Some Studies on Microbacteria from Iowa Dairy Products*

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Microbacteria have attracted attention because they are among the most heat-resistant, non-sporeforming types of bacteria found in dairy products. As they grow poorly, if at all, at 37 C, they usually were overlooked when the standard plate count procedure called for incubation of the plates for 48 hr at that temperature. However, they form small colonies when incubation temperatures of 32 to 35 C are employed. Because of their thermoduric nature, microbacteria have assumed increasing importance as contributors to high post-pasteurization counts on milk products, as the use of low incubation temperatures has become more general. Also, their common association with poorly cleaned equipment has been responsible for increased interest.

The significance of microbacteria in milk was first brought to light by Robertson (1927) in his researches on the thermophilic and thermoduric flora of pasteurized milk. Thomas *et al.* (1950) have reviewed the literature appearing up to 1950 on the incidence and thermoduric character of these organisms as they appear in dairy products and also have reviewed much of the literature in classification.

The taxonomic position of this group of bacteria has been the subject of much controversy since Orla-Jensen (1919) gave them the generic name *Microbacterium*. Other workers who were interested primarily in the taxonomic aspects of microbacteria included Jensen (1932, 1934), Wittern (1933), Speck (1943), Doetsch and Pelczar (1948) and Abd-El-Malek and Gibson (1952).

Speck (1943) concluded that *M. lacticum* and *M. flavum* constitute legitimate species of the genus *Microbacterium*, which should be classified close to the genera *Propionibacterium* and *Lactobacillus*. Later, Doetsch and Pelczar (1948) confirmed and extended the studies of Speck. They also proposed another species which was designated *Microbacterium* sp., being less heat-resistant than *M. lacticum* but more fermentative.

Abd-El-Malek and Gibson (1952) found that what they termed the saprophytic heat-resistant corynebacteria of milk were indistinguishable from *M. lacticum*. They favored discarding the genus *Microbacterium* as had been proposed by Jensen (1932, 1934). They grouped the organisms they encountered under *Corynebacterium lacticum*.

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The present studies were undertaken to obtain information relative to the occurrence of microbacteria in Iowa dairy products and to provide additional information on certain characteristics of this group of bacteria.

Experimental Procedures

In all isolation procedures the source material was streaked onto plates poured with tryptone-glucose-beef extract-milk agar (American Public Health Assn., 1948). These plates, as well as all other cultures not specifically stated to the contrary, were incubated at 32 C. Plates were incubated 2 or 3 days before isolations were attempted. A 0.01 ml loop usually was used for measuring the sample, where quantitative results were desired at the time of isolation. Where counts of pure cultures, as after heating, were desired, the usual standard plate count procedure for milk (American Public Health Assn., 1948) was followed with the sample. For primary isolations samples previously pasteurized were used without further treatment. Raw milk samples were heated at 61.7 C for 30 minutes or at 71.7 C for 2 minutes before smearing. Raw cream samples were neutralized to 0.18 to 0.20 per cent titratable acidity and 5 ml portions heated to 61.7 C for 30 minutes. Butter serum was separated by melting at 45 to 50 C, and 5 ml quantities then were heated to 61.7 C for 30 minutes. Non-fat milk solids were reconstituted 1:9 in water and pasteurized before streaking. A 1 g sample of cheese was dispersed into 9 ml of 2 per cent sodium citrate, using materials previously warmed to 45 C. These suspensions were heated at either 71.7 C for 2 minutes or 61.7 C for 30 minutes before smearing. Rinse water and swab rinses from utensils were diluted 1:1 in sterile milk and heated to 61.7 C for 30 minutes before isolations were attempted.

Colonies showing the characteristics of the known *Microbacterium* species available for comparison, as well as numerous colonies of other types, were examined to see if they were gram positive, non-sporulating short rods. Isolations of typical types were made into litmus milk. The sources of the cultures isolated and used are given in table 1.

Attempts to develop a selective medium which would permit isolation of a large percentage of microbacteria from a sample containing numerous bacteria of other types proved unsuccessful. Apparently additional in-

formation relative to the distinctive characteristics of the genus will need to be available before real progress can be made in designing a suitable selective medium by other than extensive trial and error. Attempts to isolate *M. flavum* by use of the method suggested by Wittern (1933) were unsuccessful.

In addition to original isolations, cultures 342S1 labelled *M. flavum* and H2 designated as *M. lacticum* were obtained from Dr. M. L. Speck, and cultures 3RM5 (*Microbacterium* sp.), 3RM2 (*M. lacticum*) and 531 (*M. flavum*) were supplied by Dr. R. N. Doetsch. Culture 342S1 of Speck has been designated as *Microbacterium* sp. by Doetsch and Pelczar (1948).

The procedures used in sugar fermentation tests, starch hydrolysis, nitrate reduction, catalase test and preparation of the basal medium were those suggested by Speck (1943).

TABLE 1. *Key to the sources of original isolations*

PRODUCT	CULTURE
Milk from individual producers	7/1, 14/1, 14/3, 18/3, 18/4, 42b1 42b2, 47/2
Pasteurized market milk	PC1, PC8, PM2, PM4, PM6, PM18, PM19, PM21, PH1, PH4
Cheese	S1*, S3*, S4*, C2†, Hg1‡, Hg3‡, L1§, B1¶
Non-fat-milk solids	DS1, DS2, DS4
Vacreated cream	VC2
Butter	Bu3
Milker suction hose	MS13

* From Swiss-type cheese.
† From Cheddar cheese.
‡ From Heidelberg cheese.
§ From Limburger cheese.
¶ From blue cheese.

Gelatin hydrolysis was determined by the method of Smith *et al.* (1946). Positive gelatin hydrolysis was recorded when a definite clearing around the colonies was observed after incubation at 32 C for 9 days; doubtful and negative reactions were recorded only after 20 days. Gelatin liquefaction was reported negative if no change had occurred in stabs in nutrient gelatin after incubation at 21 C for 28 days. Negative results in fermentations were recorded after incubation for 14 days.

Production of ammonia from peptone was tested on a medium containing 4 g tryptone and 0.1 g glucose in 100 ml distilled water. After incubation for 7 days, Nessler's reagent was used as the test for ammonia, using color development in an uninoculated tube as a control.

Thermal resistance was tested using organisms grown for 3 or 4 days in proteose peptone broth and then added to skim milk. The skim milk suspension of organisms was plated before and after heating to determine survival. Skim milk was used as the heating medium in preference to broth because it gave results more applicable to dairy products.

Growth temperature range was determined by inoculating proteose peptone broth tubes with one loopful of a broth culture 3 days old and incubating at 21, 37 and 45 C until growth occurred or up to 14 days. Incubation at 3 to 5 C and 12 C was prolonged, if necessary, to 28 days.

Salt tolerance was tested by using a double-strength proteose peptone broth to which enough sterile 25 per cent sodium chloride solution and sterile water were added aseptically to obtain the required salt concentration in broth of normal strength. The tubes were inoculated with one drop of culture 3 or 4 days old and then incubated up to 14 days.

Lactic acid determination was by the method of Troy and Sharp (1934).

Electron micrographs were taken from preparations made of cells centrifuged from the broth culture 3 days old. An RCA type EMU electron microscope was used.

RESULTS

Cultures of microbacteria invariably were isolated from 0.01 ml portions of pasteurized market milk, 0.01 ml or smaller quantities of cheese suspension, frequently from 0.01 ml portions of laboratory-pasteurized milk from individual producers and occasionally from other milk products. One strain was obtained from a dirty milking machine suction hose. Only a few farm utensils were examined and these did not contain the organisms in detectable numbers, possibly because hypochlorite, rather than hot water, was being used for bactericidal treatment. Since the procedures employed were not quantitative, no actual counts are presented. However, quite a few of the samples contained about 1,000 microbacteria per milliliter and the approximate counts on several samples exceeded 10,000 per milliliter or gram. In no case did the microbacteria constitute an important percentage of the countable bacteria in an unpasteurized product, but they sometimes made up more than half of the count on pasteurized products.

Some of the biochemical activities of the microbacteria strains are shown in table 2. Only three groups are recognized in the table, despite the variations in sugar fermentations, gelatin-hydrolyzing activity and nitrate reduction in the first group. Hydrolysis of starch and fermentation of maltose and lactose were general among group I, but there were differences in ability to hydrolyze gelatin and ferment the other sugars. A small percentage of the cultures reduced nitrates to nitrites. Group II was more fermentative than group I but failed to hydrolyze starch or gelatin, while the culture of *M. flavum* placed in group III was rather inert. None of the cultures used in this study produced

ammonia from tryptone in 7 or 21 days at 32 C. All cultures failed during incubation at 32 C for 10 days to show any evidence of fat hydrolysis, using the nile blue sulfate technic of Collins and Hammer (1934) with coconut oil as the fat substrate. Gelatin was not liquefied by any of the cultures in 28 days at 21 C.

When heat treatments of milk suspensions giving pretreatment counts of about 3 million were used, cultures 3RM5 and 342S1 of group II showed survivals

TABLE 2. *Some of the biochemical characteristics of microbacteria*

CULTURE	GELATIN HYDROLYSIS	NITRATE REDUCTION	STARCH HYDROLYSIS	ACID PRODUCTION FROM*			
				Glycerol	Raffinose	Xylose	Arabinose
Group I							
B1, 47/2	−	−	+	−	−	−	−
18/4	−	−	+	−	−	+	+
PC8, 3RM2, PM2, DS1, PH4, PM6 PM4, VC2, 18/3, 42b1, 42b2, DS4, H2	±	3+ 10−	+	−	−	−	−
S1, C2, PC1, Hg1, S4, 14/1, 7/1, S3	+	−	+	−	−	−	−
14/3, PH1	+	−	+	−	−	+	+
MS13, PM21, PM19	±	−	+	−	+	+	+
Bu3, L1, Hg3 DS2	+	1+ 3−	+	−	+	+	+
Group II							
3RM5, 342S1	−	1+ 1−	−	+	+	+	+
Group III							
531	−	−	−	−	−	−	−

* All cultures with the exception of 531 produced acid from lactose and only 342S1 and 531 failed to produce acid from maltose. All were catalase positive.

of slightly over 1 and 10 per cent, respectively, when heated at 61.7 C and no survival after 71.7 C for 10 minutes. Under similar conditions, five representative cultures of group I showed no definite decrease in count after heating at 71.7 C for 30 minutes. Even at 83 C, all but three of the organisms (H_2, PM2 and 14/3) of group I survived for 2.5 minutes, some in relatively large numbers. More detailed data on four of the more resistant cultures of group I heated at 84 C are presented in table 3. Survival of 11 to 33 per cent after heating at 84 C for 2.5 minutes was obtained, and two of the four strains even survived in small numbers a treatment of 15 minutes at 84 C. Organism 531 of group III failed to survive 83 C for 2.5 minutes, but no data on survival at lower temperatures were obtained.

These organisms produced acid in milk rather slowly. All of the organisms of group I had changed the reaction of litmus milk to distinctly acid in 4 days at 32 C. Twenty-eight cultures of group I produced 0.32 to 0.46 per cent developed acidity, calculated as lactic acid, and curdled the milk after 10 days, but five cultures failed to curdle the milk, one producing only

TABLE 3. *Survival data on selected cultures of group I heated at 84 C*

CULTURE	HEATED FOR 2.5 MIN			HEATED FOR 15 MIN	
	Count/ml before heating	Count/ml after heating	% survival	Count/ml before heating	Count/ml after heating
S1	1,500,000	500,000	33	1,500,000	10
B1	1,900,000	303,000	16	900,000	50
Bu3	2,100,000	350,000	17	2,200,000	20
PC1	600,000	66,000	11	500,000	10

TABLE 4. *Production of lactic acid by microbacteria in skim milk and in glucose broth**

CULTURE	TOTAL ACIDITY	DEVELOPED ACIDITY	LACTIC ACID	LACTIC ACID IN DEVELOPED ACIDITY
	%	%	%	%
Group I				
PC1	0.545	0.322	0.277	86.0
S1	0.55	0.327	0.28	85.6
L1	0.54	0.317	0.223	70.3
14/3	0.535	0.312	0.256	82.1
Bu3	0.42	0.223	0.179	90.8
DS2†	0.40	0.247	0.226	91.5
Group II				
3RM5	0.34	0.187	0.099	52.9

* Incubated for 7 days at 32 C.
† In glucose broth.

0.135 per cent developed acidity. Failure to curdle milk was not correlated with other characteristics within this group. Organisms of group II produced a definite acid reaction in litmus milk in 14 days but no coagulation; the increases in titratable acidity after 10 days were 0.185 and 0.26 per cent for 3RM5 and 342S1, respectively. Organism 531 (group III) gave no detectable change in litmus milk after 21 days and gave only 0.045 per cent developed acidity in skim milk after 10 days at 32 C. The percentage of lactic acid in the developed acidity produced in skim milk and glucose broth by representative strains of groups I and II was determined. The data in table 4 indicate that lactic acid constitutes a major portion of the acidity developed in skim milk and glucose broth by members of group I, but organism 3RM5 of group II yielded only about half of the acidity as lactic acid.

Of 32 cultures of group I tested, 17 failed to grow in proteose peptone broth in 14 days at 36.5 to 37 C, whereas all the cultures showed a slight growth at 12 C. In most cases, the growth at 37 and 12 C was not enough to cause turbidity in the broth, although typical stringy growth could be seen at the bottom of the test tubes. Of the 15 cultures which grew at 37 C, 13 gave only slight growth after 14 days. Organisms of groups II and III grew relatively well at 37 C. None of the cultures of the three groups grew at either 3 to 5 or 45 C.

Most of the cultures were fairly salt tolerant. Six cultures of group I grew when the concentration of sodium chloride was 6 but not 8 per cent; the remainder of the group grew in the presence of 8 per cent salt. Organisms of group II grew in 10 per cent sodium chloride concentration, but *M. flavum* (group III) failed to grow in broth containing 9 per cent sodium chloride, although it has been reported that it can grow in 10 per cent concentration (Doetsch and Pelczar, 1948).

Studies on colony characteristics and morphology of these organisms confirmed the findings of previous workers. Surface colonies of groups I and II grown on standard milk agar were smooth, round, glistening, convex, grayish-white, with the subsurface colonies lens-shaped. The colonies measured from less than 1 mm to 1.5 mm in diameter when incubated at 32 C for 3 days, with an average of about 1 mm. However, colonies of *M. flavum* were larger and were faintly yellow with a darker center. All the organisms were non-motile, non-sporulating and gram positive. The cells characteristically were in a palisade arrangement. Some of the cells showed granules, especially in unshadowed preparations examined with the electron microscope.

Discussion

Microbacteria of group I could be isolated from most samples of Iowa dairy products examined, by using their unusual heat resistance as a selective procedure. The procedures employed were not such that exact counts could be obtained, but in some samples the microbacteria constituted a considerable portion of the thermoduric microflora. However, in no case did these organisms cause counts to be so high as to be above usually accepted standards. Failure to find these organisms in larger numbers in or on dairy farm utensils may be a reflection of the common use of chemical bactericidal agents, rather than heat, in the sanitization of equipment on the dairy farm. A larger number of samples should be examined with more definitely quantitative procedures before definite conclusions are reached concerning incidence of these bacteria on Iowa farms and in Iowa dairy products.

Despite numerous attempts and the examination of a large number of colonial types in an endeavor to obtain microbacteria of types other than group I from Iowa dairy products, the only microbacteria isolated were members of group I. Since heating at 61.6 C for 30 minutes usually was used as an aid to isolation, rather than the considerably higher temperatures which might have been used had only organisms of group I been sought, heat destruction of the other types hardly seems an adequate explanation for their absence, even if they are not so able to withstand high temperatures as are the members of group I. The cultures of the other groups that were available for testing grew as well as or better than the members of group I on the media employed and the incubation conditions seemed satisfactory, so selective conditions of growth during primary isolation hardly seem to explain the absence of these types. Speck (1943) also found organisms corresponding to group I to be dominant among the strains he isolated and characterized. Doetsch and Pelczar (1948) observed a similar but less marked trend among their isolates. European workers seem to have found much greater proportions of other types among their isolates, but the reason for this is not apparent. The continuing failure to isolate in this country an organism which can be classified as *M. liquefaciens*, while such organisms seem relatively common in Europe, is a point of some interest.

The organisms which have been placed in group I, both the isolates and the cultures obtained from others, all seem to form an homogeneous group. There are some variations within the group, but none of the variations seem to provide a sound basis for any sub-division, even at the variety level, because of the common intergradations of characteristics. They all grow slowly on artificial media and in skim milk, show optimum growth at 32 C, are catalase positive, gram positive, aerobic, non-sporeforming, non-motile short rods of relatively small size which produce predominantly lactic acid from the lactose of milk or glucose in a broth. They are very heat-resistant, consistently ferment lactose and maltose and hydrolyse starch, and are relatively tolerant to salt. They sometimes are feebly proteolytic and are non-lipolytic. These characteristics all indicate that the members of group I belong to the species *Microbacterium lacticum* as defined by Orla-Jensen (1919), Speck (1943) and Doetsch and Pelczar (1948). Seven of the 35 cultures of *M. lacticum* produced acid from raffinose, xylose and arabinose but not from glycerol. These cultures could not be regarded as *Microbacterium* sp. of Doetsch and Pelczar because they hydrolyzed starch, failed to produce acid from glycerol, grew poorly or not at all at 37 C, and were less salt tolerant. These organisms also differed from the raffinose fermenters encountered by Abd-El-Malek and Gibson (1952) because they were able to survive in appreciable numbers heating to 71.7 C for 30 minutes and 83 C for 2.5 minutes. Three other cultures of group I were able to ferment xylose and arabinose, but not raffinose; they seemed otherwise indistinguishable from the larger

group. Doetsch and Pelczar (1948) also found one culture with this latter fermentation pattern; although they excluded this culture from the species *M. lacticum* on the basis of their description of the species, they did not provide a place in which to put the culture in their classification. Presumably the description of the species *M. lacticum* should be amended to include those cultures which occasionally are encountered with greater fermentative ability than previously has been ascribed to the species, for their other characteristics seem to indicate they definitely belong to the species.

The organisms of group II previously had been designated *Microbacterium* sp. by Doetsch and Pelczar (1948) and no new cultures were isolated in the present study. Speck (1943) earlier had classified organism 342S1 as *M. flavum*, on the basis of its inability to ferment maltose and its considerably lesser heat resistance. The two organisms of this group could not be differentiated as completely from *M. lacticum* as Doetsch and Pelczar (1948) had indicated, because a number of the strains which definitely seemed to be *M. lacticum* fermented one or more of the raffinose, xylose and arabinose group of sugars. However, the positive fermentation of glycerol by these organisms and the failure to hydrolyse starch, coupled with the much lower heat resistance, the slower acidification of milk, the lower percentage of lactic acid in the acids produced, the ability to grow at a somewhat higher salt concentration and the morphological differences seem to provide a basis for recognition of a separate species. Those who first showed this relationship should have the privilege of selecting the species name. The characteristics are sufficiently similar to those of *M. lacticum* to indicate that these bacteria probably belong in the same genus; however, the close resemblance of organisms 3RM5 and 342S1 to thermoduric members of the genus *Micrococcus* is worthy of note. The organisms of group II apparently are considerably less common than is *M. lacticum*, for Speck (1943) found only one culture out of a considerable group of isolations, Doetsch and Pelczar (1948) at least did not find them to be preponderant, and no isolations of this type were made in the present investigations.

The one culture of group III undoubtedly represents the species *M. flavum* and is easily differentiated from the two other species. Since only one culture was examined, there is no adequate basis for an opinion as to whether *M. flavum* should be retained in the same genus with *M. lacticum* and *Microbacterium* sp. Of some possible significance is the fact that this species has not been isolated by earlier investigators (Robertson, 1927; Speck, 1943; Doetsch and Pelczar, 1948) who have studied the genus *Microbacterium* as it occurs in dairy products in this country or in the present investigation.

The results presented in this paper do not provide an adequate comparative basis for expressing a satisfactorily documented opinion relative to the place in which this group of bacteria should be classified. However, the authors would like to call attention to the possible close relationship of these bacteria to the family *Micrococcaceae*. Orla-Jensen (1919) suggested this relationship more than 30 years ago. The description Abd-El-Malek and Gibson (1948) gave for their group III B of the micrococci parallels very closely the characteristics of *Microbacterium* sp. except for the slight difference in morphology. In a later publication of these same authors (1952) they point out that the organisms which might be confused with their *Corynebacterium lacticum* in their procedure for segregation of this type were thermoduric micrococci of their group III B. Certainly in any comparative study made of the genus *Microbacterium* the relationships with the genus *Micrococcus* should be examined, as well as the relationships with the saprophytic corynebacteria. Thermoduric character, salt tolerance, catalase activity, aerobic character of metabolism and many other characteristics are those of the micrococci. Morphology is hardly a valid basis for disregarding this suggestion, for both the families *Lactobacteriaceae* and *Nitrobacteriaceae*, as now recognized, contain both coccus and rod forms.

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Summary and Conclusions

Microbacteria were isolated from most of the Iowa dairy products examined for this type of bacteria. They never constituted a large percentage of the countable bacteria in unpasteurized products, but they commonly accounted for more than half of the countable colonies from pasteurized products.

Only microbacteria identifiable as *Microbacterium lacticum* were isolated during these studies. This species shows greater variability with respect to destruction by heat and to sugar fermentations than has been reported in previous studies.

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The Effect of the Carbohydrate Nutrition on Penicillin Production by *Penicillium chrysogenum Q-176*[1]

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Relatively few papers have been published on the carbon nutrition of *Penicillium chrysogenum* and its effect on penicillin yield. Jarvis and Johnson (1947) investigated the relationship between sugar utilization and penicillin formation in detail. They found that optimal penicillin yield is obtained if glucose and lactose are present in such ratios that the proper quantity of mycelium is formed from the readily available glucose before the mold is forced to use the slowly fermented lactose. Cook and Brown (1949–1950) studied the effect of various carbohydrates and salts on penicillin yields and evaluated them for the possible relation to the mechanism of formation.

The work reported here presents the results obtained when lactose is replaced by glucose, or by other carbohydrates, in such a way that there is a controlled growth of the mold during the penicillin production phase. This condition can be established by the slow addition of the sugar to the fermentation by suitable means. This study was done exclusively on a shake-flask scale. Work of a similar nature was conducted in 30-liter fermentors and will be reported elsewhere (Hosler and Johnson, 1952).

EXPERIMENTAL METHODS

Fermentation techniques. Penicillium chrysogenum Q-176 was used exclusively in these experiments. Information about the origin of this strain and previous studies on pelicillin yields by its use have already been published (Gailey *et al.*, 1946).

Inoculations were made with 5 ml of vegetative suspension 45 to 48 hours old grown on standard fermentation medium. This had been inoculated with 5 ml of a spore suspension which had been grown on the standard spore plate medium described by Gailey *et al.*, (1946). All fermentations were run on a rotary type shaker at a total volume of 100 ml in 500 ml Erlenmeyer flasks. The shaker operated at 250 rpm and described a 2-inch circle. The temperature was 25 C in all runs.

The fermentation medium used for these experiments was as follows (figures represent grams per liter): lactose, 30 (in control only); glucose, 10; ammonium acetate, 3.5; ammonium lactate, 6.0; KH_2PO_4, 6.0; $MgSO_4 \cdot 7H_2O$, 0.25; $ZnSO_4 \cdot 7H_2O$, 0.02; $FeSO_4$, 0.02; $MnSO_4$, 0.02; and Na_2SO_4, 0.5. The pH was adjusted to 6.5 before sterilization. Further additions of sugar were started when the fermentations were 24 hours old and continued thereafter, except for the lactose controls, which received no additional sugar. In the intermittently-fed runs, additions were made in 2 ml portions every 12 hours until penicillin production reached a maximum. Sodium phenylacetate at a 0.05 per cent level (calculated as the acid) was added as precursor to all fermentations every 12 hours (Higuchi *et al.*, 1946).

In the continuously-fed runs, feeding was achieved with the device used as an anti-foam control in tank fermentations (Anderson *et al.*, 1952). Feed rate was controlled by the concentration of the sugar solution

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