The Cultivation of D-Galactosamine-metabolizing Lactic Acid Bacteria

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SUMMARY

Media have been devised containing tracheal hydrolyzates or D-galactose as an economical means for the cultivation of large quantities of galactosamine-metabolizing bacteria. With these media *Lactobacillus bulgaricus* and *L. casei* showed good growth; dried preparations were able to utilize galactosamine to produce ammonia and lactic acid.

INTRODUCTION

The wide distribution and importance of glucosamine and galactosamine have been amply reviewed (Kent, 1957; Roseman, 1959), and despite the considerable work done to elucidate the metabolism of glucosamine, little has been reported about the metabolism of galactosamine (Roseman, 1959). This communication describes cultural studies resulting in the production of large numbers of galactosamine-metabolizing bacteria. These included the study of bacteria grown in a medium containing bovine tracheal hydrolyzates as a source of D-galactosamine. Ammonia production and lactic acid formation by dried preparations of lactic acid bacteria, as well as the ability to support growth, were used as criteria of galactosamine utilization.

METHODS

Cultures and media. The following organisms were tested for their ability to grow on D-glucosamine or D-galactosamine: *Streptococcus faecalis* R. ATCC 8043, *S. liquefaciens*, *S. salivarius* ATCC 9756, *Lactobacillus bulgaricus* ATCC 521, *L. buchneri*, *L. casei* ATCC 7469, *L. plantarum* ATCC 8014, *Leuconostoc mesenteroides* P-60 (*Pediococcus*; Garvie, 1959), *Pediococcus cerevisiae*. These cultures were maintained in stabs of liver powder + yeast extract + glucose agar.

The inocula were prepared by seeding Micro Inoculum Broth (Difco) from stab cultures. The cultures after growth for *c.* 18 hr. were centrifuged and washed twice with sterile water. In some cases the inocula were carried over several transfers in medium B (see below), containing glucose, glucosamine or galactosamine at 5 mg./ml. medium, before testing.

For growth studies the inocula were diluted with sterile water to an optical density of 0.4 at 660 m μ in a Beckman Model B spectrophotometer with a light path of 18 mm. One drop of this suspension was then used to inoculate tubes containing 2–6 ml. of the test medium as described in the text.

The medium B was prepared as follows: Casamino acids (Difco), 5 g.; DL-tryptophan, 0.03 g.; L-cysteine.HCl, 0.2 g.; K_2HPO_4 and KH_2PO_4 , 0.5 g. each; sodium acetate. $3H_2O$, 10 g.; NH_4Cl , 3 g.; $MgSO_4 \cdot 4H_2O$, 0.01 g.; $FeCl_3$, 0.03 g.; yeast extract, 3 g.; adenine sulphate, guanine HCl, and uracil, 0.01 g. each; distilled water, 1000 ml.; adjusted to pH 6.8.

Freshly prepared neutralized hexosamine solutions were sterilized by filtration through UF sintered glass filters and added aseptically to previously autoclaved medium.

Preparation of bovine trachea hydrolyzates. Calf trachea, obtained frozen from a slaughterhouse, was cleaned by removing superficial tissues and fat and then passed twice through a meat grinder. The ground trachea was stored at -12° until used. Eighty g. of ground trachea were suspended in 100 ml. of 2 or 4N-hydrochloric acid or 2 or 4N-sulphuric acid and heated in free-flowing steam (100°) for either 7 or 17 hr. The conditions of hydrolysis were arbitrarily chosen. The hydrolyzates were cooled to 5° and solid fat filtered off. The hydrochloric acid-digested material was adjusted to pH 6.8 with sodium hydroxide. The sulphuric acid-digested material was treated with 95% of the amount of barium hydroxide calculated to neutralize all the acid present. The precipitated barium sulphate was removed by filtration and the filtrate adjusted to pH 6.8 with sodium hydroxide.

Chemical determinations. Ammonia was determined by the microdiffusion technique of Conway & Byrne (1933) followed by nesslerization. Lactic acid was measured by the procedure of Barker & Summerson (1941).

D-Galactosamine.HCl and D-glucosamine.HCl were obtained from Mann Research Laboratories (136 Liberty Street, New York, New York, U.S.A.). Each yielded one reducing spot before and after ninhydrin treatment according to the method of Stoffyn & Jeanloz (1954).

Preparation of dried organisms. Dried *Lactobacillus bulgaricus* or *L. casei* were prepared from cultures grown in medium B which had been supplemented with glucose, glucosamine, galactose or galactosamine to $30 \mu\text{mole/ml.}$ medium. The flasks were inoculated as follows: for glucose or galactose-containing medium, organisms grown in inoculum broth were used; for flasks containing the amino sugars, organisms grown for several transfers in glucosamine or galactosamine were used. After 28 hr. of growth, the organisms were harvested by centrifugation, washed twice and dried by lyophilization. Lyophilized *L. bulgaricus* and *L. casei* were also prepared from cultures grown on trachea hydrolyzed in 4N- H_2SO_4 for 7 hr. and prepared with glucose supplements as described in the text.

RESULTS

The growth of the organisms

All of the organisms tested with glucose as substrate reached maximum growth at 24 hr.; however, the organisms which grew on the hexosamines required almost 35 hr. to achieve maximum growth. Except for *Lactobacillus buchneri* which showed no growth, all of the organisms grew well on glucosamine. With galactosamine only *L. mesenteroides*, *L. bulgaricus* and *L. casei* achieved more than minimal growth. *L. casei* and *L. bulgaricus* grew well on both amino sugars; these two organisms were studied further.

The growth-supporting activities of glucose, glucosamine or galactosamine were then investigated. Growth on each of these substrates was followed turbidimetrically during 86 hr. To decrease the lag-time for *Lactobacillus casei*, it was grown in a galactosamine-containing medium for several transfers before the test. For both lactobacilli, growth with glucose or glucosamine was superior to growth with galactosamine, at least up to 80 μ mole substrate/ml.

Since results such as these raised the question of whether growth on galactosamine was due to mutation or adaptation, a series of plating experiments were designed in which organisms from one glucose supplemented tube were plated on each of three groups of ten agar plates supplemented with 30 μ M/ml. of glucose, galactose or galactosamine. In the case of *Lactobacillus casei*, analysis of the data by Fisher's 't' test showed no significant differences between any of the substrates, indicating that the processes were adaptive. For *L. bulgaricus*, however, while glucose and galactose showed no significant differences by the 't' test, on plates containing galactosamine the mean counts were approximately one-half those found in the other two cases.

Some authors (Levene, 1916; Meyer & Rapport 1951) have reported that cartilage is rich in galactosamine. This suggested the use of tracheal hydrolyzates as an economical source for culture media containing this amino sugar. Preparations of hydrochloric acid- or sulphuric acid-hydrolyzed bovine trachea were studied to determine their efficacy as a galactosamine source for the growth of large quantities of organisms. Different conditions of hydrolysis were studied; the results obtained with sulphuric acid-hydrolyzed trachea with *Lactobacillus casei* are shown in Table 1. Hydrolyzates

Table 1. *Growth of Lactobacillus casei in media containing bovine trachea hydrolyzate*

The sulphuric acid-hydrolyzates contained 1 % (w/v) yeast extract in a final volume of 6 ml. After 64 hr. incubation, the organisms were centrifuged, washed once, resuspended in water to 6 ml. and the optical density measured. The hydrolyzates contained solids ranging from 0.082 to 0.088 g./ml.

Hydrolyzate (ml.)	Strength of sulphuric acid (N) and period of hydrolysis (hr.)			
	2N; 7	4N; 7	2N; 17	4N; 17
	Optical density			
1	0.39	0.37	0.35	0.33
2	0.64	0.59	0.55	0.50
3	0.77	0.76	0.72	0.62
4	0.94	0.86	0.80	0.73
5	1.00	1.02	0.85	0.83

were supplemented with 1 % (w/v) yeast extract. It is evident that with 2N- and 4N-sulphuric acid, the 7-hr. hydrolyzates were better than the corresponding 17-hr. hydrolyzates. For *L. bulgaricus* the growth response on the four hydrolyzate media was equivalent to that obtained for *L. casei* grown in the medium containing the 17-hr. hydrolyzates. The hydrolyzates from hydrochloric acid-hydrolyzed trachea were considerably inferior to those treated with sulphuric acid. When the different hydrolyzates containing 1 % (w/v) yeast extract were supplemented with various substances, they showed no significant increase in growth over unsupplemented media. The following substances singly, or in mixtures, were used: adenine,

guanine, uracil, xanthine, various B vitamins, sodium acetate, ammonium chloride, potassium phosphate, magnesium chloride, ferrous sulphate, tryptophan, cysteine, Casitone, Casamino acids. Although glucose supplementation of the medium increased the growth of both lactobacilli significantly, organisms grown in this manner lost much of their ability to produce ammonia and lactate in the presence of glucosamine or galactosamine.

Experiment with dried organisms

The activities of dried *Lactobacillus casei* and *L. bulgaricus* previously grown on glucose, galactose, glucosamine or galactosamine were tested by determination of their ability to produce ammonia and lactic acid from glucosamine or galactosamine. Ammonia production was taken as an indication of deamination of these amino sugars and lactate production as an indication of the metabolism of their carbon skeletons. Table 2 shows the results of such a study. It can be seen that while

Table 2. *Ammonia and lactic acid formation from glucosamine and galactosamine by dried organisms grown in medium B*

The reaction mixture contained: 0.05 ml. M-potassium phosphate (pH 7.0); 2 mg. dried organisms; 5 μ mole glucosamine or galactosamine in total volume 1 ml. Tubes were incubated at 37° for 90 min. The reaction was stopped by adding 1 ml. 5% (w/v) trichloroacetic acid.

Organism	Amino sugar substrate			
	Glucosamine		Galactosamine	
	Product (μ mole/ml.)			
	Ammonia	Lactic acid	Ammonia	Lactic acid
<i>L. bulgaricus</i> , glucose grown	2.58	3.04	0.10	0.00
<i>L. bulgaricus</i> , galactose grown	2.00	3.28	2.00	3.82
<i>L. bulgarius</i> , glucosamine grown	4.10	8.10	0.20	0.00
<i>L. bulgaricus</i> , galactosamine grown	4.10	7.30	5.49	10.60
<i>L. casei</i> , glucose grown	2.50	2.82	0.25	0.00
<i>L. casei</i> , galactose grown	2.15	3.36	3.85	7.70
<i>L. casei</i> , glucosamine grown	4.98	9.08	0.55	0.00
<i>L. casei</i> , galactosamine grown	1.54	1.10	5.54	9.28

glucose- and glucosamine-grown *L. bulgaricus* and *L. casei* had little activity toward galactosamine, they were active toward glucosamine. Galactose and galactosamine-grown *L. bulgaricus* and *L. casei* showed activity for both substrates, although *L. casei* metabolized glucosamine much less actively. With dried organisms the addition of adenosinetriphosphate to any of these assay systems did not increase ammonia or lactic acid production. When dried *L. casei* or *L. bulgaricus* grown in trachea hydrolysates were tested with glucosamine or galactosamine results like those shown in Table 3 were obtained. Supplementation of the media with glucose markedly inhibited the ability of dried preparations to metabolize galactosamine.

DISCUSSION

Glucosamine metabolism has been extensively studied and several modes for its microbial breakdown have been reported (Roseman, 1959). On the other hand, galactosamine, aside from its phosphorylation (Cardini & Leloir, 1953; Davidson,

Table 3. *Ammonia and lactic acid formation from glucosamine or galactosamine by dried organisms grown in bovine trachea hydrolyzates supplemented with glucose at different concentrations*

Assay conditions as in Table 2.

Organism	Glucose (%, w/v)	Amino sugar substrate			
		Glucosamine		Galactosamine	
		Product (μ mole/ml.)			
		Ammonia	Lactic acid	Ammonia	Lactic acid
<i>L. bulgaricus</i>	0.0	3.88	6.72	2.67	4.26
<i>L. bulgaricus</i>	0.2	4.12	7.08	1.09	1.44
<i>L. bulgaricus</i>	1.0	4.12	7.88	0.40	0.00
<i>L. casei</i>	0.0	4.22	8.46	3.05	4.70
<i>L. casei</i>	0.2	2.11	3.90	0.35	0.00
<i>L. casei</i>	1.0	2.22	3.31	0.50	0.15

1960), has been studied mostly in terms of its uridine derivatives and their subsequent conversion to glucosamine (Glaser, 1959). In the present work we were concerned with the cultural conditions for obtaining a bacterial system that would metabolize D-galactosamine. The *Lactobacillus casei* and *L. bulgaricus* strains examined have such a system, which appears to be inducible. Although preparations of dried organisms made in various ways metabolized glucosamine, only galactose or galactosamine grown-organisms attacked galactosamine. Further investigations with cell-free systems are now in progress in order to ascertain the pathway of galactosamine metabolism.

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