JOURNAL OF BACTERIOLOGY, Feb., 1967, p. 574-576
Vol. 93, No. 2

Pathway of Glucose Fermentation in Relation to the Taxonomy of Bifidobacteria

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Received for publication 7 November 1966

ABSTRACT

Cell-free extracts of 17 strains of *Bifidobacterium bifidum* (*Lactobacillus bifidus*) were examined for the presence of aldolase, glucose-6-phosphate dehydrogenase, and fructose-6-phosphate phosphoketolase. All strains turned out to lack aldolase, an enzyme unique to glycolysis, and glucose-6-phosphate dehydrogenase, characteristic of the hexosemonophosphate pathway. In all strains, fructose-6-phosphate phosphoketolase could be demonstrated. It can be concluded that bifidobacteria ferment glucose via a pathway which is different from those found in members of the genus *Lactobacillus*. The results strengthen the previous suggestions that classification of the bifidobacteria in the genus *Lactobacillus* is not justified.

The taxonomy of the bifidobacteria, a group of gram-positive, anaerobic, asporogenous bacteria, caused great controversy for several years. For instance, on purely morphological grounds bifidobacteria have been described as belonging to the genus *Actinomyces* (5, 9), *Corynebacterium* (4), and *Butyribacterium* (9). Bifidobacteria are classified in the genus *Lactobacillus* in *Bergey's Manual of Determinative Bacteriology* on the basis of biochemical characteristics. Prévot (6) considers bifidobacteria as a subgenus of the genus *Actinobacterium* in the order *Actinomycetales*. Recently, Sebald et al. (8) and Werner and Seeliger (10) found, in addition to several morphological and biochemical differences, a difference in deoxyribonucleic acid (DNA) base composition between bifidobacteria and species of the genera *Lactobacillus*, *Corynebacterium*, and *Propionibacterium*. They concluded that bifidobacteria constitute a separate genus, named *Bifidobacterium*. In agreement with Dehnert (1), they distinguished one species, *Bifidobacterium bifidum*, in this genus. Chiefly on the basis of behavior towards about 20 carbohydrates, Dehnert (1) divided strains of *B. bifidum* into five groups, named biotypes. Reuter (7), however, found more than five biochemical types, and proposed to distinguish eight species in the genus *Bifidobacterium*.

The occurrence of a specific catabolic route in a group of bacteria may be another argument to differentiate them from other groups. Our recent investigations (W. de Vries, S. J. Gerbrandy, and A. H. Stouthamer, Biochim. Biophys. Acta, *in press*) have shown that *B. bifidum* type 5 (1) ferments glucose via a pathway which is different from those found in homo- and heterofermentative lactic acid bacteria. Because of the absence of aldolase and glucose-6-phosphate dehydrogenase from cell-free extracts of this strain of *B. bifidum*, the glycolytic system and hexosemonophosphate shunt, pathways common to lactic acid bacteria (C. J. A. van den Hamer, Thesis, State University, Utrecht, The Netherlands, 1960), are not operating. The key reaction in the fermentation of glucose by this strain appeared to be a phosphoketolase cleavage of fructose-6-phosphate into acetylphosphate and erythrose-4-phosphate. Pentosephosphates are then formed by the action of transaldolase and transketolase. Xylulose-5-phosphate phosphoketolase splits pentosephosphates into acetylphosphate and glyceraldehyde-3-phosphate. Through reactions which also occur in glycolysis, lactate is formed from glyceraldehyde-3-phosphate.

The purpose of the present investigation was to establish whether other biochemical types of *B. bifidum* also ferment glucose via the phosphoketolase route.

MATERIALS AND METHODS

Organisms, media, and identification of the strains. Strains of *Bifidobacterium* were kindly supplied by K. C. Winkler (Laboratory of Microbiology, State University, Utrecht), J. de Waart (Central Institute of Nutrition and Food Research, Zeist), A. R. Prévot (Institut Pasteur), H. Beerens (Institut Pasteur de

TABLE 1. *Specific activity of some enzymes in cell-free extracts of different strains of Bifidobacterium*

Name and no. under which the strain was obtained	Obtained from	Biochemical type according to Dehnert (2)	Specific activity		
			Aldolase	Glucose-6-phosphate dehydrogenase	Fructose-6-phosphate phosphoketolase
Lactobacillus bifidus 128	K. C. Winkler	5	—[a]	—	18.1 ± 1.4 (7)
B. bifidum	J. de Waart	5	—	—	6
B. bifidum 2921A	A. R. Prévot	5	—	—	10
B. bifidum 3028B	A. R. Prévot	5	—	—	4
B. bifidum 3275	A. R. Prévot	3	—	—	16
B. bifidum B5	H. Beerens	3	—	—	7
B. bifidum B9	H. Beerens	5	—	—	10
B. bifidum B144	H. Beerens	1 or 2[b]	—	—	7
B. bifidum B420	H. Beerens	1 or 2[b]	—	—	23
B. adolescentis var.a.E194a.Ia[c]	G. Reuter	5	—	—	4
B. parvulorum var.a.S50.IV	G. Reuter	3	—	—	14
B. lactentis 659.X	G. Reuter	4	—	—	9
B. bifidum var.a.E319f.IIIa	G. Reuter	1	—	—	12
B. breve var.a.S1.VII	G. Reuter	3	—	—	7
B. infantis S12.V	G. Reuter	3	—	—	10
B. breve var.b.S46.VIII	G. Reuter	3	—	—	6
B. liberorum 76c.IX	G. Reuter	4	—	—	8
L. casei	K. C. Winkler		20	4	—
L. fermenti	K. C. Winkler		—	35	ND

[a] Values lower than 0.05 are indicated as negative.
[b] The types 1 and 2 cannot be distinguished from each other on account of fermentation of sugars.
[c] Roman numerals indicate the type according to Reuter (7).

Lille), and G. Reuter (Institut für Lebensmittelhygiene der Freien Universität, Berlin). The strains were maintained as stab-cultures in tomato-agar (Oxoid) containing 2% glucose and 0.2% cysteine. They were subcultured weekly. Identification of the strains was performed by testing their behavior towards 15 carbohydrates as described by Dehnert (1). Carbohydrates (2%) were added to a liquid medium of the following composition (per liter of water): peptone (Oxoid), 10 g; beef extract (Oxoid), 10 g; yeast extract (Oxoid), 5 g; Tween 80, 1 ml; K_2HPO_4, 2 g; Na acetate · $3H_2O$, 5 g; diammonium citrate, 2 g; $MgSO_4$ · $7H_2O$, 0.2 g; and $MnSO_4$ · $4H_2O$, 0.05 g; *p*H of the medium was 6.8. A McIntosh anaerobic jar was used to obtain anaerobic conditions. The gas phase was N_2 plus CO_2 (95:5). Cultures were incubated at 37 C. For mass cultures, the above-mentioned liquid medium was used with 2% glucose added.

Preparation of cell-free extracts. A 300-ml amount of a well-grown culture (2% glucose) was centrifuged in an MSE 18 centrifuge (Measuring & Scientific Equipment, Ltd., London, England) at 23,000 × *g* for 20 min. Cells were washed three times with 0.85% KCl, and were resuspended in 20 ml of 0.85% KCl. Cell extracts were prepared by submitting cell suspensions to ultrasonic oscillation in an MSE ultrasonic disintegrator (60 W at 20 kc/sec) for 10 min.

The resulting homogenate was centrifuged at 12,000 × *g* for 15 min at 4 C. Cell-free extracts contained 1 to 6 mg of protein per ml, as determined by the method of Lowry et al. (5).

Enzyme assays. Spectrophotometric assays were performed at 25 C in quartz cuvettes (1-cm light path) with a Unicam Sp 820 constant-wavelength scanner. Colorimetric measurements were performed with a Unicam Sp 600 spectrophotometer.

Aldolase was determined by means of a Biochemica Test combination (C. F. Boehringer und Soehne GmbH, Mannheim, Germany), in which triosephosphates formed from fructose-1,6-diphosphate are trapped with phenylhydrazine and colored with 2,4-dinitrophenyl-hydrazine. The specific activity was expressed as micromoles of fructose-1,6-diphosphate converted per milligram of protein per hour.

Glucose-6-phosphate dehydrogenase was assayed by following the increase in absorbance at 340 mμ due to the reduction of either nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP). The assay mixture contained, per 3 ml: tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.8), 200 μmoles; $MgCl_2$, 10 μmoles; glucose-6-phosphate, 5 μmoles; NAD or NADP, 4 μmoles; cell-free extract, about 1 mg of protein. The specific activity was expressed as micromoles of reduced NAD ($NADH_2$) or reduced NADP ($NADPH_2$) formed per milligram of protein per hour.

The determination of fructose-6-phosphate phosphoketolase was based on the formation of acetylphosphate from fructose-6-phosphate. The reaction mixture contained, per 0.75 ml: histidine buffer (*p*H 6.0), 45 μmoles; potassium phosphate buffer (*p*H 6.0), 18 μmoles; thiamine pyrophosphate, 0.25 μmole; fructose-6-phosphate, 15 μmoles; cell-free

extract, 0.2 to 0.5 mg of protein. After incubation at 30 C for 30 min, the reaction was stopped by addition of 0.75 ml of 2 M hydroxylamine (*p*H 5.4). Fructose-6-phosphate was added to control flasks after addition of hydroxylamine. Acetylphosphate was measured according to Lipmann and Tuttle (2). A standard curve was prepared with acetylphosphate obtained from C. F. Boehringer und Soehne GmbH, Mannheim, Germany. The specific activity was expressed as micromoles of acetylphosphate formed per milligram of protein per hour.

Chemicals. NAD, NADP, glucose-6-phosphate, and fructose-6-phosphate were obtained from C. F. Boehringer und Soehne GmbH, Mannheim, Germany.

RESULTS AND DISCUSSION

Table 1 shows the specific activities of aldolase, glucose-6-phosphate dehydrogenase and fructose-6-phosphate phosphoketolase in cell-free extracts of various strains of *Bifidobacterium*. For comparison, cell-free extracts of *Lactobacillus casei*, a homofermentative lactic acid bacterium, and of *L. fermenti*, a heterofermentative lactic acid bacterium, were run in parallel.

For all strains of *Bifidobacterium* and for *L. fermenti* the specific activity of aldolase, an enzyme unique to glycolysis, turned out to be less than 0.05. In Table 1, values less than 0.05 are indicated as negative. The specific activity of aldolase in *L. casei* was 20. It can be concluded that the glycolytic system does not operate in any strain of *Bifidobacterium* tested.

Glucose-6-phosphate dehydrogenase, characteristic of the hexosemonophosphate pathway, was completely absent from all strains of *Bifidobacterium* tested. This rules out the operation of the hexosemonophosphate pathway. In *L. fermenti*, the specific activity of glucose-6-phosphate dehydrogenase was 35. In agreement with the data of van den Hamer (Thesis, State University, Utrecht, The Netherlands), small amounts of glucose-6-phosphate dehydrogenase could be detected in *L. casei*.

In all strains of *Bifidobacterium*, fructose-6-phosphate phosphoketolase could be demonstrated, with specific activities ranging from 4 to 23. In cell-free extracts of *L. casei*, fructose-6-phosphate phosphoketolase could not be detected.

It can be concluded that all strains of *Bifidobacterium* tested ferment glucose via the phosphoketolase route previously found to be operating in *B. bifidum* type 5 (*L. bifidus* 128). The glycolytic system or hexosemonophosphate shunt, pathways involved in the degradation of glucose by lactic acid bacteria, could not be detected in any strain. The presence of fructose-6-phosphate phosphoketolase, and the absence of aldolase and glucose-6-phosphate dehydrogenase, seem to be distinguishing characteristics of bifidobacteria. Therefore, our experiments strengthen the conclusions of Sebald et al. (8) and Werner and Seeliger (10) that bifidobacteria form a separate group and do not belong to the genus *Lactobacillus*. Because of the complete agreement in the fermentation route of glucose in all strains of *Bifidobacterium* tested, it seems to us more justified to distinguish one species, *Bifidobacterium bifidum*, in the genus *Bifidobacterium* (8, 10) than to divide the genus *Bifidobacterium* into eight species as Reuter (7) does.

ACKNOWLEDGMENTS

The technical assistance of M. G. G. Schotanus is gratefully acknowledged. Thanks are also due to K. C. Winkler, J. de Waart, A. R. Prévot, H. Beerens, and G. Reuter for supplying strains.

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