Stimulation of Lactic Streptococci in Milk by β-Galactosidase

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Acid production in milk by lactic streptococci was stimulated by added β-galactosidase. Both glucose and galactose accumulated rapidly in the presence of this enzyme. Glucose accumulation ceased as the culture entered the most rapid period of acid production, whereas galactose accumulation continued. In cultures without added β-galactosidase, a low concentration of galactose accumulated in the milk, whereas glucose was not detected after 2 hr of incubation. Cultures grew and produced acid faster in broth containing glucose rather than galactose or lactose. These observations suggest that the lactic streptococci do not metabolize the lactose in milk efficiently enough to permit optimum acid production and that a phenomenon such as catabolite repression functions to allow for a preferential use of glucose over either galactose or lactose. In addition to providing the culture with a more readily available energy source, it is possible that the culture produced more acidic metabolites as a result of preferentially utilizing the glucose released by the action of the β-galactosidase.

The natural habitat of the lactic streptococci is generally accepted as being plants which do not contain lactose. Although milk is the medium mostly used for propagating the lactic streptococci for commercial use, there have been no reports which indicate that the lactose in milk supports optimum growth of these organisms. Also, no information is available evaluating the effect of altering the available energy source(s) on the acid production and growth of lactic streptococci in milk. However, Shahani and Vakil (9) observed little difference in growth rate and acid production when Streptococcus lactis was grown in other media containing glucose, galactose, or lactose.

This study was undertaken to determine whether the lactose-metabolizing system of the lactic streptococci imposes restrictions on acid production in milk cultures of the organism.

**MATERIALS AND METHODS**

**Source and maintenance of cultures.** S. lactis (strains AC1 and C4) and S. cremoris (strains AC2, AC6, AC11, and C10) obtained from the stock culture collection of the food microbiology laboratories and three commercial multiple-strain cheddar cheese starter cultures (A, B, and C) were used in this study. Stock cultures were maintained by propagation in sterile litmus milk with a 1% inoculum and incubation for 16 to 18 hr at 22 C. Cultures were stored at 3 C between subcultures. Three or more transfers were made before using a culture in an experiment.

**Preparation of β-galactosidase.** β-Galactosidase (EC 3.2.1.23, isolated from Escherichia coli K-12) was obtained from Worthington Biochemical Corp. (Freehold, N.J.). The enzyme was suspended in 0.1 M Na2HPO4-KH2PO4 buffer at pH 7.0 to give a concentration of 1 mg (50 IU) per ml of buffer. The enzyme solution was prepared daily and passed through a sterile membrane filter with an exclusion limit of 0.45 μm and placed in an ice-water bath. Inactivated β-galactosidase was prepared by heating a sample of the stock enzyme solution for 15 min at 121 C.

**Acid production in milk.** Measurements of the effect of added β-galactosidase on lactic streptococci in milk were based on acid production by the culture. Eleven per cent reconstituted nonfat milk solids (NFMS) were steamed for 30 min at 100 C. After tempering at 32 C, 2.2 ml of a fresh 16- to 18-hr milk culture was inoculated into 200 ml of the milk. Samples (18 ml) of inoculated milk were added to tubes (2.8 by 10.4 cm) containing 2 ml of stock β-galactosidase (1 mg/ml) or 2 ml of sterile 0.1 M Na2HPO4-KH2PO4 buffer. The samples were incubated at 32 C in a thermostatically controlled water bath, and the pH was continuously monitored by the method described by Gilliland and Speck (4).

**Growth in broth.** The effect of varying the sugar
source (glucose, galactose, and lactose) on the growth of lactic streptococci was measured in broth. The basal medium was prepared by dissolving 20 g of tryptone (Difco), 5 g of yeast extract (BBL), 2.5 g of gelatin (Difco), 4 g of sodium chloride, and 0.5 g of ascorbic acid in approximately 400 ml of distilled water, adjusting to pH 7.0 with 1 N NaOH, and adjusting the final volume to 500 ml with distilled water. It was sterilized by autoclaving for 15 min at 121 C. After tempering at 32 C, a 1% inoculum of a fresh broth culture (containing the appropriate sugar) was added. A 10-ml amount of the inoculated broth was added to tubes (2.8 by 10.4 cm) containing 10 ml of 2% sterile aqueous solutions of the desired sugar. The broth was incubated at 32 C, and the pH was continuously monitored or growth was followed turbidimetrically at 600 nm.

Plate counts. Colony counts were done by the procedures described by Peebles et al. (7).

Sugar analyses. Milk cultures were assayed for glucose and galactose at 1-hr intervals during growth at 32 C. The reconstituted NFMS was prepared in the same manner as described for measuring acid production. Samples for sugar analysis were adjusted to pH 4.6 with 5 N HCl followed by centrifugation at 20,000 x g for 10 min at 3 C. The supernatant fluid was removed, adjusted to pH 7.0 with 1 N NaOH, and centrifuged at 20,000 x g for 10 min. The whey was collected and stored at 3 C until analyzed for sugars.

Glucose was quantitatively measured with the glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). Galactose was quantitated spectrophotometrically by the method of Finch et al. (2) by using galactose dehydrogenase (Sigma Chemical Co., St. Louis, Mo.). The assay mixture consisted of the following: 0.2 ml of 2.5 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.6); 0.2 ml of 0.0025 M nicotinamide adenine dinucleotide; and 0.5 ml of sample containing 0 to 0.20 µmoles of D-galactose. The optical density (OD) at 340 nm was measured on a Beckman DU spectrophotometer against a reagent blank. A 0.1-ml amount of galactose dehydrogenase (0.25 unit) was added, and the reaction was allowed to go to completion at room temperature (25 C). The OD was again measured at 340 nm, and the change in OD was determined. Galactose concentration in the samples was then determined by comparing the OD value to a standard curve prepared with known concentrations of galactose.

RESULTS

The acid production by milk cultures of six single-strain and three multiple-strain lactic streptococcus cultures was stimulated by the addition of β-galactosidase. The time required for the cultures to reach pH 5.0 in milk with and without the added enzyme is shown in Table 1. The amount of stimulation was computed by subtracting the time required for the cultures to reach pH 5.0 in milk containing the enzyme from that required in the control milk.

S. cremoris C6 exhibited the greatest response with 1.2 hr of stimulation, and S. lactis AC2 exhibited the smallest response with 0.4 hr of stimulation. An examination of acid production curves for cultures growing in milk with and without the enzyme indicated that the primary effect of β-galactosidase on acid production was to shorten the lag period. The addition of heat-inactivated enzyme to milk cultures of lactic streptococci failed to stimulate acid production.

Growth of the lactic streptococci in reconstituted NFMS at 32 C with and without added β-galactosidase was measured by plate counts (Table 2). Portions of each culture were plated when the samples containing the enzyme reached pH 5.0. Growth of S. cremoris AC11 appeared to be stimulated by the addition of the enzyme, but no differences were observed for S. cremoris AC1, S. cremoris AC5, or S. lactis C10.

Growth of lactic streptococcus cultures in broth containing either glucose, galactose, or milk was quantitated and compared with the addition of β-galactosidase. The results are presented in Table 1. The acid production by the cultures was measured at 32 C using the glucostat reagent. The time required for the cultures to reach pH 5.0 in milk with and without the added enzyme is shown in Table 1. The amount of stimulation was computed by subtracting the time required for the cultures to reach pH 5.0 in milk containing the enzyme from that required in the control milk.

| Cultures | Time (hr) to reach pH 5.0 at 32 C | Stimulation (hr) |
|----------|-------------------------------|-----------------|
| Control  | β-Galactosidase               |                 |
| Streptococcus cremoris AC1      | 7.8                         | 7.0             | 0.8            |
| S. lactis AC2                    | 6.8                         | 6.4             | 0.4            |
| S. cremoris AC1                  | 7.1                         | 6.2             | 0.9            |
| S. cremoris AC11                 | 8.7                         | 8.1             | 0.6            |
| S. cremoris C6                   | 7.0                         | 5.8             | 1.2            |
| S. lactis C10                    | 5.9                         | 5.0             | 0.9            |
| Cheddar cheese starter A         | 6.7                         | 6.0             | 0.7            |
| Cheddar cheese starter B         | 7.1                         | 6.0             | 1.1            |
| Cheddar cheese starter C         | 7.0                         | 6.2             | 0.8            |

| Culture | Plate count/ml at 32 C | β-Galactosidase |
|---------|------------------------|-----------------|
| Control |                        |                 |
| Streptococcus cremoris AC1      | 2.4 x 108         | 2.6 x 108       |
| S. cremoris AC1                  | 8.5 x 108         | 8.8 x 108       |
| S. cremoris AC11                 | 3.6 x 108         | 6.0 x 108       |
| S. lactis C10                    | 2.0 x 108         | 1.9 x 108       |
lactose was followed turbidimetrically. The times required for three single-strain lactic streptococci to reach an optical density of 0.300 are presented in Table 3. All cultures exhibited fastest growth in the medium containing glucose and slowest growth in that containing galactose. S. cremoris AC, exhibited the widest range, requiring 2.5 hr to reach an optical density of 0.300 in the glucose broth and 7.5 hr in the galactose broth. The same relationships were observed with respect to acid production in broth containing the three sugars.

The concentrations of glucose and galactose in the whey fraction from milk cultures of S. lactis C10, determined at 1-hr intervals during the first 7 hr of growth at 32 C are shown in Fig. 1. Although a low level of glucose (0.05 mg/ml) was detected initially in the control sample, none was detected after 2 to 3 hr of incubation. The concentration of galactose in the control culture at 0 hr was also low (0.25 mg/ml) and appeared to decrease slightly during the first 2 hr of incubation. However, after 5 hr of incubation, it began to accumulate slowly (0.43 mg/ml at 7 hr). The accumulation of galactose occurred during the period of most rapid acid production. The data suggest that S. lactis C10 does not utilize all of the galactose resulting from the catabolism of lactose in milk. In the presence of β-galactosidase, both glucose and galactose accumulated very rapidly during the first 3 hr of incubation (Fig. 2). It is apparent that the accumulation of glucose ceased as the culture entered the most rapid period of acid production. Galactose continued to accumulate, reaching a concentration of 26 mg/ml or 2.6% after 7 hr of incubation. Since milk contains approximately 5.0% lactose (which, if completely hydrolyzed, would yield about 2.5% each of the two hexoses glucose and galactose), it appears that little if any galactose was utilized by the culture growing in milk containing added β-galactosidase. Presumably the lack of glucose accumulation beyond the first 3 to 4 hr of incubation was due to its preferential utilization by the culture. By comparing the data from Fig. 1 (6 hr to reach pH 5.2) and Fig. 2 (5 hr to reach pH 5.2), it is apparent that the culture produced acid more rapidly in milk containing free glucose than in the control milk which contained lactose as the sugar source.

**Table 3. Growth of lactic streptococci in broths containing 1% glucose, 1% galactose, or 1% lactose**

| Culture             | Time (hr) required to reach OD of 0.300 |
|---------------------|----------------------------------------|
|                     | Glucose  | Galactose | Lactose |
| Streptococcus lactis C10 | 4.1      | 5.5       | 4.7     |
| S. lactis AC4       | 2.4      | 3.5       | 2.9     |
| S. cremoris AC1     | 2.5      | 7.5       | 2.7     |

Fig. 1. Acid production and sugar residues for milk cultures of Streptococcus lactis C10. Symbols: Δ, pH; O, glucose; □, galactose.

Fig. 2. Acid production and sugar residues for milk culture of Streptococcus lactis C10 in the presence of 0.1 mg of β-galactosidase per ml. Symbols: Δ, pH; O, glucose; □, galactose.
DISCUSSION

The initial hydrolysis of lactose by the lactic streptococci apparently does not function at a rate that permits maximum acid production by the cultures growing in milk. The addition of $\beta$-galactosidase to lactic streptococcus milk cultures resulted in increased acid production by all single- and multiple-strain cultures included in this study. The major portion of the stimulation resulted from a decreased lag period in acid production. Of the four single-strain cultures assayed, stimulation of growth (as measured by plate count) was observed for only S. cremoris AC$^{11}$, whereas acid production was increased for all cultures assayed. The stimulation of acid production in milk resulting from added $\beta$-galactosidase may be the result of one or more of the following mechanisms: (i) elimination of steps in a catabolic pathway by providing a more readily available energy source; (ii) faster growth resulting in more acid production; or (iii) an alteration in the carbohydrate metabolism of the cells so that a greater percentage of the energy source is converted to acid end products.

The addition of $\beta$-galactosidase to milk cultures rapidly hydrolyzes a portion of the lactose to glucose and galactose, providing the culture with a choice for an energy source of glucose, galactose, or lactose, whereas a normal milk culture has only lactose. The lactic streptococci subsequently produced acid faster when provided with free glucose than when lactose was the sugar source. The data suggested that the lactose hydrolysis is limiting with respect to acid production by lactic streptococci and that their action in producing cultured milk products could be accelerated by adding $\beta$-galactosidase.

The increased acid production by S. cremoris AC$^{11}$, in the presence of $\beta$-galactosidase can be attributed in part to increased growth. However, for the most part acid production by the streptococci was stimulated without a corresponding increase in colony-forming units. Garvie and Mabbitt (3) observed a similar phenomenon in that the addition of peptone to a slow acid-producing variant of S. cremoris in milk stimulated the acid production to the level of the fast parent strain without any change in the growth of the slow variant. It is also possible that increased growth of the other cultures may have resulted in greater cell size or chain length which would not necessarily appear as increases in plate count. Shahani and Vakil (9) reported that glucose, galactose, and lactose were equally as effective in supporting growth and acid production for broth cultures of S. lactis UN. However, in the present study, growth was greatest in broth containing glucose as the energy source, followed by lactose and galactose. Such differences probably can be attributed to use of different cultures and basal media.

In the presence of glucose as an energy source, the lactic streptococci may convert a larger percentage of the sugar to acid end products as a result of altered carbohydrate metabolism. This could contribute to increased acid production by milk cultures containing added $\beta$-galactosidase without an apparent corresponding increase in growth. Even though there was some accumulation of galactose in the control milk culture (Fig. 1), a portion of this moiety of lactose was apparently utilized by the culture. The metabolism of the galactose by milk cultures may result in the production of less acid end products. White et al. (11) reported that 90% of the glucose metabolized by S. pyogenes was converted to lactic acid, whereas only 25 to 57% of the galactose metabolized by the same organism was converted to lactic acid. Similar results have been reported by other workers (5, 8, 9). These observations suggest that the streptococci may be more homofermentative when growing in a medium containing glucose rather than galactose or lactose alone.

Although several mechanisms have been proposed for the hydrolysis of lactose by the lactic streptococci (1, 6, 9, 10), little mention has been made regarding the fate of the resulting galactose moiety. In the present study, milk cultures of S. lactis C$^{10}$ accumulated some galactose during growth with no accumulation of glucose, indicating that milk cultures of lactic streptococci do not utilize all of the galactose resulting from lactose catabolism. In the presence of added $\beta$-galactosidase, very little galactose was utilized by the streptococci, whereas glucose appeared to be utilized as rapidly as it was hydrolyzed by the enzyme during the most rapid period of acid production.

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