MICROCOCCUS CRYOPHILUS, SPEC. NOV.; A LARGE COCCUS ESPECIALLY SUITABLE FOR CYTOLOGIC STUDY

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An unusually large coccus, isolated in the course of a study by Sulzbacher and McLean (1950) of the bacterial flora of fresh pork sausage, was observed in hanging drop preparations to contain possible nuclear material. In preparations by the DeLamater (1951) technic for cytological study, DeLamater and Woodburn (1951) have shown that the organism is a peculiarly favorable one for the exhibition of mitosis. Mudd *et al.* (1951) have utilized it in mitochondrial studies. It has, therefore, seemed important to record a taxonomic description of the bacterium, for which we propose the name *Micrococcus cryophilus*, spec. nov.

Habitat. The coccus was isolated from a finished package of pork sausage prepared from frozen meat. In a survey covering 2 years, in which 300 separate colonies were studied from plated sausage, sausage ingredients, and plant equipment, this organism was encountered only once.

Morphology and staining. The organism occurs in chains, clusters, and diploforms, and as single spheroidal cells (figure 1). The coccus reacts irregularly when gram stained, i.e., some organisms on every smear from veal infusion agar, regardless of age of the culture, are gram-positive, while a predominant number of the cells are gram-negative. As may be seen (table 2), a few very large and a smaller number of extraordinarily large cells occur under optimal growth conditions in young cultures of this bacterium. A high percentage of giant cells is found in old cultures in yeast extract broth, pH 5.5. In any case, they may occur singly or in diploform and may stain uniformly gram-positively or gram-negatively or show a dense gram-positive central region, occupying one-half to three-fourths of the area in a cell, with gram-negative surrounding cytoplasm. The origin and fate of the giant cells have not been traced in living preparations.

Cells grown on Morton-Engley (1945) yeast-extract, glucose agar stain uniformly, reacting negatively to Gram's stain. In preparations from cultures (18 to 24 hours old, grown at 9.8 C or 20 C) on Difco veal infusion agar and stained by Gram, a great many cells of both average and giant size contain material which is gram-positive and which is surrounded by a peripheral gram-negative area. The gram-positive areas are present whether acetone-alcohol mixture, 35 per cent and 65 per cent, respectively, or 95 per cent alcohol is used for decolori-

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zation. The fact that such gram-positive areas are not present in all cells and that the pattern of such areas when present is not identical, indicates a cell differentiation that could be associated with function or could be a result merely of the relatively complete decolorization of the more peripheral protoplasm.

In the electron-micrograph (figure 2), the "electron-opacity" of the cells is seen not to extend to the periphery. This is in contradistinction to the cells of the usual fully gram-positive staphylococci and streptococci in which the electron-opacity extends to the periphery of the protoplast (Mudd and Anderson, 1944). The semitransparent cell-wall connecting adjoining cells is clearly seen at one site in figure 2.

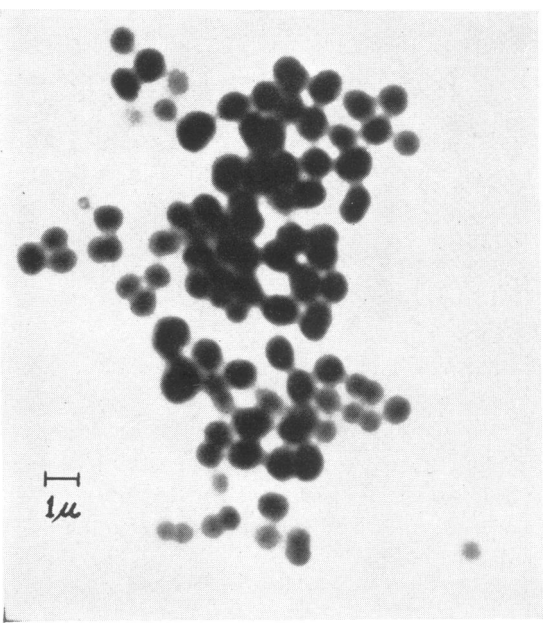

Figure 1. Photomicrograph of gram-stained preparation of *Micrococcus cryophilus* grown on Difco veal infusion agar, 24 hours at 20 C. Photomicrograph taken by Loren C. Wintersheid, Department of Microbiology, University of Pennsylvania Medical School.

Peritrichous flagella have been demonstrated on a very occasional cell by Kulp's modification of Loeffler's stain. Likewise, an occasional motile cell has been observed in hanging drop preparations.

The Periodic-acid-Schiff stain, a modification of the Hotchkiss-McManus procedure by Kligman and Mescon (1950), indicates that no cellulose or chitin is present in the cell walls of the organism.

Size. Since the unusual size of the coccus makes it useful for cytological studies, an effort has been made to obtain an accurate record of measurements. Cognizant of the work of Knaysi (1941) and of Dubin and Sharp (1944), a series of measurements was undertaken on cells prepared in different ways. An effort was made to determine whether or not cells attached to the slide by means of Mayer's

albumin fixative, dried in air and stained with Loeffler's methylene blue, would measure the same as cells dried in air, passed through the flame three times, and stained with Loeffler's methylene blue or by Gram (Hucker's ammonium oxalate-crystal violet). In addition, cells attached to the slide with albumin fixative and stained with methylene blue, without previous drying, were measured. Measurements of cells in such preparations were compared with those made on 100 living cells suspended in physiological saline in a hanging drop by means of the light microscope utilizing Wratten filters: Bausch and Lomb neutral density 1.2, K-1 no. 6 and B no. 58. Measurements were made of 100 cells in each of groups

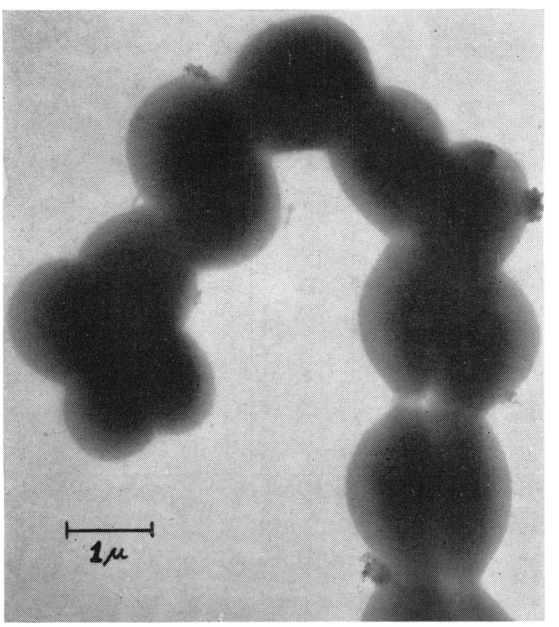

Figure 2. Micrococcus cryophilus grown 24 hours on collodion over M. and E. agar containing 2 per cent glucose. No fixation. Stored over "drierite." Electron micrograph taken by Mrs. Jean Minkin, Research Engineer, through the courtesy of the Franklin Institute, Laboratories for Research and Development.

1, 2, and 4 (table 1), the cells being taken from a single culture on veal infusion agar grown for 18 hours at 20 C. Measurements were also made of 100 cells in group 3 (table 1), these cells being taken from a culture grown on the same medium at 20 C for 48 hours. Care was exercised to measure cells as encountered rather than selected cells of any type or size. Forty-six cells in electron micrographs were measured.

Measurements of stained cells prepared in four different ways (table 1) were subjected to an analysis of variance[5] by the method of Snedecor (1934). Frequency distribution of cell size as affected by method of staining is recorded in

[5] The authors are indebted to Dr. John R. Preer, Department of Zoology, University of Pennsylvania, for making the analysis of variance.

TABLE 1

Treatment of smears of suspensions of cells in physiological saline according to groups for analysis of variance

GROUP NO.	AGE OF CULTURE (HOURS)	FIXATIVE APPLIED TO SLIDE PRIOR TO CELL SUSPENSION	CELL SUSPENSION ALLOWED TO DRY ON SLIDE PRIOR TO STAINING	FLAMING OF SMEAR	STAINING OF SMEAR
1	18	None	Yes	3 times through Bunsen burner	Gram-Hucker's ammonium oxalate-crystal violet
2	18	None	Yes	3 times through Bunsen burner	Loeffler's methylene blue—one minute
3	48	None	Yes	3 times through Bunsen burner	Gram-Hucker's ammonium oxalate-crystal violet
4	18	Mayer's albumin fixative	Yes	None	Loeffler's methylene blue—one minute
5	24	Mayer's albumin fixative		None	Loeffler's methylene blue—one minute

TABLE 2

Frequency distribution of cell size as affected by method of staining

CLASS—AS TO SIZE (×)	SIZE RANGE OF CLASSES IN MICRONS	FREQUENCY OF OCCURRENCE IN 100 CELLS OF THOSE IN A GIVEN CLASS AS TO SIZE			
		f	f	f	f
		Group 1*	Group 2	Group 3	Group 4
1	1.0 –1.2	2	0	0	0
2	1.21–1.4	0	0	0	0
3	1.41–1.6	73	83	72	74
4	1.61–1.8	17	10	14	17
5	1.81–2.0	0	0	0	0
6	2.01–2.2	6	7	7	6
7	2.21–2.4	0	0	0	0
8	2.41–2.6	0	0	4	1
9	2.61–2.8	0	0	0	0
10	2.81–3.0	1	0	3	2
11	3.01–3.2	0	0	0	0
12	3.21–3.4	0	0	0	0
13	3.41–3.6	1	0	0	0

* See table 1 for description of treatment by groups.

table 2. As analysis of variance showed that size of cells in groups 1, 2, 3, and 4 is essentially the same, the conclusions are that: (1) the use of albumin fixative to ensure adherence of cells to the slide during the staining process results, if the cells are allowed to dry in air before staining, in stained cells the same size as those on slides that are flamed, (2) once the cytoplasm has become shrunken as the result of drying in air, the more drastic treatment of flaming and the Gram's stain shrink it no more than methylene blue staining, and (3) the age of the culture (within 18 to 48 hours) does not affect the measurements obtained for cells in gram-stained preparations. Though not included in the analysis of variance, measurements on cells not dried before staining, group 5 (table 1), indicate that such treatment results in cells with visible outlines in the same size range as for those in groups 1, 2, 3, and 4 (table 1).

Since the sizes of cells measured in groups 1, 2, 3, and 4 (table 1) do not differ significantly, size data on the 400 cells have been used for calculation of frequency distribution as to size of the cells of the organism. For the 400 cells in question, the arithmetic means of diameters is 1.6 μ and the standard deviation 0.03.

Average sizes (based on greatest diameter) for living and stained cells measured with the light microscope, and cells in electron micrographs (mounts dried for 2 weeks in desiccator over "drierite") are, respectively, 2.7 μ, 1.69 μ, and 1.15 μ. It is assumed that the measurements on living bacteria are accurate to within the error of 0.36 μ inherent in estimating the distance between divisions of the ocular micrometer. This assumption is based on the fact that the bacterial cell wall, though invisible with the optics used, is known to be very thin.

Cultural characteristics (at optimum temperature) and temperature relations. The organism produces a moderately heavy, glistening, filiform, creamy-white growth on nutrient agar, and the same type but heavier growth on veal infusion and yeast extract agars. Growth has not been obtained under anaerobic conditions. In cultures 10 days' old a very faint pink color is noticeable on veal infusion agar while on yeast extract agar old cultures appear brownish-yellow. A ring is formed in yeast extract or nutrient broth in 24 hours. Gelatin is not liquefied, indole and H_2S are not formed, and nitrates are not reduced. The organism produces no hemolysis on blood agar. It is not capable of growth in sodium citrate or in $NH_4H_2PO_4$ as sole sources of carbon and nitrogen, respectively. Ulrich milk becomes alkaline in 2 days with slight reduction of the methylene blue indicator in 4 days. There is no fermentation of xylose, glucose, lactose, sucrose, maltose, cellibiose, mannitol, dulcitol, or salicin. Urease and catalase are formed but no oxidase.

Growth occurs at -4.0 C, is good at 9.8 C, maximum between 23 and 24 C and nil above 25 C. Growth is best at pH 6.8 to 7.2 but occurs in the range 5.5 to 9.5.

SUMMARY

The organism is apparently a new species and definitely belongs in the genus *Micrococcus*, although it forms no acid in glucose broth. This conclusion has been

reached as the result of a careful search of the appendix attached to the section on the Micrococcaceae in Bergey's Manual (Breed, Murray, and Hitchens, 1948), with special attention to descriptions of unusually large cocci and cocci listed under meat and milk in the habitat index. *Micrococcus cryophilus* (frost-loving) is suggested as a name for the organism since it grows only in a low temperature range. The minimal and maximal growth temperatures are, in our experience, as mentioned in the discussion of temperature relations, −4.0 C and 25 C, respectively.

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