OCCURRENCE OF MUCOID VARIANTS OF LACTOBACILLUS BIFIDUS. DEMONSTRATION OF EXTRACELLULAR AND INTRACELLULAR POLYSACCHARIDE[1]

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A mucoid variant of *Lactobacillus bifidus* was first described by Malyoth and Bauer (1950, 1951). Colonies of this variant incubated anaerobically on solid media were said to be hygroscopic and to become watery on exposure to atmospheric conditions at room temperature. In the first paper, attempts to demonstrate capsules by staining and by suspension in India ink were reported as unsuccessful. If the organisms were stained following suspension in freshly ground Chinese ink, however, capsules were seen. In the second paper, it was reported that capsules may be demonstrated also by means of a phase-contrast microscope when suspensions of organisms are exposed to specific immune serum.

In our laboratory the occurrence of mucoid variants or mutants of *L. bifidus* often has been observed. Occasionally a rare mucoid colony may be found in growth from the original inoculum. When strains of *L. bifidus* isolated from the stools of breast-fed infants are carried in the chemically defined medium of Norris *et al.* (1950) by periodic transfers, mucoid colonies may appear among the smooth parent colonies. Growth in broth is likewise more viscid than that of the parent type. So far as is now known, the cultural requirements of the organisms appear to be the same as those of the parent type, and differences in agglutination reactions between them have not been detected by Williams *et al.* (1953). Although the mucoid organisms appear to be a variant of the smooth parent type, they have not been observed to revert to the parent type. Like the parent type, however, a change to aerobic straight rods, so-called *Lactobacillus parabifidus*, frequently occurs. At the same time the mucoid character of the colonies is lost.

In view of these observations it is considered important to confirm the occurrence of such variants and to describe in more detail the characteristics of the organisms.

MATERIALS AND METHODS

Strain "Jackson" was selected for the present study. This organism was isolated originally in April, 1949, from the stool of a breast-fed infant, aged 2½ months, and was characterized by a typical bifid morphology and a porcelain-like smooth colony. For about a year the organism was carried in stock culture, and then a single cell clone was isolated which gave rise to aerobic straight-rod variants as previously reported by Norris *et al.* (1950). These are designated as "Jackson SC-8" and "Jackson B-6", respectively. Subsequently, mucoid variants designated "Jackson Mucoid" also were isolated from cultures of Jackson SC-8. These 3 strains have been maintained as separate stock cultures by transfers every 48 hours in liquid medium described by Norris *et al.* (1950). This medium has been modified by reduction of lactose from 7 per cent to 3.5 per cent and by omission of pancreatin. Ascorbic acid is added aseptically. Unless otherwise indicated cultures were incubated at 37 C anaerobically with the addition of 10 per cent CO_2 to the atmosphere. Platings were made once a week to check on the purity of the cultures.

For viscosity studies 5 ml of broth in test tubes 15 by 1.5 cm were inoculated with the appropriate strain and incubated for 48 hours. Viscosity was measured in an Ostwald viscosimeter at 35 to 37 C. The supernatant was used for the measurements. Although the same viscosity tube was used for all tests, the tube was not cleaned between duplicate or triplicate readings on the same specimen. Between tests on different specimens, however, the tube was washed with tap

water and then with a dilute solution of alkaline detergent followed by repeated washings with distilled water. The tube was dried with acetone.

When stained for polysaccharide, the organisms were attached to glass slides coated with a thin layer of egg albumin. The Coleman-Schiff reagent was used in the Hotchkiss technique according to Glick (1949). The organisms were fixed in Carnoy's fluid for two minutes and were treated with the nonalcoholic solution of periodic acid for 5 minutes. The spreads were treated with iodide thiosulfite solution for 5 minutes and with the Schiff reagent for 20 minutes. Between steps, washing with water or a solution of 70 per cent ethanol gave equally good results. Empirically it was observed that Carnoy's fluid was superior for fixation either to heat or to 10 per cent formalin. Acetylation of hydroxyl groups and the removal of acetyl groups were carried out by the method of McManus and Cason (1950).

RESULTS

Plate cultures of Jackson Mucoid, when first removed from the anaerobic jar after incubation for 48 hours at 37 C, resemble those of the non-mucoid bifid strains but are distinctly more viscous (figure 1). Following exposure to atmospheric conditions at 37 C for 24 hours, individual colonies enlarge and coalesce and at the same time become less viscid (figure 2). If a duplicate plate culture is kept under anaerobic conditions with the addition of 10 per cent CO_2, there is no further growth during this 24 hour period nor is there any gross change in the degree of viscosity. Since growth will not occur on plates in the presence of atmospheric oxygen or in the absence of additional CO_2, the change described in the colonies does not appear to be due to continued multiplication of the organisms. The loss of viscosity and increased volume of the colonies appear to be accelerated by increasing temperatures up to the melting point of the agar in the presence of oxygen, but not in its absence. At lower temperatures, the reaction appears to be slowed. At room temperatures, however, changes in the character of the colonies are observed readily in an hour or so.
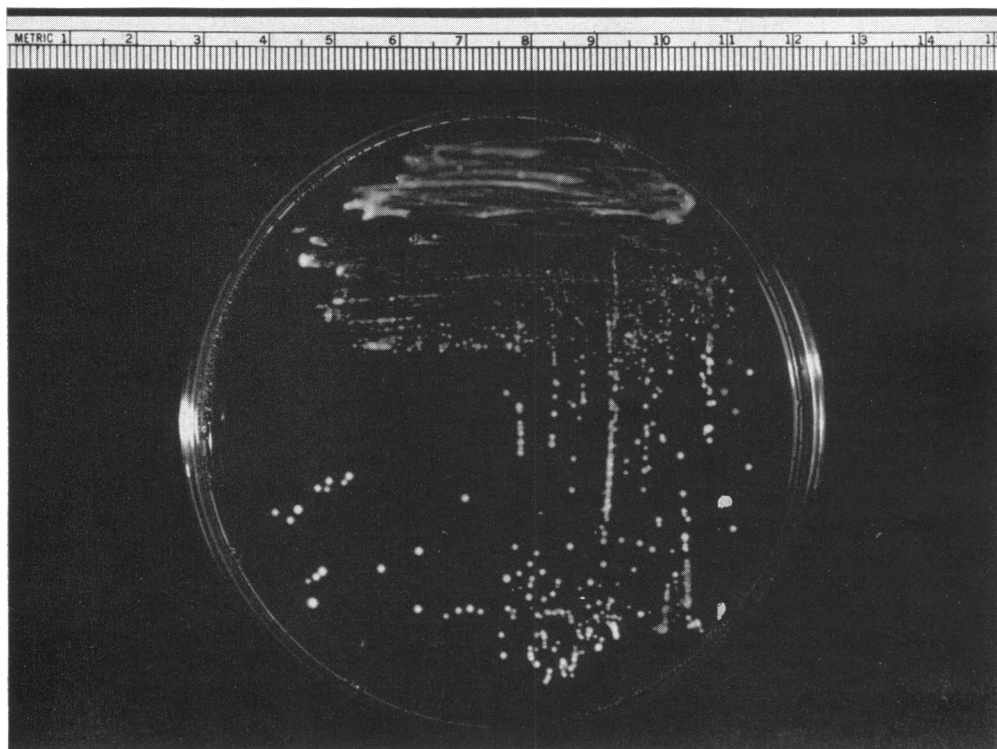
The viscosity of liquid cultures of mucoid

Figure 1. Lactobacillus bifidus, strain Jackson Mucoid. Plate culture following 48 hours' incubation at 37 C anaerobic plus 10 per cent CO_2. Note mucoid character of growth.

Figure 2. Same following 24 hours' exposure to air at 37 C. Note enlargement of individual colonies and their watery appearance.

strains is likewise greater than that of either ordinary bifid strains or of the unbranched mutants. Following exposure to air, the viscosity gradually diminishes until it approaches that of the nonmucoid strains and of uninoculated broth. In table 1 are shown the data of a typical experiment in which tube cultures of uninoculated broth, Jackson B-6, Jackson SC-8, and Jackson Mucoid first were incubated anaerobically with the addition of 10 per cent CO_2 to the atmosphere for 48 hours and then were incubated aerobically for the periods indicated. Meanwhile aliquot cultures of Jackson Mucoid, incubated under the original anaerobic conditi.ns, were used for comparison with those tubes which subsequently were incubated aerobically. The results indicate that the viscosity of the cultures of Jackson Mucoid when maintained anaerobically for 144 hours does not alter, whereas when exposed to atmospheric conditions the viscosity decreases to that of the controls within 96 hours. The viscosities of the nonmucoid bifid strain and the unbranched strain approximate that of the uninoculated broth.

When the Seitz filtrate of a previously dialyzed 48 hour broth culture was treated with two and one-half volumes of 95 per cent ethanol, a white stringy precipitate was isolated which gave a positive Molisch test and stained faintly with the Hotchkiss periodic acid technique. The ethanol precipitate did not cause reduction of copper ions, but when hydrolyzed with hydrochloric acid, the presence of reducing substances was detected. The precipitate was not soluble in fat solvents and did not give positive Biuret, Millon, Xanthoproteic, or Heller tests. The mucoid substance was not precipitated from aqueous solution by alkaloidal reagents or heavy metals.

The precipitate obtained by the preceding method did not give a viscid solution in water. In order to obtain a viscid preparation, it was necessary immediately upon removal from the anaerobic jar following incubation for 48 hours to bubble nitrogen through the liquid culture while precipitation was carried out with two volumes of 95 per cent ethanol at room temperature. The final concentration of the latter was brought to 60 per cent. The precipitate was

TABLE 1

Reduction in viscosity of liquid cultures of mucoid Lactobacillus bifidus on exposure to air at 37 C

TIME IN HOURS	ANAEROBIC MEDIUM CONTROL	AEROBIC MEDIUM CONTROL	ANAEROBIC UN-BRANCHED JACKSON B-6	AEROBIC UN-BRANCHED JACKSON B-6	ANAEROBIC NONMUCOID BIFID JACKSON SC-8	AEROBIC NONMUCOID BIFID JACKSON SC-8	ANAEROBIC BIFID JACKSON MUCOID	AEROBIC BIFID JACKSON MUCOID
0	1'29" 1'32" 1'30"		1'21" 1'20"		1'53" 1'41"		4'58" 4'26" 4'20"	
24		1'21" 1'23"		1'27" 1'25"		1'39" 1'35"	4'45" 4'39" 4'36"	3'30" 3'24"
48		1'19" 1'18"					4'48" 4'42"	2'7" 2'4"
96		1'23" 1'22"					4'56" 4'40" 4'30"	1'51" 1'48" 1'48"

Figures represent time in minutes and seconds for viscosimeter chamber to empty. Duplicate or triplicate readings are shown for each specimen. Media control and cultures were incubated anaerobically with the addition of 10 per cent CO_2 at 37 C for 48 hours before the initial readings. Aliquot tubes were incubated thereafter aerobically as indicated except for set of tubes of Jackson Mucoid which were maintained under conditions of anaerobiosis.

washed several times with ethanol and ether. When the papers of Malyoth and Vogel (1952) and Vogel (1952) were brought to our attention, a precipitate obtained by the above method was resuspended in 85 per cent formic acid, according to their procedure, for periods varying from 2 to 10 hours. The mixture of bacterial cells and precipitate was centrifuged then at 20,000 rpm for 30 minutes in a Spinco ultracentrifuge. The supernatant which was now cell-free was precipitated several times with 95 per cent ethanol and was hydrolyzed with 2 N H_2SO_4 at 100 C for 4 hours. The acid ions were removed with BaOH. Paper chromatography was carried out with a solvent of one part pyridine, two parts ethyl acetate, and two parts water. Galactose, glucose, and fucose were identified. There were also uronic acids and one unidentified pentose which was not arabinose, rhamnose, ribose, or xylose. These findings are in keeping with those of Vogel (1952), but the precipitate before hydrolysis again did not give a viscid aqueous solution.

Accordingly, in place of the formic acid procedure the original ethanol precipitate and bacterial cells were suspended in a 50 per cent aqueous solution of NH_4OH at 60 C for 3 hours, while bubbling of nitrogen through the solution was continued. Following centrifugation and reprecipitation with ethanol, the precipitate was dialyzed against running tap water for 48 hours. The residue when evaporated to dryness was now gelatinous and produced a colorless viscous aqueous solution in a concentration of as little as 0.4 per cent. By means of an ultracentrifuge, the polysaccharide was shown to be homogeneous. At a concentration of 1.0 per cent by weight in 0.15 M NaCl + 0.02 M sodium phosphate at pH 7.40, the materials exhibited a single, very sharp peak with a sedimentation constant of 0.851 S.[2] Since the shape of the molecule was unknown, the molecular weight was not determined. When the precipitate or residue was hydrolyzed as in the preceding paragraph, the same sugars and the unidentified pentose were present, but now there was no uronic acid. The ash-free precipitate gave as little as 0.3 per cent nitrogen and in aqueous solution gave an optical rotation of I α I$_D^{25}$ +90°.

Attempts on our part to demonstrate the

[2] Kindly measured for us by Dr. S. Sorof, Lankenau Hospital Research Institute, Philadelphia.

Figure 3. Lactobacillus bifidus, strain Jackson SC-8. Hotchkiss periodic acid-Feulgen stain, × 1,500. Note large fuchsinophilic granules and globules within cells.

presence of capsules have been unsuccessful. Methods which failed included suspension in India ink and freshly ground China ink, staining by the Hiss method and the more recent procedure of Gray (1943), and exposure of the organisms to immune rabbit serum while under observation in a Bausch and Lomb bright field dark phase contrast microscope according to the method of Malyoth and Bauer (1951). In the last instance the serum employed was antiserum for Jackson Mucoid and was that used in the study of agglutination reactions reported by Williams *et al.* (1953). Although the organisms were clumped rapidly upon exposure to the serum, there was no evidence of separation by interposed capsular substance.

For staining by the Hotchkiss periodic acid-Feulgen procedure, spreads of Jackson SC-8, the parent type nonmucoid *L. bifidus*, and Jackson B-6, the unbranched mutant, so-called *L. parabifidus*, were made on the same slide with Jackson Mucoid and thus were stained under identical conditions. None of these strains was stained by the Schiff reagent before treatment with periodic acid, but the cell walls of all three

were stained lightly with the Schiff reagent following periodic acid. As may be seen in the case of Jackson SC-8 (figure 3), large globules of more intensely stained substance are distributed unevenly in the organisms. Whether the substance is entirely within the cytoplasm or is deposited in the cell wall is uncertain. This appearance, however, suggests the former interpretation. In the case of Jackson Mucoid (figure 4), a similar substance is demonstrable but is distributed more often as cross striae or bands than as globules. No such material is stained in the case of the unbranched mutant Jackson B-6 (figure 5). When the organisms were acetylated before treatment with the periodic acid-Schiff reagents, staining did not occur, but when the acetyl group was removed with potassium hydroxide followed by treatment with the periodic acid-Schiff reagents, the organisms were stained in the previous manner. Contrary to the report of Stout and Koffler (1951), the presence of egg albumin on the slide was not observed to interfere with acetylation. The evidence is believed, therefore, to indicate that the stainable material is polysaccharide in character.

Figure 4. Lactobacillus bifidus, strain Jackson Mucoid. Hotchkiss periodic acid-Feulgen stain, × 1,500. Note bands of fuchsinophilic substance within cells.

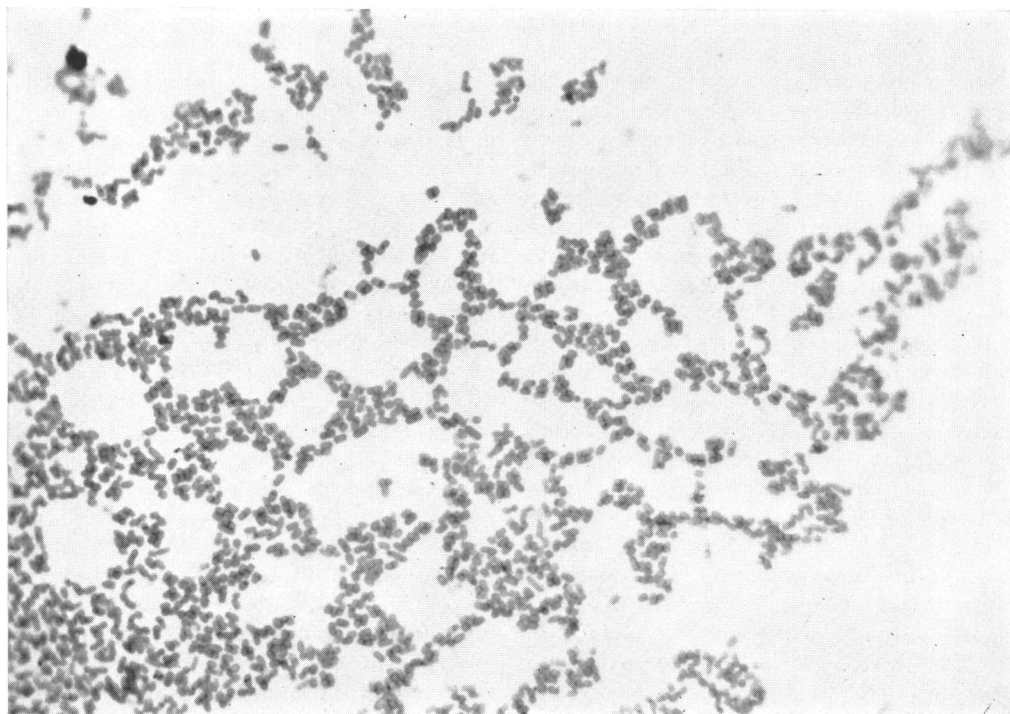*Figure 5. Lactobacillus parabifidus*, strain Jackson B-6. Hotchkiss periodic acid-Feulgen stain, × 1,500. Note absence of fuchsinophilic granules within cells. In each strain, the cell walls are stained lightly.

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DISCUSSION

It appears that L. bifidus may occur in a mucoid phase and that the extracellular mucoid substance is a polysaccharide which on hydrolysis yields monosaccharides including glucose. The reports of Malyoth and Bauer (1950, 1951), Malyoth and Vogel (1952), and Vogel (1952) thus are confirmed. In addition, our findings indicate that mucoid strains are derived from nonmucoid strains and thus are to be considered as mutants or variants of the latter. Furthermore mucoid strains themselves may change by mutation or variation to aerobic nonmucoid unbranched rods which appear to be identical in all respects, so far investigated, with similar mutants, so-called L. parabifidus, derived from nonmucoid strains of L. bifidus.

Upon hydrolysis of the extracellular polysaccharide, in addition to glucose we have identified galactose, fucose, and a pentose which is not xylose that Vogel (1952) reports to be present. When the precipitated polysaccharide is treated with formic acid, uronic acid also is present after hydrolysis as previously reported by Malyoth and Vogel (1952). The precipitate resuspended in solution, however, is no longer viscous. For this reason it is possible that uronic acid may be an artifact produced by treatment with acid. This belief is supported by the fact that alkali treated precipitate is viscous and that following hydrolysis uronic acid is not present. The loss of viscosity on exposure of the polysaccharide to air may be explained by depolymerization. A more detailed study of the structure of the polysaccharide is planned for the future.

Although the presence of polysaccharide by staining with the Hotchkiss periodic acid-Schiff method has been demonstrated within the cell bodies of both mucoid and nonmucoid strains of L. bifidus, extracellular polysaccharide has not been found in the case of nonmucoid strains nor in the case of the unbranched mutants, L. parabifidus. The lack of intracellular polysaccharide in the case of L. parabifidus constitutes another distinguishing feature between this organism and L. bifidus. Whether the polysaccharide within the cells is identical with the extracellular polysaccharide of the mucoid strain has not been determined. That no significant serologic difference between the nonmucoid and mucoid strains of Jackson by agglutination reactions was demonstrated by Williams et al. (1953) may indicate either that the polysaccharide within the cell and that without the cell are immunologically identical or that as Malyoth and Vogel (1952) and Vogel (1952) have found the polysaccharide is not by itself antigenic. In this connection, the latter authors demonstrated precipitins for the polysaccharide in rabbits immunized with suspensions of whole organisms.

The failure on our part to demonstrate the presence of capsules for mucoid strains of L. bifidus and thus to confirm the reports of Malyoth and Bauer (1950, 1951) may be due to the character of the strains examined since these workers likewise were unable to detect capsules in all strains.

SUMMARY

The occurrence of mucoid variants or mutants of Lactobacillus bifidus has been described. Although these strains have not been observed to revert to a nonmucoid type, mutation to the aerobic unbranched Lactobacillus parabifidus frequently occurs and, in this case also, reversion to L. bifidus has not been demonstrated.

Evidence is presented which indicates that the extracellular mucoid substance is a highly polymerized polysaccharide which undergoes depolymerization with loss of relative viscosity when exposed to atmospheric conditions. The polysaccharide is not ordinarily demonstrable as a true capsule.

Within the cell, globules or masses of polysaccharide also can be stained in both mucoid and nonmucoid strains of L. bifidus but not in L. parabifidus.

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