JOURNAL OF BACTERIOLOGY, Sept. 1972, p 841-844

Vol. 111, No. 3
Printed in U.S.A.

Influence of Nutrition on the Morphology of a Strain of *Bifidobacterium bifidum*

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Received for publication 1 May 1972

A mucoid variant of *Bifidobacterium bifidum* was converted from its normal curved rod or bifid form to a highly branched form when grown in a chemically defined minimal medium. Branching could be prevented by the addition of a mixture of DL-alanine, DL-aspartic acid, L(+)-glutamic acid, and DL-serine, but not when any one of these four amino acids was omitted. Although sodium chloride induced pleomorphism, calcium ions were ineffective in suppressing the appearance of these pleomorphic forms. None of the cell wall precursors tested, viz., *N*-acetyl-D-glucosamine, α-ε-diaminopimelic acid, and muramic acid, inhibited branching.

The variable structure of *Bifidobacterium bifidum* has interested investigators since the organism was described by Tissier in 1899 (11). *B. bifidum* will be used throughout this report although in some of the literature cited it is referred to as *Lactobacillus bifidus*. The occurrence of straight rods, forked or bifid rods, and branched or globular swelling of rods in a single strain remained unexplained biochemically for many years, adding to the confusion in studying this organism. Sundman and Björksten (10) and Glick et al. (2) suggested that defects of the cell wall were responsible for branching and swelling of bacillary forms and were due either to absence of essential precursor substances in the medium or to interference with the synthesis of cell wall compounds.

Working with a mucoid variant of *B. bifidum*, we observed morphological differences when it was cultured in two different media. One of these was a complex medium which was only partially defined chemically. The second was a less complex but chemically defined medium in which the organisms were greatly branched as contrasted with the curved rod and bifid forms characteristically found in the more complex medium. In the present report, conversion of the branched organisms to curved or bifid rods by the addition of certain amino acids to the chemically defined medium will be reported. Effects of certain salts and cell wall precursors on the structure of the organism were also studied and are reported.

The organism used in this study was a mucoid variant of strain Jackson SC-8 of *B. bifidum*, which was isolated from the feces of a breast-fed infant (7, 8). It was maintained as a stock culture by transfers every 48 hr in a liquid medium described by Norris et al. (8). Its characteristic property of rendering the growth medium highly viscous remained unchanged over the years as did its ability to ferment certain carbohydrates and to form lactic and acetic acids from glucose (estimated for us at the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, through the courtesy of W. E. C. Moore and Elizabeth P. Cato). The media used were: regular medium (RM) which was a semisynthetic medium developed by Norris et al. (8) for the cultivation of *B. bifidum*; minimal medium (MM) which was the same as proposed by Hassinen et al. (3) and shown by them to permit growth of certain strains of *B. bifidum*; modified minimal medium (MMM) was MM to which was added a mixture of the following 17 crystalline amino acids: DL-alanine, L(+)-arginine-hydrochloride, L-asparagine, DL-aspartic acid, glycine, L(+)-glutamic acid, DL-isoleucine, L(+)-leucine, DL-lysine, DL-methionine, L(−)-proline, DL-phenylalanine, DL-serine, DL-threonine, DL-tryptophan, L(−)-tyrosine, and DL-valine. Each amino acid was added in the amount of 667 mg/liter. The various media were sterilized by autoclaving at 121 C for 10 min. Cultures were incubated at 37 C in an

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anaerobic atmosphere produced by a Gas Pak System (Baltimore Biological Laboratory) which included carbon dioxide.

Aqueous solutions of substances to be tested for their effect on structure were sterilized by passage through a 0.22-μm membrane filter (Millipore Corp.) and were added aseptically to each tube to give the desired final concentration. Growth promoting effect of the various constituents of the medium was found either by withdrawing one constituent at a time from the otherwise complete medium (RM or MMM) or by the addition of the constituents to the minimal medium (MM) and the measuring of growth indirectly by titrimetric assay of the acid produced.

Microscopic appearance of the cells grown in the two media, RM and MM, showed differences in structure. Cells from RM were slightly curved. A few had bifid ends (Fig. 1A). Cells from MM were pleomorphic and so profusely branched that they ceased to bear any resemblance to rods (Fig. 1B). Addition of the four amino acids to the medium resulted in a reversal of the branched state to the curved state or bifid form (Fig. 1C). Organisms transferred from RM to MM start to branch 3 hr after inoculation and retain the branched structure on repeated subculture in MM. Growth in MM as measured by acid production was less than in RM (Table 1). The organism has been maintained in a branched state in the minimal medium for over 6 months.

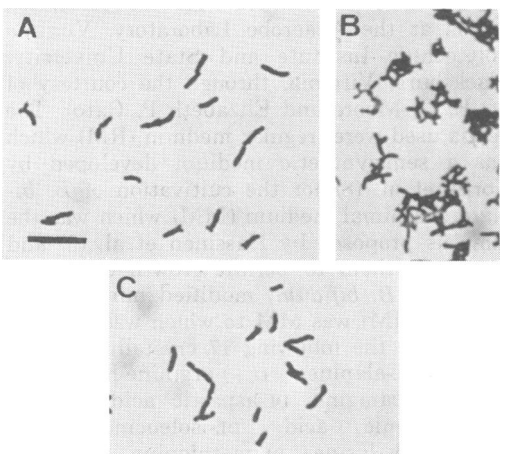

FIG. 1. *Gram-stained preparations of Bifidobacterium bifidum, 44-hr culture. A, Bifid and curved rod form grown in RM; B, branched form grown in MM; C, reversal of the branched to an unbranched form by the addition of certain amino acids. Bar is equal to 10 μm.*

This observation does not support the contention of those who consider branching to be a degenerative phase preceding death of the organism (9).

Constituents of the RM, in which MM was lacking, were added individually to the MM in the same concentration as they occur in RM, and their effect on cellular structure and growth was observed. Addition of N-Z-Case (pancreatic digest of casein, Sheffield Chemical, a Division of National Dairy Products Corp., Norwich, N.Y.) or of a mixture of 17 amino acids to MM restored growth to the degree observed in RM and also suppressed branching (Table 1). The addition of DL-alanine, L-asparagine, DL-tryptophan, and Tween 80 stimulated growth, but the branched structure of the cells remained unchanged. The other supplements neither improved growth nor altered the appearance of the cells. Ammonium acetate and cysteine-hydrochloride were the sole sources of nitrogen in MM, and both were needed by the organism to grow in this medium (Table 1). Their addition to RM, in which they are not present, did not affect the structure of the cells.

These results suggested a possible role of some of the amino acids present in the MMM in maintaining the organism in the unbranched phase. This was tested by withdrawing one amino acid at a time from the otherwise complete MMM and by observing subsequent growth and cellular structure of the organism.

Omission of DL-alanine, DL-aspartic acid, L(+)-glutamic acid or DL-serine from the MMM resulted in less growth than in the complete MMM, but the structure of the cells remained unchanged. The deletion of other amino acids neither affected growth nor produced any visible changes in the structure of the organism. Addition of all four of these amino acids to the MM increased acid production only slightly and did not restore the degree of growth observed in RM or MMM (Table 1). The highly branched structure of the organism characteristically seen in MM, however, was changed to the unbranched form in the MM containing all four amino acids (Fig. 1C), but this change did not occur when any one of the four was not added to the medium. Thus, the presence of all four amino acids appeared to be essential to keep the organism in the unbranched phase. Addition of any of the remaining amino acids present in the MMM to the MM, alone or in combination, stimulated growth but did not affect the structure of the cells which continued to remain

TABLE 1. *Growth and morphological appearance of B. bifidum in different media*[a]

Media	Acid (0.1 N) produced per 5.5 ml of medium at 44 hr (ml)	Morphological appearance
Regular medium (RM)	15.0	Rods, curved, and few bifids
Minimal medium (MM)	6.2	Highly branched and pleomorphic
MM + N-Z-Case[b]	14.9	Same as in RM
MM + 17 amino acids (MMM)	14.8	Same as in RM
MM + alanine	7.8	Branched and pleomorphic
MM + asparagine	8.0	Branched and pleomorphic
MM + Tryptophan	7.7	Branched and pleomorphic
MM + Tween 80	8.0	Branched and pleomorphic
MM − ammonium acetate	1.0	No growth
MM − cysteine HCl	0.7	No growth
MMM − alanine	11.1	Same as in RM
MMM − aspartic acid	11.1	Same as in RM
MMM − glutamic acid	10.9	Same as in RM
MMM − serine	10.6	Same as in RM
MM + Ala + Asp + Glu + Ser	8.8	Same as in RM

[a] Substances which on addition or omission affected acid production by less than 1.0 ml, or did not change the appearance of the cells, are not included in the table.

[b] +, Added; −, omitted.

branched and pleomorphic. It seems obvious that, under the conditions of the experiment, DL-alanine, DL-aspartic acid, L(+)-glutamic acid, and DL-serine are essential for maintaining the cells in the curved rod form. This observation may be significant since we have not found reports that amino acids per se have an important role in cell wall synthesis and in changing a pleomorphic phase to the straight rod form. The presence of each of these amino acids, however, has been reported in the cell walls of several strains of this organism (1, 12).

In order to investigate further a possible mechanism of branching, several cell wall precursors were tested for their effect on the structure of this organism. *N*-acetyl-D-glucosamine, α-ϵ-diaminopimelic acid, and muramic acid failed to reverse branching when added to MM. Addition of NaCl (0.35 M) to RM caused multiple branching of cells which appeared similar in character to those in MM. $CaCl_2$ (0.01 to 0.02 M) stimulated growth slightly but did not inhibit pleomorphism produced by NaCl in RM, nor did its addition to the MM change branched forms to straight rods. Induction of pleomorphism in *B. bifidum* by the addition of NaCl to the growth medium thus confirmed the observations of Kojima et al. (4). However, the reversal of this effect by the addition of Ca^{2+} ions, as reported by the above workers (5), did not occur in our experiments. Contrary to their reports, sodium acetate, which is present in our regular medium in 0.3 M concentration, does not appear to induce branching and pleomorphism. These unexplained discrepancies may be due to differences in individual strains or in the culture medium used. The suggestion of these authors that branching in *B. bifidum* is principally due to its inability to produce crosswalls when grown in a medium deficient in Ca^{2+} ions (6) seems to be an oversimplification of a rather complex phenomenon which is perhaps caused by a variety of factors. It is apparent that the organism can be maintained indefinitely in either a branched or in a bifid and curved form simply by manipulating the composition of the growth medium.

The authors wish to acknowledge the technical assistance of Mollie Gallagher.

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