glycogen, dextrin and starch, were selectively attacked. Some strains produced acid from glycogen while none fermented inulin. Starch was hydrolyzed with the production of acid by all but one strain of M. lacticum. Acid was produced slowly (15 days at 30 C) by cultures OJ3, OJ6, 516, 30-3 and S-2. Acid production by these strains was weak. Culture 8181 failed to produce acid from starch in 15 days at 30 C. The remainder of the strains of M. lacticum were able to produce acid from starch in 4 to 8 days at 30 C. Glycerol, adonitol, dulcitol and sorbitol were not fermented by any of the M. lacticum strains used in this study. Most strains were able to produce acid from mannitol. Cultures 513 and 1PM3 were the exceptions. Aesculin was not split by any of the M. lacticum strains except 3PMb-9 and acid was produced from salicin by cultures S-2, 1PM3, SRM2 and 3PMb-9.

M. lacticum generally produced from 0.20 to 0.40 per cent acid (calculated as lactic) from skim milk. Cultures OJ3, 516, 8180, S-2, 1PM3 and SRM2 produced a weak curd in skim milk. Cultures OJ6, 513 and 8181 produced negligible amounts of acid from skim milk. The reactions in litmus milk correlated well with those produced in skim milk. Most of the cultures of M. lacticum produced an acid reaction in 4 days at 30 C.

Nitrate reduction was variable. Six of the ten strains of M. lacticum tested gave a positive test for nitrite.

The final pH was found to range from 5.10 to 6.10. This finding correlated well with the methyl-red test. Eight of ten strains of

M. lacticum were methyl-red positive.

None of the cultures of M. lacticum grew in 10 per cent salt broth, hydrolyzed sodium hippurate or produced acetyl-methyl-carbinol.

Three of the cultures survived heating at 85 C for $2\frac{1}{2}$ min. in yeast-extract-protose-peptone-glucose-phosphate broth (pH 5.6).

Physiological Characteristics of Microbacterium flavum:

M. flavum was found to be fermentatively less active than M. lacticum. The pentoses were not fermented. Cultures 534 and 342-S1 were able to produce acid from xylose and arabinose. The hexose sugars were generally attacked but only culture 342-S1 was found to produce acid from galactose. Rhamnose was not fermented. The disaccharides were not attacked but cultures 342-S1 and 534 produced acid from the six compounds tested. These two cultures were also able to produce acid from raffinose. Only culture 534 produced acid from starch and none of the other polysaccharides were attacked by the strains of M. flavum tested. Mannitol was fermented by all the cultures examined but no other alcohol was fermented. Cultures 342-S1 and 534 were able, however, to produce acid from glycerol.

Acid production in skim milk and litmus milk was negligible. Litmus milk cultures showed no visible change after 8 days' incubation at 30 C.

Culture 342-S1 was the only strain of M. flavum that reduced nitrate to nitrite.

The final pH of the M. flavum cultures was found to range from 5.6 to 7.0. Culture 342-S1 was the only strain which produced a relatively low pH (5.6). All other strains of M. flavum had a final pH of 6.65 or above. Culture 342-S1 was the only strain found to be methyl-red positive.

Cultures OJ8, OJ9 and 531 were able to grow in 10 per cent salt broth. The bulk of growth appeared as a viscous sediment in the bottom of the tube.

None of the strains of M. flavum used in this study were able to survive 85 C for 2½ min. in yeast extract-proteose-peptone-glucose-phosphate broth (pH 6.6).

Physiological Characteristics of Other Microbacterium-like cultures:

Cultures 738 and 750 (B. acidophilus) were not as active physiologically as M. lacticum. They were able to produce acid from dextrin and culture 738 produced acid from starch. Both 738 and 750 were unable to attack the disaccharides and to this extent were similar to the M. flavum cultures. Strains 738 and 750 also produced acid from mannitol. No acid was formed in skim milk and the reaction of litmus milk remained unchanged after 8 days at 30 C. Their aerobic growth and catalase production indicate that they are not to be thought of as similar to Lactobacillus acidophilus. Many of their physiological reactions, viz., growth at 30 C and fermentation of mannitol, further support this view.

Cultures SRM5 and SRM5-1 were found to be similar in their reactions to cultures 342-S1 and 534. All of these cultures were of morphological type 2. The physiological reactions of these cultures, as well as 534 and 342-S1, are sufficiently different from those of M. lacticum and M. flavum to place them in an intermediate group (Microbacterium species). The outstanding characteristics of this intermediate group were acid production from arabinose, xylose, raffinose and glycerol and their morphological similarity.

Table VI shows the physiological differentiation of these cultures from M. lacticum and M. flavum based upon the fermentation of arabinose,

xylose, raffinose, glycerol and starch. It can be seen that M. lacticum does not produce acid from pentoses but does produce acid from starch (with the exception of culture 8181). M. flavum cannot ferment pentoses or starch. The intermediate group (including 342-S1 and 534) is sharply differentiated by acid-production from the pentoses as well as from glycerol and raffinose. Culture 513 (labeled M. lacticum) was not considered to be morphologically or physiologically related to other organisms encountered in this study.

The significance of these findings is discussed in terms of the identification of these organisms as well as the natural relationships of this genus to other genera in another section.

TABLE VI

Differential biochemical reactions of M. lacticum, M. flavum and M. species

| Substrate Species | Arabinose | Xylose | Raffinose | Glycerol | Starch |
|----------------------|-----------|--------|-----------|----------|--------|
| <u>M. lacticum</u> | | | | | |
| OJ3 | 0 | 0 | 0 | 0 | A |
| OJ6 | 0 | 0 | 0 | 0 | A |
| 8180 | 0 | 0 | 0 | 0 | A |
| SP7b-9 | 0 | 0 | 0 | 0 | A |
| SRM2 | 0 | 0 | 0 | 0 | A |
| 1PM3 | 0 | 0 | 0 | 0 | A |
| S-2 | 0 | 0 | 0 | 0 | A |
| 30-3 | 0 | 0 | 0 | 0 | A |
| 8181 | 0 | 0 | 0 | 0 | 0 |
| <u>M. flavum</u> | | | | | |
| OJ8 | 0 | 0 | 0 | 0 | 0 |
| OJ9 | 0 | 0 | 0 | 0 | 0 |
| 581 | 0 | 0 | 0 | 0 | 0 |
| <u>M. species</u> | | | | | |
| 534 | 0 | A | A | A | A |
| 342-81 | A | A | A | A | 0 |
| SRM5 | A | A | A | A | 0 |
| SRM6-1 | A | A | A | A | 0 |

Legend:

0 = No change

A = Acid

CHAPTER VI

VITAMIN REQUIREMENTS OF THE GENUS MICROBACTERIUM

Orla-Jensen, Otte and Snog-Kjaer (1936) concluded their studies on the vitamin requirements of 2 strains of M. lacticum with the following statement:

Im gegensatz zu den echten Milchsäurebakterien einen gedeihen die unechten Milchsäurebakterien -- die Coli- und Aerogenesbakterien -- gleich gut in kohlenbehandelter und in nicht kohlenbehandelter Milch; was damit übereinstimmt dass diese Bakterien sich in rein synthetischen Nährlösung entwickeln können; in ähnlicher Weise verhält sich Microbacterium lacticum.¹

In 1943 Orla-Jensen reiterated his belief that M. lacticum does not require "lactoflavin" or "milk-bios" for growth. A search of the literature disclosed no other experimental data on the vitamin requirements of members of this genus.

In a previous section it was shown that a medium of the following composition would support good growth of the microbacteria:

| | |
|--------------------------------------|----------|
| Yeast extract..... | 1.0 g |
| Proteose-peptone..... | 0.5 g |
| Glucose..... | 0.1 g |
| Potassium phosphate (monobasic)..... | 0.2 g |
| Potassium phosphate (dibasic)..... | 0.2 g |
| Water to..... | 100.0 ml |

pH 6.6

The organisms used as inocula for the experiments reported in this and following sections were grown in this yeast-extract, proteose-peptone, glucose medium (abbreviated Y.E.P.P.). The preparation of the

¹"The coli and aerogenes group of "pseudo-lactic acid" bacteria are just the opposite of the true lactic acid bacteria -- they grow equally well in charcoal treated milk or non-treated milk; this means that these organisms can grow in a purely synthetic medium, this is also true for Microbacterium lacticum."

Inoculum was carried out as follows: Fifteen ml centrifuge tubes containing 5 ml Y.E.P.P. were inoculated from stock slants with a straight wire needle and incubated 4 days at 30 C. The cells were then centrifuged, the supernatant discarded, and the sedimented cells washed with 5 ml of 0.85 per cent saline. The suspension was again centrifuged, the supernatant discarded, and the sedimented cells resuspended in 5 ml of 0.85 per cent saline. One loopful (inside diameter 4 mm) of this suspension served as the inoculum for 10 ml of test medium.

Several strains of microbacteria were tested for growth on a medium of the following composition:

| | | | |
|--|----------|------------------------------------|-------------|
| Casein hydrolysate, salt free, vitamin free ² | 1.0 g | Thiamine hydrochloride..... | 100.0 μ |
| Glucose, o.p..... | 1.0 g | Riboflavin..... | 100.0 μ |
| Sodium acetate, o.p..... | 1.4 g | Nicotinic acid..... | 100.0 μ |
| l-tyrosine..... | 0.01 g | Pyridoxine hydrochloride..... | 100.0 μ |
| l-tryptophane..... | 0.01 g | β -Calcium pantothenate..... | 100.0 μ |
| Asparagine..... | 0.025 g | Para-aminobenzoic acid..... | 100.0 μ |
| Choline chloride..... | 0.005 g | Glutamine..... | 100.0 μ |
| Guanine hydrochloride..... | 1.0 mg | Folic acid ³ | 20.0 μ |
| Adenine sulfate..... | 1.0 mg | Biotin..... | 10.0 μ |
| Xanthine..... | 1.0 mg | Salt A ⁴ | 0.5 ml |
| Uracil..... | 1.0 mg | Salt B ⁵ | 0.5 ml |
| Water to..... | 100.0 ml | | |

pH 6.9 \pm 0.2

²Casein hydrolysate obtained from Dr. M. L. Speck, National Dairy Research Laboratories, Baltimore, Maryland. Designated as Lot #5.

³Synthetic folic acid designated as "Folvite." Obtained from Dr. Benjamin Carey, Lederle Laboratories, Pearl River, New York.

⁴Contains monobasic potassium phosphate 25 g, dibasic potassium phosphate 25 g, boric acid 25 mg, potassium iodide 2.5 mg, water 250 ml.

⁵Contains magnesium sulfate \cdot 7H₂O 10 g, sodium chloride 0.5 g, ferrous sulfate \cdot 7H₂O 0.5 g, manganese sulfate \cdot 4H₂O 0.5 g, copper sulfate \cdot 5H₂O 25 mg, zinc sulfate 2.5 mg, water 250 ml.

The above medium was prepared in the concentrations indicated and sterilized by filtration through a Jena 1G5 auf 3 glass filter. The medium was dispensed aseptically into sterile test tubes⁶ in 10 ml amounts. This medium supported the growth (visible turbidity) of M. lacticum (cultures OJ3, 30-3) but not M. flavum (culture OJ8). Growth was discernable in 3 days at 30 C. The same cultures also grew when the medium was sterilized by autoclaving 12½ min. at 121 C.

Omission of glutamine and sodium acetate did not alter the amount of growth obtained and hence these substances were eliminated from further consideration. Casein hydrolysate in 0.5 per cent concentration was found to be as satisfactory as 1.0 per cent. Consequently, the former concentration was used throughout the remainder of these experiments.

Comparison of the amount of acid formed in the last-mentioned medium (by electrometric titration to pH 6.8) with additions of 0.1 per cent liver extract (Wilson's fraction L), peptonized milk or an aqueous extract of carrots (also in 0.1 per cent concentration) showed no significant differences.

⁶All glassware used in these experiments was prepared as follows: The test tubes and flasks were washed in tap water with tri-sodium phosphate (5 g per liter) and "Orvus" (1 ml per 5 liters). They were then rinsed 10 times in distilled water and dried in an oven at 110 C. The tubes and flasks were then plugged with gauze-covered cotton, autoclaved 15 min. and held 12 hours at 110 C before use. The pipettes were thoroughly rinsed with tap water and immersed in concentrated sulfuric acid (containing 1 g sodium nitrate per liter) for 30 min. They were thoroughly washed, dried and treated as the test tubes and flasks.

The constituents of the medium were then arbitrarily divided into four groups as follows:

| <u>Group A</u> | <u>Group B</u> | <u>Group C</u> | <u>Basal Substances</u> |
|------------------------|-------------------------|---------------------------|-------------------------|
| Thiamine . HCl | 1-Inositol | Adenine . SO ₄ | Casein hydrolysate |
| Riboflavin | Choline chloride | Guanine . HCl | Glucose |
| Nicotinic Acid | Para-amino-benzoic acid | Uracil | dl-tryptophane |
| d-Calcium pantothenate | | Xanthine | l-cystine |
| Pyridoxine . HCl | | | Asparagine |
| Biotin | | | Salts A and B |
| Folic acid | | | |

The substances contained in groups A, B, and C were sterilized individually through a Jena 165 auf 3 glass filter and stored under refrigeration. The dl-tryptophane, l-cystine and asparagine were treated in like manner. Casein hydrolysate, glucose and salt solutions were sterilized individually by autoclaving 15 min. at 121 C. The substances under test were added aseptically in proper concentrations to a sterile flask and dispensed in 10 ml amounts in sterile test tubes. In this way any combination of vitamins and accessory substances could easily be prepared without resorting to laborious and time-consuming filtrations and at the same time avoiding unknown chemical reactions introduced by autoclaving them in combination.

For preliminary work the type culture M. lacticum (OJ3) was studied. Four media were prepared, viz. (1) basal substances only, (2) #1 plus group A, (3) #2 plus group B, and (4) #3 plus group C. It was found that culture OJ3 would grow in media #2, #3, and #4 but not #1 and thus it was concluded that the required growth factors for this organism were present in group A. The growth in media #2, #3 and #4 appeared to be of the same density. To determine which of the vitamins in group A were needed, seven media were prepared each of which was deficient in one vitamin of group A. The results of this experiment are presented in Table VII.

TABLE VII

Effect of elimination of individual vitamins from group 1 on the growth of Microbacterium lacticum (O. J. #5)

| Basal Medium + Group 1 Substances Eliminated Individually | Serial Transfers | | | |
|---|--|------------------|------|----------------|
| | Ml. 0.009N NaOH Required to Neutralize 10 ml. of Culture | | | |
| | 1 | 2 | 3 | 4 |
| No Omission | 17.0 | Good * Growth | 17.4 | 17.1 |
| Less Thiamin Hydrochloride | 16.8 | 13.7 | 12.3 | Good Growth |
| Less Riboflavin | 16.5 | 15.6 | 15.8 | 18.5 |
| Less Pyridoxine Hydrochloride | 16.1 | 13.9 | 14.8 | 15.6 |
| Less Nicotinic Acid | 14.5 | Good Growth | 15.1 | 15.4 |
| Less d-Calcium Pantothenate | No Growth | | | |
| Less Folic Acid | 15.7 | 13.5 | 13.9 | 16.6 |
| Less Biotin | 10.6 | 12.5 | 15.6 | 10.8 |

* Growth observed, but cultures not titrated.

The data presented in this table indicate that M. lacticum (OJ3) was unable to grow in the absence of d-calcium pantothenate. When this vitamin was added to the medium good growth resulted. In addition the results indicate that culture OJ3 was able to grow through four serial (loop) transfers when each vitamin of group A, other than d-calcium pantothenate, was eliminated.

To determine the essentiality of d-calcium pantothenate for other strains of M. lacticum, 14 strains were tested against graded amounts of this vitamin. The results of this experiment are presented in Table VIII. Growth was much heavier in the tubes containing 0.1 μ /ml of d-calcium pantothenate than in those containing 0.01 μ /ml of this vitamin. It can

be seen that the titration values (ml 0.009 N sodium hydroxide per 10 ml medium neutralized to pH 6.8) differed only slightly. It was decided to measure growth response turbidimetrically using a photoelectric-colorimeter (Fisher AC model) in order to obtain a greater difference in the measurement of growth response to various vitamin doses. Filter 425 (blue) was used throughout this work.

TABLE VIII

Response of 14 strains of M. lacticum to graded amounts of d-calcium pantothenate as measured by acid production

| Media | Culture Designation | | | | | | | | | | | | | |
|-----------------------------------|--|------|------|------|------|------|------|------|------|------|------|------|------|------|
| | 1411 | 1412 | 1413 | 1414 | 1415 | 1416 | 1417 | 1418 | 1419 | 1420 | 1421 | 1422 | 1423 | 1424 |
| | ml. 0.009 N Sodium Hydroxide per 10 ml Medium* | | | | | | | | | | | | | |
| 1. Basal | 2.9 | 2.6 | 2.4 | 2.3 | 2.5 | 2.7 | 2.5 | 2.2 | 2.4 | 2.3 | 2.2 | 2.0 | 2.4 | 2.4 |
| 2. #1 + 0.0001 µ/ml d-Ca.pant. | 3.8 | 3.2 | 2.0 | 2.8 | 2.4 | 2.7 | 2.0 | 2.7 | 2.1 | 2.6 | 2.4 | 2.9 | 2.2 | 2.2 |
| 3. #2 + 0.001 µ/ml d-Ca.pant. | 3.5 | 2.2 | 2.4 | 2.3 | 2.3 | 2.2 | 2.0 | 2.1 | 2.4 | 2.7 | 2.8 | 2.7 | 2.4 | 2.4 |
| 4. #3 + 0.01 µ/ml d-Ca.pant. | 3.5 | 2.6 | 2.7 | 2.8 | 2.7 | 2.4 | 2.9 | 2.9 | 2.3 | 2.5 | 2.0 | 2.3 | 2.1 | 2.1 |
| 5. #4 + 0.1 µ/ml d-Ca. pant. | 4.6 | 2.8 | 2.8 | 2.2 | 2.3 | 2.2 | 2.6 | 2.2 | 2.5 | 2.6 | 2.2 | 2.5 | 2.2 | 2.2 |

* Incubated 7 days at 30 C.

The presence of thiamine in the medium appeared to increase the amount of growth obtained with culture OJ3. To determine whether or not this held true for other strains of M. lacticum the response of 13 strains to d-calcium pantothenate and d-calcium pantothenate plus thiamine was tested. Table IX shows the results obtained. These results indicated that whereas d-calcium pantothenate was necessary for the

inhibition of growth, thiamine plus d-calcium pantothenate gave more growth than d-calcium pantothenate alone.

TABLE IX

Response of Microbacterium laeticum to thiamine • HCl and d-calcium pantothenate

| Composition of Media | Per Cent Light Absorption | | | | | | | | | | | | |
|--|---------------------------|-----|------|------|------|------|------|------|------|------|-----|-----|-----|
| | OJ3 | OJ6 | 1PM1 | 1PM3 | 3RM2 | 3RM3 | 3RM6 | 6180 | 6181 | 80-5 | 9-2 | 513 | 516 |
| Basal Only | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13 | 0 |
| Basal + 1.0 μ /ml Thiamine • HCl | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 15 | 31 | 0 |
| Basal + 1.0 μ /ml d-calcium pantothenate | 2 | 0 | 20 | 7 | 3 | 6 | 0 | 11 | 0 | 0 | 16 | 15 | 0 |
| Basal + 1.0 μ /ml each of Thiamin Hydrochloride and d-calcium pantothenate | 57 | 0 | 44 | 50 | 44 | 47 | 0 | 40 | 0 | 57 | 40 | 35 | 0 |

It was desirable to know whether or not dl-tryptophane, asparagine, l-cystine and salts A and B were necessary for the growth of M. laeticum. Fourteen strains were tested in two media. One medium contained the above-mentioned substances, the other did not. Table X shows the results of this experiment. It can be seen that these compounds did not appreciably affect the amount of growth and they were omitted from the test medium.

Experience had shown that 0.2 per cent each of mono and dibasic potassium phosphate added to Y.F.P.P. gave good growth. Hence these salts were included in the test medium as buffer salts. The test medium

(Basal medium) at this point had the following composition:

| | |
|--|-------------|
| Casein hydrolysate, salt-free, vitamin-free..... | 0.5 g |
| Glucose, c.p..... | 1.0 g |
| Potassium phosphate (monobasic)..... | 0.2 g |
| Potassium phosphate (dibasic)..... | 0.2 g |
| Thiamine hydrochloride..... | 100.0 μ |
| d-calcium pantothenate..... | 100.0 μ |
| Water to..... | 100.0 ml |

pH 6.6

TABLE X

Influence of l-asparagine, l-cystine, l-tryptophane and salts on the growth of M. lacticum

| Culture Designation | Compounds Included * | Compounds Omitted |
|---------------------|----------------------|-------------------|
| OJ3 | 18 ⁷ | 21 |
| OJ6 | 16 | 5 |
| 3RM2 | 45 | 41 |
| 3RM3 | 43 | 36 |
| 3RM6 | 40 | 15 |
| 1PM1 | 68 | 56 |
| 1PM3 | 50 | 39 |
| 8180 | 40 | 26 |
| 8181 | 6 | 0 |
| 3-2 | 36 | 42 |
| 30-3 | 35 | 29 |
| 738 | 34 | 28 |
| 513 | 69 | 60 |
| 516 | 0 | 11 |

*1-Asparagine, l-cystine, l-tryptophane and salts, plus basal medium.

⁷Per cent light absorption after 4 days at 30 C.

The response of 4 strains of M. lacticum to graded amounts of d-calcium pantothenate in the above medium is shown in table XI. Curves drawn with these data are shown in figures 8a, 8b, and 8c. These results appear to establish the essentiality of d-calcium pantothenate for M. lacticum.

TABLE XI

Response of Microbacterium lacticum to graded amounts of d-calcium pantothenate

| Medium | Per Cent Light Absorption | | | |
|--|---------------------------|------|------|-----|
| | 0J3 | 3RM2 | 8180 | 8-2 |
| 1. Basal + 1.0 μ /ml Thiamin . HCl & d-Calcium pantothenate | 0 | 0 | 0 | 0 |
| 2. 1 + 0.001 μ /ml d-Calcium pantothenate | 0 | 0 | 0 | 0 |
| 3. 1 + 0.01 μ /ml d-Calcium pantothenate | 0 | 0 | 2 | 12 |
| 4. 1 + 0.05 μ /ml d-Calcium pantothenate | 11 | 25 | 32 | 34 |
| 5. 1 + 0.1 μ /ml d-Calcium pantothenate | 25 | 35 | 41 | 40 |
| 6. 1 + 0.5 μ /ml d-Calcium pantothenate | 31 | 45 | 45 | |
| 7. 1 + 1.0 μ /ml d-Calcium pantothenate | 35 | 47 | 46 | 45 |

All of the cultures used in this study were then tested for growth through 5 serial (loop) transfers at 48 hour intervals (30 C) in the above medium. The results of this experiment are presented in table XII. It can be seen that only the cultures designated as M. lacticum grew. (Culture 738 designated as B. acidophilus also grew through 5 transfers).

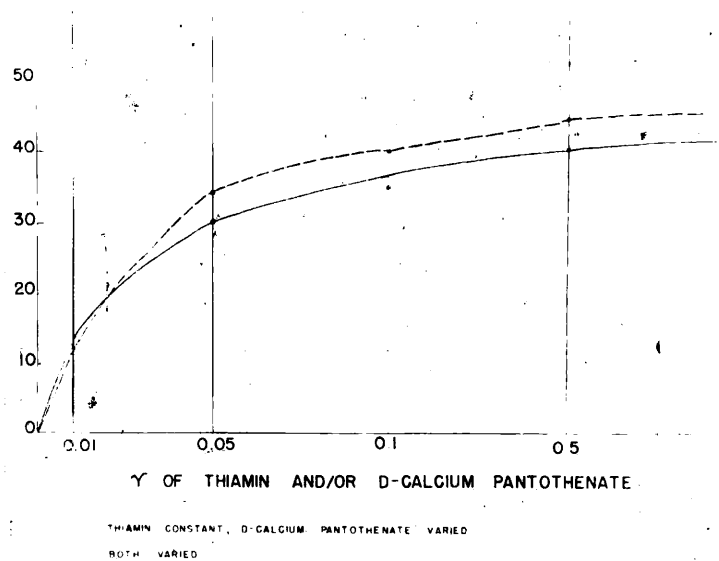


Fig. 8a Response of Microbacterium lacticum (S-2) to graded amounts thiamine . HCl and d-calcium pantothenate

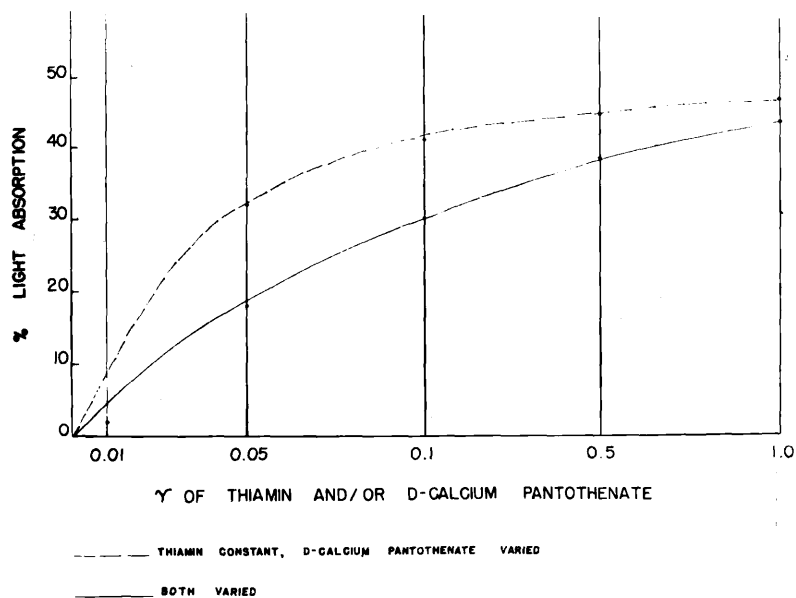


Fig. 8b Response of Microbacterium lacticum (8160) to graded amounts of Thiamine . HCl and d-calcium pantothenate

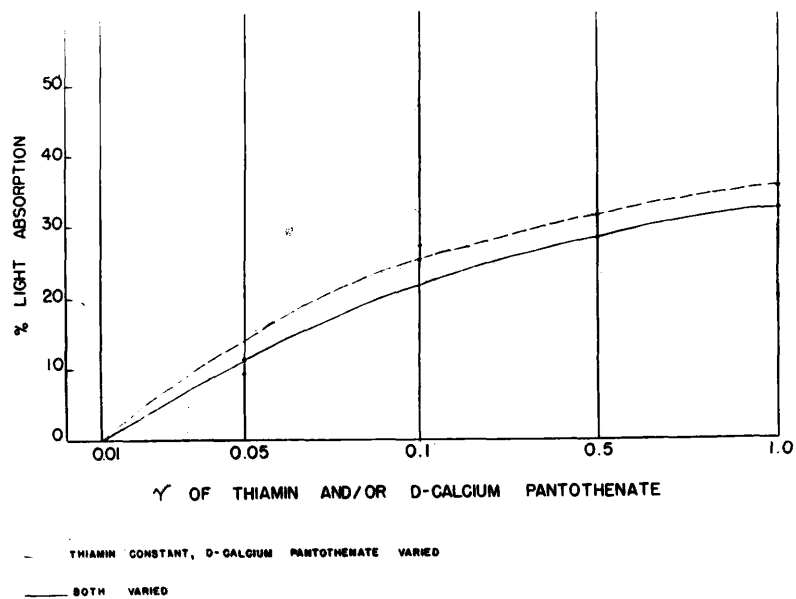


Fig. 8c Response of Microbacterium lacticum (OJ3) to graded amounts of thiamin HCl and d-calcium pantothenate

M. flavum, and other microbacteria described in Chapter V, did not grow through 5 serial transfers and evidently have more complex nutritional requirements. It was found that addition of 0.1 per cent carrot or liver extract to this medium would support the growth of M. flavum.

TABLE XII

Growth of members of the genus Microbacterium in basal medium through five serial transfers

| Culture Designation | Serial Transfers | | | | |
|---------------------|------------------|-----|-----|-----|-----|
| | 1st | 2nd | 3rd | 4th | 5th |
| OJ3 | + | + | + | + | + |
| OJ6 | 0 | | | | |
| 513 | + | + | + | + | + |
| 516 | 0 | | | | |
| 8180 | + | + | + | + | + |
| (8181) | not tested | | | | |
| 30-3 | + | + | + | + | + |
| S-2 | + | + | + | + | + |
| 1PM3 | + | + | + | + | + |
| SRM2 | + | + | + | + | + |
| SRM-9 | + | + | + | + | + |
| OJ8 | + | + | + | 0 | |
| OJ9 | 0 | | | | |
| 531 | + | 0 | | | |
| 534 | 0 | | | | |
| 342-S1 | 0 | | | | |
| 738 | + | + | + | + | + |
| 750 | + | + | 0 | | |
| SRM5 | 0 | | | | |
| SRM-1 | 0 | | | | |

Legend:

+ = growth (visible turbidity) in 48 hours 30 C.
0 = no growth

Cultures which grew through 5 serial transfers in the above experiment were tested for growth in the various media shown in table XIII. These results showed that, with the exception of culture 513, all of the strains of M. lacticum which would grow in the basal medium required d-calcium pantothenate for initiation of growth.

TABLE XIII

Effect of d-calcium pantothenate and thiamine on the growth of 9 strains of M. lacticum

| Media | Culture Designation | | | | | | | | |
|----------------------------------|---------------------|-----|------|------|-----|------|------|-----|--------|
| | CJ3 | 513 | 5180 | 30-3 | S-2 | 1PM3 | ERM2 | 738 | SPM6-9 |
| 1. Basal, No Vitamins | 0 | 21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2. #1 + 1.0 μ /ml Thiamine | 0 | 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3. #1 + 1.0 μ /ml d-Ca pant. | 18 | 22 | 15 | 30 | 31 | 39 | 31 | 26 | 32 |

Pantoic lactone (α , β dihydroxy, β dimethyl butyrolactone) and β -alanine are the constituent parts of pantothenic acid. To determine whether M. lacticum could use either moiety alone or in combination, an experiment was devised where these substances were included separately and in combination in place of d-calcium pantothenate. The results presented in table XIV show that M. lacticum cannot use these compounds in place of d-calcium pantothenate.

TABLE XIV

Effect of substituting pantoic lactone and β -alanine for d-calcium pantothenate on the growth of 9 strains of M. lacticum

| Media | Culture Designation | | | | | | | | |
|---|---------------------|-----|------|------|-----|------|------|-----|--------|
| | CJ3 | 513 | 5180 | 30-3 | S-2 | 1PM3 | ERM2 | 738 | SPM6-9 |
| 1. Basal + 1.0 μ /ml Thiamine + Pantoic lactone (no d-Ca pant) | 0 | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2. Basal + 1.0 μ /ml Thiamine + β alanine (no d-Ca pant) | 0 | 28 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3. Basal + 1.0 μ /ml Thiamine + Pantoic lactone and β alanine | 0 | 23 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

A summary of the preparation and composition of the final medium used in this study is given as follows: A solution of vitamin-free hydrolyzed casein, neutralized to pH 6.6 (with 0.1 N sodium hydroxide), and solutions of glucose and phosphate salts were prepared and sterilized individually by autoclaving 15 min. at 121 C. The d-calcium pantothenate and thiamin hydrochloride were sterilized individually by filtration through a Jena 1G5 auf 3 glass filter. Appropriate volumes of these ingredients were mixed together and added to sterile test tubes in 10 ml amounts so that the final concentrations were as listed on page 49.

The results presented indicate that under the described conditions d-calcium pantothenate was a nutritional requirement for most strains of M. lacticum used in this study. Further evidence was presented that whereas d-calcium pantothenate was essential for initiation of growth, thiamin hydrochloride was, in many instances, stimulatory. The stimulation by thiamin hydrochloride was observed to be non-linear from 0.01 μ /ml to 0.1 μ /ml. From table XI it can be seen that the optimum concentration of d-calcium pantothenate for 4 strains of M. lacticum was approximately 0.1 μ /ml. The minimum lies between 0.01 and 0.02 μ /ml of d-calcium pantothenate.

Total growth of M. lacticum in the medium listed on page 49 was less than in Y.E.P.F. or in a medium which had all the group A growth factors added, which indicated that an additional factor or factors are required for maximum growth. In addition, visible turbidity appears in Y.E.P.F. in 2 days at 30 C. Visible turbidity in the medium presented on page 49 appears in 3 $\frac{1}{2}$ to 4 days at 30 C.

CHAPTER VII

THE GROWTH OF M. LACTICUM IN CHEMICALLY DEFINED MEDIA

Orla-Jensen, Otte and Snog-Kjaer (1936) stated that M. lacticum would grow in a chemically defined medium. These workers apparently did not test their hypothesis, and a search of the literature revealed no other data on the subject.

To determine whether M. lacticum would grow in a synthetic medium, the casein hydrolysate used in the experiments described in chapter VI was omitted and nineteen pure amino acids were substituted for it.

Table XV shows the composition of the first medium employed. The amino acids were dissolved in distilled water in a concentration of 10 mg per ml and autoclaved 12 min. at 121 C. Amino acids not soluble in water (cystine and tyrosine) were dissolved in a minimum amount of hydrochloric acid and made to volume with distilled water. In studies where only a few amino acids were used they were neutralized to pH 7.0 with 1 N sodium hydroxide before use.

TABLE XV

A chemically defined medium for M. lacticum

| | | | |
|---------------------------------|-------------------------------|-----------------------|---------|
| Glucose, c.p. | 1.0 g | dl-Isoleucine | 40.0 mg |
| Potassium phosphate (monobasic) | 0.2 g | l-Tyrosine | 20.0 mg |
| Potassium phosphate (dibasic) | 0.2 g | dl Serine | 40.0 mg |
| d-Calcium pantothenate | 100.0 μ | l (+) Arginine | 20.0 mg |
| Thiamine hydrochloride | 100.0 μ | l (-) Proline | 20.0 mg |
| Aminoacetic acid | 20.0 mg | dl Threonine | 40.0 mg |
| l (+) Glutamic acid | 20.0 mg | l (-) Leucine | 20.0 mg |
| dl Phenylalanine | 40.0 mg | l (+) Lysine | 20.0 mg |
| β -alanine | 4.0 mg | dl Tryptophane | 40.0 mg |
| l (+) Histidine | 20.0 mg | dl Aspartic acid | 40.0 mg |
| l Cystine | 10.0 mg | dl α - Alanine | 40.0 mg |
| dl-Methionine | 40.0 mg | dl Valine | 40.0 mg |
| | Distilled H ₂ O to | | 100 ml |

pH 6.6 \pm 0.1

Cultures OJ3, S13, S180, SO-3, S-2, 1PMS, 3RM2, 738 and 3Pwb-9 were studied since they were the only ones which grew in the casein hydrolysate medium described in chapter VI. The inoculum was prepared in the manner employed in the investigations on vitamin requirements.

When cultures S-2, 3RM2, OJ3 and S180 were tested for growth in the medium listed in table XV, it was found that they would all grow through five serial transfers. If riboflavin, pyridoxine, nicotinic acid (1.0% per ml) and folic acid and biotin (0.1% per ml) were added to this medium, growth appeared in 2 days at 30 C and was much heavier at the end of 4 days than in the medium given in table XV. Omission of d-calcium pantothenate and thiamine hydrochloride from this medium resulted in no growth of any of the strains tested.

To determine whether any amino acids were essential for the growth of M. lacticum, they were arbitrarily divided into three groups as follows:

| <u>Group 1</u> | <u>Group 2</u> | <u>Group 3</u> |
|---------------------|------------------|----------------------|
| 1 (+) glutamic acid | 1 (-) tyrosine | amino acetic acid |
| dl-valine | 1 (+) histidine | dl-aspartic acid |
| 1 (-) leucine | dl-threonine | 1 (+) lysine |
| dl-isoleucine | dl-phenylalanine | dl-serine |
| dl-methionine | 1 (-) proline | dl- α alanine |
| 1 (+) arginine | | β -alanine |
| dl-tryptophane | | |
| l-cystine | | |

Culture 3RM2 was used as an "indicator organism," and it was assumed that most of the remaining strains would have similar requirements.

When the groups of amino acids were tested singly or in combination it was found that group 1 amino acids would support growth of M. lacticum whereas groups 2 or 3 alone would not. If groups 2 and 3 were combined, growth was obtained. These results are summarized in table XVI. It was concluded that the amino acids present in group 1 were sufficient to

support growth, whereas groups 2 and 3 did not contain adequate amino acids or else some of them were inhibitory for M. lacticum. It should be noted that inorganic nitrogen (0.5 per cent ammonium chloride) would not serve as a nitrogen source for M. lacticum.

Further experiments showed that any amino acids from group 1 or from groups 2 plus 3 could be eliminated without materially altering the amount of growth. In all cases, however, the growth was poorer than when nineteen amino acids were employed.

TABLE XVI

Growth of M. lacticum (SRM2) with amino acids (Groups 1, 2 and 3) and in 0.5 per cent ammonium chloride

| Serial Transfers | Group 1 | Group 2 | Group 3 | Groups 1 + 2 | Groups 2 + 3 | Groups 1 + 3 | 0.5 per cent NH ₄ Cl |
|------------------|---------|---------|---------|--------------|--------------|--------------|---------------------------------|
| 1st | + | 0 | 0 | + | + | + | 0 |
| 2nd | + | | | + | + | + | |
| 3rd | + | | | + | + | + | |
| 4th | + | | | + | + | + | |
| 5th | + | | | + | + | + | |

*Each medium contained: 1 per cent glucose
 0.37 per cent potassium phosphate (dibasic)
 0.13 per cent potassium phosphate (monobasic)
 100 μ each of d-calcium pantothenate and thiamine
 Amino acid groups 1,2,3 singly or in combination
 pH 7.0 \pm 0.1

It was found possible to "train" some strains to grow in a very simple synthetic medium. This was accomplished by repeatedly sub-culturing the organisms in a medium containing only groups 2 plus 3 and dropping out one amino acid from these groups after every five sub-cultures. Thus, 1PMS, OJS, 8180, and 3PMS-9 could be made to grow in a medium containing 500 mg phenylalanine per 100 ml as the only nitrogen source. Growth was visible but poor after 6 days at 30 C. The organisms listed

would grow on continued transfers in this medium.

Additions of 0.5 mgm per 100 ml each of adenine sulfate, guanine hydrochloride, uracil and xanthine to groups 2 plus 3 had no significant effect on growth.

From these studies it appears as if the following conclusions may be made regarding the growth of M. lacticum in a chemically defined media: (1) inorganic nitrogen as ammonium chloride will not satisfy the nitrogen requirements for M. lacticum, (2) a mixture of 19 amino acids and 2 vitamins supports good growth of strains S-2, SEM2, 8180, and OJ3, (3) d-calcium pantothenate is absolutely necessary for the growth of M. lacticum in a synthetic medium, (4) there appears to be no essential amino acid for M. lacticum; growth is proportional to the amount and variety of amino acids present, and (5) the purines, adenine, xanthine, and guanine and the pyrimidine uracil do not increase the growth of M. lacticum.

CHAPTER VIII

DISCUSSION AND SUMMARY

Morphology and Physiology:

In general, the microbacteria form small, round, smooth, convex, surface colonies of 0.25 to 2.0 mm in diameter after 2-3 days at 30 C. Microbacterium lacticum usually forms white colonies which may be described as "dew drop." Microbacterium flavum and some Microbacterium species form colonies which are pigmented citron or canary yellow although some are white.

Microbacterium lacticum may be considered to be a small, thin, (0.5 to 0.7 by 1.4 to 2.0 μ), gram positive, non-motile, non-sporing rod. When stained preparations are prepared from solid media, they exhibit a characteristic angular and side-by-side arrangement. The cells of some cultures are very slightly swollen giving them a slight but definite club-shape. Microbacterium flavum is usually larger than Microbacterium lacticum (0.7 to 0.9 by 1.4 to 2.5 μ). Both species are found to contain granules when stained with Loeffler's methylene blue. The Microbacterium species group of organisms was always found to be cocco-bacillary or wedge-shaped. They differ greatly from the classical morphology of Microbacterium lacticum and Microbacterium flavum. Several cultures of these latter species were found to contain cocco-bacillary or wedge-shaped cells; and consequently, one cannot in all instances identify these organisms on their morphological appearance. In most cases, however, Microbacterium lacticum was of the classical morphology as described above.

Some of the organisms designated as Microbacterium species had an appearance very similar to micrococci. However, they were found to be biochemically distinct from Micrococcus epidermidis, Micrococcus flavus,

Micrococcus aurianticus, Micrococcus canidicans, Micrococcus candicus,
Micrococcus conglomeratus, Micrococcus varians and Micrococcus luteus.

The group designated as Microbacterium species attacks the widest range of carbohydrates with the production of acid. Microbacterium lacticum is comparatively less active in this respect, while Microbacterium flavum attacks the least number of carbohydrates. None of the microbacteria produce gas from carbohydrates detectable by the usual cultural techniques.

The microbacteria are not proteolytic since they neither liquified gelatin nor digested the casein of skim milk. Hydrogen sulphide and indole were not produced.

The members of this genus were found to be facultatively anaerobic. Growth under anaerobic conditions was very meager after prolonged incubation. All of the organisms studied produced catalase and were variable in their ability to reduce nitrate to nitrite.

Although Microbacterium lacticum was reported able to survive 95 C for 2½ minutes in milk, only 3 of the cultures were able to survive this treatment in yeast-extract-proteose-peptone broth (pH 6.6). Undoubtedly, the milk itself enhances the thermal resistance of these organisms. This characteristic should not be used as a routine means of species identification because of the necessity for standardization of age of culture, number of cells, pH, dimensions of test tubes, etc. Some of these difficulties have been mentioned by Rahn (1945).

The above considerations lend themselves to the following characterization of the microbacteria:

(1) Microbacterium species. Gram positive, non-sporing, non-motile, wedge-shaped or coccobacillary rods, (0.7 to 1.4 μ diameter). Non-acid fast.

Form small surface colonies about 1 mm in diameter. Colonies smooth, round, convex, glistening, white or grey. Internal structure amorphous.

Catalase positive.

Acid in litmus milk.

Gelatin not liquefied.

Indole not formed.

Hydrogen sulfide not formed.

Acid from arabinose, xylose, dextrose, galactose, fructose, mannose, cellobiose, lactose, melibiose, sucrose, trehalose, raffinose and glycerol.

Aerobic, facultatively anaerobic.

Optimum temperature 30 C.

(2) Microbacterium lacticum. Gram positive, non-sporing, non-motile, short rods (0.5 to 0.7 by 1.4 to 2.1 μ). Non-acid fast.

Granulated when stained with methylene blue. Characteristic angular and side-by-side arrangements in preparations made from solid media. At times appear coccobacillary or wedge-shaped.

Colonies same as Microbacterium species.

Catalase positive.

Acid in litmus milk; coagulation variable.

Gelatin not liquefied.

Indole not formed.

Hydrogen sulfide not formed.

Acid from dextrose, galactose, fructose, mannose, maltose, dextrin and usually starch. Fail to ferment arabinose, rhamnose, xylose, melibiose, raffinose and glycerol.

Aerobic, facultatively anaerobic.

Optimum temperature 30 C.

(3) Microbacterium flavum. Gram positive, non-sporing, non-motile, short rods (0.7 to 0.9 by 1.4 to 2.6 μ). Non-acid fast. Granulated when stained with methylene blue. May appear cocco-bacillary or wedge-shaped.

Form small surface colonies about 2.5 mm in diameter. Colonies smooth, round, convex, glistening, citron or canary yellow.

Catalase positive.

Litmus milk unchanged.

Gelatin not liquefied.

Indole not formed.

Hydrogen sulfide not formed.

Acid from dextrose, fructose, mannose, and mannitol. No acid from arabinose, rhamnose, xylose, maltose, raffinose, dextrin, starch or glycerol.

Aerobic, facultatively anaerobic.

Optimum temperature 30 C.

Vitamin and Amine Acid Requirements:

It has been found that d-calcium pantothenate is absolutely necessary for the growth of Microbacterium lacticum. Further, it can utilize neither pantoic lactone or β -alanine alone or in combination and thus is similar to the lactic acid bacteria, propionic acid bacteria and certain strains of Corynebacterium diphtheriae (Porter, 1946). The amount of d-calcium pantothenate necessary for growth is also of the same order of magnitude as is required by the above mentioned organisms. The exact function of d-calcium pantothenate (or pantothenic acid) in the metabolism of bacteria or higher organisms is as yet unknown.

It may possibly function in several metabolic processes (McIlwain and Hughes, 1944; Sevag and Green, 1944).

It is of interest to note that most of the strains which are of similar physiology and identified as Microbacterium lacticum require d-calcium pantothenate.

Microbacterium lacticum has been found able to grow in a synthetic medium composed of 19 amino acids and 2 vitamins. The amount of growth decreases with a decrease in the amount of variety of amino acids used. There are evidently no essential amino acids. The lactic acid bacteria also require a variety of amino acids for optimum growth (viz. alanine, arginine, aspartic acid, cystine, glutamic acid, histidine, leucine or isoleucine, lysine, methionine, phenylalanine, serine, threonine, tryptophane, tyrosine and valine). Microbacterium lacticum does not appear to be nutritionally as fastidious as some of the lactic acid bacteria. Under the conditions used, adenine, xanthine, guanine, and uracil do not stimulate Microbacterium lacticum. This species is unable to use 0.5 per cent ammonium chloride as a sole source of nitrogen.

Microbacterium flavum and Microbacterium species were found unable to grow in a synthetic medium which supported the growth of Microbacterium lacticum.

Taxonomy:

The microbacteria are undoubtedly closely allied to the lactobacilli and propionibacteria. The microbacteria do not seem to be biochemically related to the diphtheroid group since they are more active fermentatively and have lower optimum temperatures. Since they possess catalase they are probably more closely related to the propionibacteria than the lactobacilli. The only points of difference are the

types of fermentation products and oxygen relationships. The latter property is dependent (at least in the case of microaerophilic organisms) on the chemical composition of the medium and hence is not too important as a taxonomic criterion.

Undoubtably other species of microbacteria remain to be described. In this work only three species have been studied. Since Microbacterium mesentericum has been proven to be a mycobacterium (Wittern, 1933), and is now classified as Nocardia mesenterica, and since Microbacterium liquefaciens is quite difficult to isolate, and perhaps nonexistent, the above three species are the only microbacteria known today.

They are differentiated biochemically and seem to form a firm base for the genus. In the light of present knowledge, it would be foolish to abandon the genus on the grounds that it is a "miscellaneous collection of gram positive rods". One need only think of the micrococci or the genus Bacterium to realize that the genus Microbacterium is fairly well defined.

From the data presented, the following key has been constructed which may aid in the identification of the microbacteria.

I. Acid from glycerol and raffinose

A. Acid from arabinose and xylose

1. Microbacterium species

II. No acid from glycerol and raffinose

A. Acid from maltose and starch

2. Microbacterium lacticum

B. No acid from maltose and starch

3. Microbacterium flavum

This key differs somewhat from that in the 6th edition of Bergey's Manual (1948). The latter differentiates Microbacterium lacticum from

Microbacterium flavum on the basis of starch fermentation and heat-resistance. While the key presented in this study retains starch fermentation as a distinguishing characteristic between these species, heat-resistance has not been employed for reasons given earlier in this chapter. The key is similar to that given by Orla-Jensen (1919) in that maltose is employed as a second differential characteristic. Wittern (1933) and Speck (1943) have reported that Microbacterium flavum may ferment maltose and the latter investigator has recommended that it not be used for differential purposes. None of the strains of Microbacterium flavum used in this study were able to ferment maltose. Bergey's Manual (1939) uses the fermentation of starch and maltose as differential characteristics between these two species. In addition, the key presented in this study employs glycerol and raffinose fermentation to differentiate these species from the organisms indicated as Microbacterium species. It should be understood that Microbacterium species is morphologically distinct from Nocardia mesenterica.

The 5th edition of Bergey's Manual (1939) placed Microbacterium as the second genus in the family Bacteriaceae. In the 6th edition (1948) this genus has been transferred to the family Lactobacteriaceae and is the second genus of the tribe Lactobacilleae. Microbacterium liquefaciens has been placed in an appendix to the genus.

The data in the preceding sections may be of aid in identifying Microbacterium lacticum, Microbacterium flavum and certain other organisms possessing morphological and physiological properties which ally them to these species.

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