A STUDY OF THE GENUS MICROBACTERIUM

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Orla-Jensen (6) described seven strains of rod-shaped bacteria, occurring chiefly in dairy products, which manifested sufficient peculiar properties in common to be placed in a separate genus, to which he gave the name Microbacterium. Since that time but little information has been added regarding these organisms, and their relationships to other more well-defined groups have remained questionable. In Bergey's Manual (2) this genus was placed in the family Bacteriaceae, a "heterogeneous collection of genera whose relationships to each other and to other groups are not clear."

The cultures of Orla-Jensen (6) were small rods, weak acid-formers in milk; they reduced nitrates to nitrites, and usually split hydrogen peroxide. He believed they constituted a genus entirely separate from the other lactic acid rod forms which were discussed; i.e., Thermobacterium, Streptobacterium, and Beta bacterium. Three species, viz., Mbm. lacticum, Mbm. flavum, and Mbm. mesentericum, were definitely recognized and less definitely a fourth, Mbm. liquefaciens.

Robertson (7) described several thermoduric cultures from pasteurized milk and concluded that they were probably identical with Microbacterium lactis, (probably Mbm. lacticum of Orla-Jensen), although some of their properties were different from those described by Orla-Jensen for this species. These organisms were the most heat resistant of a number of non-spore-forming species isolated by him from pasteurized milk. He was not convinced that Lactobacillus thermophilus described by Ayers and Johnson (1) was not actually Microbacterium lacticum.

Wittern (9) considered the genus Microbacterium and the tetracocci as marking the boundary of the lactic acid bacteria on one side as do the coli-aerogenes group on the other. Wittern was able to isolate the three species originally described by Orla-Jensen, but as was true with Robertson's cultures, the properties of her organisms did not conform entirely with the properties described by Orla-Jensen. Wittern was particularly concerned with Mbm. mesentericum which showed numerous similarities to the Actinomycetes and the genus Mycobacterium. After a detailed comparison of the properties of these various groups, she concluded that Mbm. mesentericum showed decidedly more similarities to the genus Mycobacterium than to the lactic acid bacteria and should more rightfully be considered a member of that genus.

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1 Now with Sealtest, Inc., Research Laboratories, Baltimore, Md.
Jensen (5) believed that the morphology of the microbacteria warranted their being considered as corynebacteria and mycobacteria. He designated *Mbm. lacticum* as *Corynebacterium lacticum*, *Mbm. flavum* as *Mycobacterium flavum*, and *Mbm. liquefaciens* as *Corynebacterium liquefaciens*. Jensen pointed out that physiological properties of *Mbm. lacticum* and *Mbm. flavum* are considerably different from those of the true corynebacteria and mycobacteria, but believed that the morphological similarities of these groups should receive precedence in classification studies.

The present study is an effort to define more clearly the genus *Microbacterium*, and to study the relationships of these organisms to other well-defined groups.

**EXPERIMENTAL AND RESULTS**

**Isolation and Sources of Cultures Studied**

Many of the organisms were first encountered as contributing materially to high thermoduric plate counts of milk. Subsequent studies showed that these organisms were common on milking equipment. Milk stone contained large numbers of organisms belonging to the genus *Microbacterium*, and this material may be considered the immediate source of those which appeared in milk in particularly large numbers. The *Microbacterium* cultures used in this study were isolated from the following sources: milk pasteurized in the laboratory at 161° F. for 16 seconds, 1 sample, 9 cultures; milk pasteurized in the laboratory at 145° F. for 30 minutes, 1 sample, 3 cultures; milk pasteurized in a dairy plant at 161° F. for 16 seconds, 1 sample, 4 cultures; raw milk as delivered by farmers to the dairy, 8 samples, 8 cultures; dairy farm strainer, 1 sample, 1 culture; milking machines, 3 samples, 14 cultures; milk stone on a milk pail, 1 sample, 8 cultures; cheddar cheese, 3 samples, 25 cultures.

Isolations from equipment were made by rinsing the particular piece with sterile skim milk. A sample of the rinsing was pasteurized at 161° F. for 16 seconds and plated on tryptone-glucose-meat-extract-skim milk agar. Raw milk arriving at the dairy was pasteurized and plated as were the rinsings of the equipment. The pasteurized samples were also plated and incubated in the same manner. Milk stone was removed from pails by a cotton swab moistened with sterile skim milk. The swab was pressed out in 5 ml. of sterile skim milk and the milk was then heated at 161° F. for 2 minutes. Plates were poured with tryptone-glucose-meat-extract-skim milk agar and incubated at 30° C. for 3 days.

Isolations from cheese samples were made by triturating 1 g. of ripe cheddar cheese and 0.1 g. of sodium citrate in about 2 ml. of sterile water. Finally 8 ml. more water were added and mixed with the dissolved cheese. This solution was then heated at 161° F. for 2 minutes and plated with tryptone-glucose-meat-extract-skim milk agar and incubated at 30° C. for 3 days.
In the majority of the samples it was found that 3 days at 30°C should be allowed for the development of *Microbacterium* colonies. The colonies may not be visible or they may be easily overlooked after only 2 days’ incubation, specially at 37°C. For this reason the microbacteria may be overlooked when samples are incubated for this time and temperature in routine milk plate counts.

In the present investigation 48 cultures representing all of the sources listed previously were studied. In addition, two cultures, Nos. 8180 and 8181, which were received from the American Type Culture Collection, Washington, D. C., were studied. These cultures were believed to represent two original cultures of Orla-Jensen.

After a culture was purified it was carried in litmus milk, or on agar slants of the following composition: Bacto proteose-peptone 0.5 g.; beef extract 0.30 g.; glucose 0.1 g.; K$_2$HPO$_4$ 0.4 g.; KH$_2$PO$_4$ 0.125 g.; distilled H$_2$O 100 ml.; agar 1.5 g.; final pH approximately 7.0. A clear broth which supported good growth of the microbacteria could be obtained by omitting the agar from the formula of the foregoing agar medium.

**Morphology**

The morphology of the microbacteria has been described extensively by Orla-Jensen (6), Wittern, and Jensen, but several further observations have been made in the present investigation. The appearance of *Microbacterium* organisms in milk when stained by the Newman-Lambert stain may often be misleading as to their actual morphology. In such preparations the irregular staining of the cells and their frequent grouping often make them appear as small cocci in groups. But when grown on agar slants and stained by the Gram method the cells usually stained evenly and were gram positive with the exception of one culture, the cells having characteristic angular and palisade arrangements. Occasionally some cells would show a darker polar staining giving the appearance of short-chained streptococci. When grown on tryptone-glucose-skim milk-meat agar for 3 days at 30°C and stained by the Gram stain, the individual cells measured 0.4–0.5 × 0.6–1.0 μ, the average being about 0.4 × 0.8 μ.

None of the cultures was found to form spores and all were non-motile. The colonies on standard tryptone-glucose-skim milk-meat-extract agar were very small and smooth, the surface colonies round, and the sub-surface ones lens-shaped. After 7 days’ incubation at 30°C on this medium, the colonies averaged about 0.7 mm. in diameter, although individually they varied from 0.2 mm. to 1.0 mm. Frequently the colonies would be hardly more than visible after 3 days at 30°C on this medium.

**Oxygen Relationships**

Orla-Jensen (6) observed that the microbacteria were aerobes and were able to split hydrogen peroxide. Wittern (9) found that all cultures of
the genus *Microbacterium* which she tested were able to grow in broth when the atmosphere was reduced to 14–15 mm. mercury pressure. In a study of the rod-shaped lactic acid bacteria, Hansen (4) found that *Mbm. lacti-
cum* and *Mbm. flavum* utilized oxygen much more rapidly in the presence of
chocolate or lactate than did organisms of the *Thermobacterium, Streptobo-
cacterium, and Betabacterium* genera. The strong inhibiting effect of
hydrocyanic acid on the *Microbacterium* cultures indicated that they dif-
ered from other lactic acid rods not only by having catalase but in their
hemin content.

All of the microbacteria in this study were characteristic aerobes as they
grew well on agar slants and produced catalase. The ability of the or-
genisms to grow in anaerobic conditions was determined by inoculating the
cultures into tubes of the proteose-peptone-broth medium which had been
heated in the steamer for 20 minutes just prior to inoculation to aid in
eliminating dissolved oxygen from the broth. The cultures were then in-
cubated in a vacuum oven and the temperature held at 32° C. Air was
evacuated from the oven and replaced with CO₂. Evacuation and replace-
ment with CO₂ was repeated several times and finally the oven was filled
with CO₂ and the cultures were incubated for 5 days. The cultures grew
under these conditions, although the growth was not so abundant as under
aerobic conditions, which indicated that the microbacteria are facultatively
anaerobic.

*Acid Produced in Milk*

Although many of the microbacteria were relatively weak acid formers
in milk, a number of the cultures formed sufficient acid in 7 days at 30° C.
to produce a typical acid curd in the milk. It remained to learn to what
extent lactic acid was responsible for the acidity, since it was only assumed
previously that it was lactic acid, as based on Orla-Jensen’s observation
that d-lactic acid was usually formed.

Eighteen representative cultures were used to study the amount of lactic
acid produced in milk. The cultures were inoculated from a 3 day litmus
milk culture into 75 ml. of sterile skimmilk and incubated at 30° C. usually
for 7 days. The initial acidity and total acidity were determined by titrating
a 9 g. sample with N/10 NaOH using 3 drops of 1 per cent phenol-
phthalein (in 50 per cent alcohol) as indicator. The lactic acid was deter-
dined by the method of Troy and Sharp (8) in which a 25 g. sample was
used for assays in this investigation. This method is particularly adapted
for determining lactic acid in milk. The procedure consists of the pre-
cipitation of interfering substances by copper hydroxide at 45° C., direct
oxidation of the filtrate with potassium permanganate after acidifying
with a sulfuric acid-manganese sulfate mixture, distillation of the acetalde-
hyde into sulfite solution, and titration of the bound sulfite with iodine.
The results of these analyses showed that the acid formed by most of the cultures was predominantly lactic acid (Table 1). Culture 342S1, which showed very weak acid production and produced but little lactic acid, possessed other properties which were different from most of the cultures studied and will be discussed later. The percentage of the acid as lactic acid, produced by these cultures in milk, is similar to that found by Troy and Sharp (8) to be produced by *Lactobacillus bulgaricus* and *Leuconostoc paracitrovorus* when grown in milk. It would seem that the microbacteria should be considered as lactic acid bacteria as regards the kind of acid produced.

**Thermal Resistance**

Orla-Jensen (6) and Wittern (9) used the unusually high thermal resistance of the microbacteria in isolating these organisms from various products. Robertson (7) has also noted this property in the *Microbacterium* cultures which he isolated from pasteurized milk. In the present study the organisms were first encountered as contributing to high counts of pasteurized milk, and it became obvious that the high heat-resistance was one of the most outstanding characteristics of the microbacteria. It seemed desirable to determine the thermal resistance of a number of these cultures and ascertain whether they could be eliminated from a product by any reasonable heat treatment.
Ten representative cultures were selected and grown in sterile litmus milk for 4 days at 30° C. Tubes containing 5 ml. of sterile litmus milk at room temperature were inoculated with one drop of the 4-day culture immediately before being subjected to each heat treatment. The tubes were then immersed in a water-bath with the water level about 2–3 inches above that of the milk. The tubes were shaken continuously during the time required to reach temperature, and less frequently during the holding period. One and one-half minutes were required to reach 62.5° C. and approximately 2 minutes to reach 71.6° C. and 85° C.; the holding period was begun only after the tubes reached the proper temperature. After heating the tubes were cooled quickly to about 30° C. and incubated at 30° C. to determine the viability of the cultures.

All of the cultures withstood 62.5° C. for 30 minutes and all but one (342S1) survived 71.6° C. for 10 and 30 minutes. These results indicate that the microbacteria would survive in milk pasteurized by the usual holder-process or by the high-temperature-short-hold process, as was found to be true during the earlier part of this investigation, when a number of the cultures were first isolated. All but culture 342S1 withstood 85° C. for 2½ minutes, and seven survived heating to 85° C. for 10 minutes, while none survived 85° C. for 30 minutes. In a separate experiment, two of the cultures remained viable in skimmilk for approximately 12 hours at 62.5° C., although no multiplication occurred during this time. Like the true lactic acid bacteria, the microbacteria have been found to be non-pathogenic (Wittern (9)) and their occurrence in milk and dairy products should be no public health menace.

Production of CO₂

The microbacteria do not produce sufficient gas when grown in carbohydrate media to be detected by visual inspection. Since it is recognized that the amount of carbon dioxide production is of considerable value in characterizing the propionic acid bacteria and certain of the lactobacilli, it seemed desirable to measure the CO₂ produced by the microbacteria.

The amount of CO₂ produced by 15 typical Microbacterium cultures was determined in improvised Eldredge tubes. Into one tube was placed 20 ml. of broth containing: glucose, 1 per cent; yeast extract, 1 per cent; proteose-peptone (Difco), 0.5 per cent; K₂HPO₄, 0.4 per cent; KH₂PO₄, 0.1 per cent; the pH was adjusted to 7.0. To the other tube was added 25 ml. N/10 Ba(OH)₂ to absorb the CO₂ produced. In another series of tubes skimmilk was substituted for the glucose broth.

The tubes were incubated at 30° C. for 7 days in a slanting position to give a maximum absorption surface to the Ba(OH)₂. The amount of Ba(OH)₂ converted into BaCO₃ by the CO₂ produced was determined by titrating the residual Ba(OH)₂ with N/10 HCl (Table 2).
The microbacteria produced relatively small amounts of CO₂ from both the milk and glucose broth, although the volume produced from the glucose broth was slightly larger than that from the milk. Owing to the small amount of CO₂ produced, further studies on the relationship of sugar fermented and CO₂ produced were considered unnecessary. The fact that the cultures were grown under a limited oxygen supply probably accounted in some measure for the limited CO₂ production, although the growth in all the cultures was abundant. For comparative purposes, however, it can be concluded that the microbacteria, with a limited oxygen supply, produce markedly less CO₂ than do the propionic acid bacteria.

**TABLE 2**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Gm. CO₂ per 20 ml. medium</th>
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<tr>
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</tr>
<tr>
<td>H3</td>
<td>0.002</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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<tr>
<td>Cb1</td>
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</table>

**General Biochemical Characteristics**

**Litmus milk.** All of the cultures produced an acid reaction in litmus milk, although several cultures were decidedly weaker acid producers than were the majority. Eighteen cultures produced sufficient acid in 7 days at 30° C. to curdle the milk, a typical acid curd being produced. A number of the cultures were found to curdle the milk after prolonged laboratory cultivation in milk, whereas this property was lacking when the culture was freshly isolated. Litmus milk was a very satisfactory medium for carrying the cultures, as they usually remained viable for several months in this medium when stored in the refrigerator.

**Action on gelatin.** The organisms were grown in the proteose-peptone medium to which was added 4 per cent Bacto-Gelatin. None of the cultures liquefied the gelatine after incubation at 30° C. for about 14 days.

**Catalase production.** The cultures were grown in the proteose-peptone medium for 7 days at 30° C. After this period 3 ml. of 1 per cent H₂O₂ was added to each tube. All of the cultures produced catalase, usually a
considerable amount. Catalase was formed in much larger amounts in this medium than in sterile skimmilk.

_Nitrate reduction._ There are conflicting reports describing the ability of the microbacteria to reduce nitrate. Orla-Jensen (6), Jensen (5), and Bergey’s Manual (2) describe the organisms as able to reduce nitrate to nitrite, while Robertson (7) found that his cultures usually failed to reduce nitrates. In the present investigation the cultures were grown in broth containing 0.5 per cent Bacto-proteose-peptone, 0.3 per cent meat extract, and 0.1 per cent \( \text{KNO}_3 \) (pH adjusted to 7.0–7.2) for 10–14 days at 30°C. Forty-four of the cultures failed to reduce nitrate, while five were able to reduce it, indicating that, in general, the microbacteria are unable to reduce nitrate.

_Starch hydrolysis._ The organisms were streaked onto starch agar plates, the medium containing 0.5 per cent Bacto-proteose-peptone, 0.3 per cent meat extract, 0.2 per cent soluble starch, and 1.5 per cent agar (pH adjusted to 7.0–7.2). After 10–14 days at 30°C, 48 of the cultures had hydrolyzed the starch, while one culture failed to attack it. This culture, 342S1, also possessed other peculiar characteristics which will be mentioned later. These results are in accord with those of Dull (3), who isolated, from the intestines of adult persons, cultures of _Mbm. lactis_ which produced diastase.

_Fermentation of sugars._ The sugars which have been described by others as valuable for distinguishing between species of microbacteria were used in this work (i.e., glucose, maltose, and raffinose). The media used contained 0.5 per cent proteose-peptone, 0.3 per cent meat extract, brom thymol blue, and the pH adjusted to 7.0–7.2. This was tubed and sterilized, and then sufficient 10 per cent sterile sugar solution (sterilized by filtration through a Seitz filter) was added to give approximately 1 per cent concentration of the sugar. Prior to inoculation, the tubes were incubated for 24 hours to ascertain their sterility. All of the cultures produced acid from glucose and maltose; all failed to ferment raffinose except culture 342S1.

With the exception of its ability to ferment raffinose, culture 342S1 possessed the properties of _Mbm. flavum_. The key to the species of the genus _Microbacterium_ in Bergey’s Manual (Bergey et al. (2)) designates the failure of _Mbm. flavum_ to ferment maltose as an important identifying characteristic of this species. In the present investigation the _Mbm. flavum_ cultures was found to ferment maltose. Likewise, Wittern (9) found 13 out of 21 cultures of _Mbm. flavum_ fermented maltose. These data indicate that the inability to ferment maltose is not a constant characteristic of _Mbm. flavum_ and should not be used as an identifying characteristic of this species.

DISCUSSION

The microbacteria form a well-defined group of bacteria. They have some properties in common with the genus _Propionibacterium_, but differ
mainly in that they produce chiefly lactic acid, are more aerobic, and produce relatively little carbon-dioxide from carbohydrates in an atmosphere containing a limited oxygen supply. This is the genus to which the microbacteria show the closest relationship, both morphologically and physiologically. Differing chiefly from the lactobacilli by producing catalase, the microbacteria possess some similarities to this group, such as producing chiefly lactic acid in milk, being non-proteolytic, gram positive, and non-motile, and usually failing to reduce nitrates to nitrites. Members of the genus Microbacterium are found in human and animal feces, milk, dairy products, and dairy farm equipment, along with the true lactic acid bacteria. Morphologically, the microbacteria show similarities to organisms of the genus Corynebacterium, particularly the "diphtheroids," but differ greatly from them physiologically. To place the microbacteria in the genera Corynebacterium and Mycobacterium, on the basis of morphology as proposed by Jensen (5), would undoubtedly prove confusing. The characteristics shown by the microbacteria in the present investigation seem to support the view that the genus Microbacterium shows close relationships to the genera Propionibacterium and Lactobacillus, and should be placed close to these genera in a system of classification.

The genus Microbacterium seems to contain two well-defined species; viz., *Mbm. lacticum* and *Mbm. flavum*. *Mbm. lacticum* forms much more acid in milk, chiefly lactic acid, does not ferment raffinose, hydrolyzes starch, usually does not reduce nitrate to nitrite, and forms no distinct pigment on agar. *Mbm. flavum* (culture 342S1 in this study) is somewhat larger in size (approximately 0.5 x 1 - 2 μ), forms relatively small amounts of acid in milk, of which lactic acid forms a smaller part, fails to hydrolyze starch, reduces nitrate to nitrite, forms a yellow pigment on agar, and is less heat resistant than *Mbm. lacticum*. Although Orla-Jensen (6) included in the genus *Mbm. mesentericum*, Witten (9) demonstrated that this organism by its morphology, its ability to use petroleum as a carbon source, and by other differences from the microbacteria, should be allocated more properly to the genus Mycobacterium. No culture of this organism was encountered in the present investigation.

The distinct differences between Lactobacillus thermophilus and any members of the genus Microbacterium should be emphasized. Robertson (7) suggested that this organism was possibly the same species as *Mbm. lacticum*. *L. thermophilus*, however, does not form catalase, is a true thermophile in that it grows best from 55° C. to 63° C., has a lower thermal death time (i.e., killed immediately when heated to 82.2° C.) and is distinctly different morphologically (cells measure 0.5-1.0 x 2.5-6 μ) from *Mbm. lacticum*. From the data available it would appear that *L. thermophilus* is, as proposed by Ayers and Johnson (1), a true lactobacillus, and that its properties separate it entirely from the genus Microbacterium.
One of the outstanding characteristics of the Microbacterium cultures is their high heat resistance. The organisms evidently have their origin primarily in the intestines and feces of animals (see Orla-Jensen (6), Dull (3), and Wittern (9)) and gain entrance into milk by means of dairy equipment. Their ability to withstand high temperature enables them to remain on dairy farm equipment between milkings if the equipment is not thoroughly cleaned with water above 200° F., or if it is not chemically sterilized. In this manner they can contribute materially to high thermoduric plate counts. Once the organisms have gained entrance into the milk, the usual pasteurization temperatures for market milk and cooking temperatures for cheeses permit them to survive in these products.

CONCLUSIONS

Organisms belonging to the genus Microbacterium may be characterized as short, gram positive, non-motile, non-sporulating, diphtheroid-like rods which form catalase, produce predominantly lactic acid in milk, fail to liquefy gelatine, usually fail to produce nitrite from nitrate, and are very thermoduric.

Two well-defined species, Microbacterium lacticum and Microbacterium flavum are recognized in this genus.

The microbacteria gain entrance into milk chiefly by means of dairy farm milking equipment. The high heat resistance of these organisms enables them to occur in pasteurized milk and milk products. The metabolism of the microbacteria suggests that their role in these products is very similar to that of the true lactic acid bacteria. Owing to their presence in dairy products in smaller numbers, their activity probably represents only a small part of the microbiological activity occurring in these products.

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REFERENCES


