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Improved Medium for Lactic Streptococci and Their Bacteriophages

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Incorporation of 1.9% β-disodium glycerophosphate (GP) into a complex medium resulted in improved growth by lactic streptococci at 30 C. The medium, called M17, contained: Phytone peptone, 5.0 g; polypeptone, 5.0 g; yeast extract, 2.5 g; beef extract, 5.0 g; lactose, 5.0 g; ascorbic acid, 0.5 g; GP, 19.0 g; 1.0 M $MgSO_4 \cdot 7H_2O$, 1.0 ml; and glass-distilled water, 1,000 ml. Based on absorbance readings and total counts, all strains of *Streptococcus cremoris*, *S. diacetilactis*, and *S. lactis* grew better in M17 medium than in a similar medium lacking GP or in lactic broth. Enhanced growth was probably due to the increased buffering capacity of the medium, since pH values below 5.70 were not reached after 24 h of growth at 30 C by *S. lactis* or *S. cremoris* strains. The medium also proved useful for isolation of bacterial mutants lacking the ability to ferment lactose; such mutants formed minute colonies on M17 agar plates, whereas wild-type cells formed colonies 3 to 4 mm in diameter. Incorporation of sterile GP into skim milk at 1.9% final concentration resulted in enhanced acid-producing activity by lactic streptococci when cells were inoculated from GP milk into skim milk not containing GP. M17 medium also proved superior to other media in demonstrating and distinguishing between lactic streptococcal bacteriophages. Plaques larger than 6 mm in diameter developed with some phage-host combinations, and turbid plaques, indicative of lysogeny, were also easily demonstrated for some systems.

Lactic streptococci are nutritionally fastidious and require complex media for optimum growth (9, 10, 11, 14, 16). In synthetic media, all strains require at least six amino acids and at least three vitamins (2, 27). Their homofermentative acid-producing nature requires that media be well-buffered for reasonable growth response; in this regard Hunter (12) observed that more growth and larger colonies (0.7 to 1.0 mm in diameter after 48 h) resulted in a medium containing lactose, yeast extract, peptone, and beef extract to which 0.05 M sodium phosphate had been added.

Bacteriophages for lactic streptococci usually are assayed by the agar overlay technique described by Adams (1), using one of the several complex media cited above. During a study of the plating efficiency of lactic streptococcal phages, Lowrie and Pearce (19) observed that not all bacterial strains, especially those of *Streptococcus cremoris*, grew well when inoculated into the most widely used of the complex media then available. They devised a new medium, designated M16, which overcame this problem; it was unique in containing a plant protein extract (Phytone) but lacked phosphate, relying on peptone and acetate for buffering capacity. The omission of phosphate was intentional to allow calcium supplementation for phage assays. However, Thomas et al. (30) incorporated phosphate into this medium for their study of streptococcal proteinases, calling the more-buffered medium T5.

In the present investigation, a correlation was obtained between restriction in sizes of bacterial colonies and phage plaques and a rapid decline in pH with the M16 medium. Attempts were made, therefore, to improve the buffer strength of the medium without resorting to the use of phosphate, well known for precipitation problems in bacteriological media due to its ability to sequester alkaline earth metals (5). The recent reports by Douglas et al. (8) and Douglas (7) suggested that glycerophosphate (GP) would be suitable for this purpose, especially since its use allowed large plaques on *S. lactis* to develop (8). The present report describes the resulting new medium, designated

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M17, and its use in demonstrating improved growth of lactic streptococci and their bacteriophages.

MATERIALS AND METHODS

Medium. M17 broth medium is made by adding the following ingredients to 1,000 ml of glass-distilled water in a 2-liter flask: polypeptone (BBL, Cockeysville, Md.), 5.0 g; Phytone peptone (BBL), 5.0 g; yeast extract (BBL), 2.5 g; beef extract (BBL), 5.0 g; lactose (May and Baker Ltd., Dagenham, England), 5.0 g; ascorbic acid (Sigma Chemical Co., St. Louis, Mo.), 0.5 g; β-disodium GP (grade II, Sigma Chemical Co.), 19.0 g; and 1.0 M $MgSO_4 \cdot 7H_2O$ (May and Baker, Ltd.), 1.0 ml. This concentration was optimum for growth and prevented the pH of *S. cremoris* cultures from falling below 5.9 after growth for 15 h at 30 C. Broth is dispensed (10 ml) into tubes and autoclaved at 121 C for 15 min; the pH of the broth (22 to 25 C) is 7.15 $\pm$ 0.05. Bottom agar used for assay of bacterial colonies or phage plaques is prepared by adding 10.0 g of Davis agar (Davis Gelatine Ltd., Christchurch, N.Z.) to 940 ml of glass-distilled water and heating the mixture to boiling to dissolve the agar. The remaining ingredients, except lactose, are added to the dissolved agar and the mixture is autoclaved at 121 C for 15 min. After cooling to 45 C in a temperature-controlled water bath, a sterile solution of lactose (5.0 g in 50.0 ml of glass-distilled water and sterilized at 121 C for 15 min) to which has been added 10.0 ml of sterile 1.0 M $CaCl \cdot 6H_2O$ is gently added to the melted agar basal medium. The calcium addition is necessary only when the bottom agar plates are to be used for growing phage, but its addition has no adverse effect on use of the medium for plating bacteria; usually, slight cloudiness develops when the calcium is added. After mixing carefully to avoid bubbles, 15- to 18-ml quantities are added to sterile petri plates. The bottom agar plates are held overnight (15 to 18 h) at 22 to 25 C to dry and then checked for any contaminating colonies; they then are stored at 2 to 5 C until used. Top overlay agar is prepared by adding 4.5 g of Davis agar to 1,000 ml of glass-distilled water and heating to boiling until the agar is dissolved. The remaining broth ingredients, including lactose but excluding calcium chloride, are then added and the medium is dispensed (50-ml quantities) into prescription bottles and autoclaved (121 C, 15 min). Top agar is used for carrying diluted phages and bacteria to bottom agar plates for determining titers of virus preparations and colony counts in bacterial cultures. M16, T5, and lactic broth were prepared as described previously (9, 19, 30).

GP-SM. Severe protein denaturation and browning occurred when the GP was autoclaved with skim milk (SM). Therefore, a stock solution containing 9.5 g of GP per 10.0 ml of glass-distilled water was sterilized (121 C, 15 min) separately, and 0.2 ml was added per 10 ml of sterile SM, providing a final concentration of 1.9% GP.

Bacterial strains. Most strains were available from the culture collection maintained in the Dairy Research Institute. These included *S. cremoris* AM_1, AM_2, ML_1, P_2, R_1, 158, 368 (a derivative of AM_1), and AM_1 (799) (AM_1 lysogenized with phage 799) and *S. diacetilactis* DRC_1. *S. lactis* strains used were ML_8, C_2, and C_2*lac*$^-$. The C_2 strains were obtained from T. Molskness of Oregon State University.

Bacteriophage strains. Two bacteriophages were used, both isolated from cheddar cheese whey. Phage 799 is virulent for *S. cremoris* AM_2, and strain 690 is virulent for *S. cremoris* SK_{11}; each phage, however, will form plaques on hosts other than those on which they were originally isolated.

Growth measurement. Bacterial growth was assayed by recording absorbance readings (600 nm) at 30- to 60-min intervals of the various strains inoculated (1.0%) into 10.0 ml of the various media in flasks fitted with a side arm accommodated by a Bausch & Lomb Spectronic 20 colorimeter. Colony-forming units per milliliter of culture were determined after blending (60 s) of 1:100 dilutions in 10% M17 broth (21) followed by serial dilution, as appropriate; aliquots (0.1 ml) were poured on the surface of M17 bottom agar plates after being mixed with 2.5 ml of top agar as described below for the phage assay procedure, except calcium chloride was omitted.

Culture activity in milk. The influence of daily subculturing in M17, M16, and LB for 10 days on acid-producing activity in SM was measured. Strains were maintained in the three broth media by inoculation at 1% and incubation at 30 C for 24 h. Each day, the 24-h broth cultures were each inoculated in duplicate (1%) into 10 ml of SM containing 9.5% solids (100 g of powder plus 910 ml of distilled water; sterilized at 121 C for 15 min) and incubated, one tube at 30 C and the other at 22 C. Tubes at 30 C were tested for pH after 6 h, and tubes at 22 C were tested for ability to coagulate milk when held for 15 h. Studies also were carried out to determine the influence of culturing strains in SM containing GP on their subsequent acid-producing activity when inoculated into sterile SM. Strains AM_1, AM_2, ML_1, and ML_8 were incubated at 22 C for 15 h in GP-SM and SM. Each strain was subcultured from these two types of milk into SM and incubated at 30 C in a temperature-controlled water bath; pH measurements were taken at hourly intervals.

Bacteriophage assays and stocks. To ensure homogeneity, bacteriophage stocks were renewed by single-plaque isolation (3, 4, 22). Aliquots (0.1 ml) of an overnight (15-h) M17 broth culture of the appropriate bacterial host were placed in sterile test tubes (10 by 75 mm) fitted with aluminum caps. One drop (0.05 ml) of sterile calcium chloride (1.0 M) was then added to each tube followed by 0.1 ml of phage previously serially diluted in 10% M17 broth so that about 20 plaques per plate resulted. After 3 to 10 min at 22 to 25 C (room temperature) to allow for phage adsorption, melted and cooled (45 C) M17 top agar (2.5 ml per tube) was then added, and the tube contents were immediately poured on the surface of hardened M17 bottom agar in sterile plastic petri plates (10 by 90 mm). Plates were incubated at 30 C and observed periodically for isolated plaques from 3 h onward. When they appeared, usually between 3 to 5 h, two or three well-isolated plaques were

picked by touching the top layer with sterile 152-mm applicator sticks; the plaque was then transferred to 0.5 ml of chilled (2 to 5 C) M17 broth contained in a test tube (10 by 75 mm) and held in the refrigerator overnight. (The titer of these young plaques is 10^5 to 10^6/ml.) Incubation of phage plaque plates was continued until the next morning when they were examined to ensure that no other plaques developed which were partially coincidental with the plaques originally selected and that the plaques were typical in morphology and size for the particular phage-host system. One or more of the M17 broth phage-containing tubes were then used to prepare the phage stock. This was done by adding the entire contents of the tube to 10.0 ml of a 3.5-h M17 broth culture (absorbance = 0.10 to 1.15 at 600 nm) of the appropriate bacterial host growing at 30 C; 0.1 ml of $CaCl \cdot 6H_2O$ (1.0 M) also was added. With continued incubation, lysis occurred from 2 h onward, usually by 4 h. If lysis did not occur, the stock was discarded and prepared from another plaque isolate. Overnight (15- to 18-h) incubation of phage-infected cultures would sometimes yield turbid cultures due to emergence of phage-resistant mutants. After lysis, the phage-laden culture was centrifuged at 4,500 rpm for 10 min in a bench-top clinical centrifuge. The supernatant was then filter sterilized by passing through a sterile syringe-mounted membrane filter (0.45 μm; Millipore Corp.) into a sterile screw-capped tube. Titer of the stock was determined by counting plaques that developed in M17 top agar when the serially diluted sterile lysates were plated as described above. Stocks were stored at 2 to 5 C. Titers ranged from 10^8 to 10^{10} plaque-forming units per ml and would occasionally increase two- to threefold during the first week of storage. The phages were relatively stable when stocks were prepared in this manner, declining in titer only 5 to 10% over 6 months of storage.

RESULTS

Bacterial growth. Figure 1 shows the buffering capacity of M17 broth in comparison to three other media. Although T5 medium was almost as well buffered as M17, it was unsuitable for bacteriophage assays because of calcium precipitation and, therefore, was excluded from further study. The well-buffered nature of M17 under growth conditions also was apparent. For example, five lactic streptococcal strains tested gave pH values ranging from 5.78 to 6.10 after 24 h of growth at 30 C in M17 broth; these strains grown in M16 and LB, however, gave pH values from 4.70 to 4.87 and 4.42 to 4.70, respectively. When cells of *S. cremoris* AM_1 were grown either in M16 or M17 broth (Fig. 2) and inoculated into M16, M17, or LB media, the best growth response occurred in M17 medium. By 9 h, the absorbance readings of M16-grown cells revealed 49 and 52% more growth in M17 as compared to M16 and LB, respectively; for M17-grown cells, these increases were 30 and 45%, respectively. Also, a 1- to 2-h lag occurred when M16-grown cells were used, whereas except in LB the lag was eliminated with M17-grown cells. These data are typical for all 12 *lac*$^+$ strains included in the study. In support of the absorbance data, M17 consistently allowed higher total cell counts in each case. For example, with *S. cremoris* AM_1, total counts after 15 h at 30 C in M16, M17, and LB were 1.0×10^8, 1.6×10^8, and 5.6×10^7, respectively, and for *S. cremoris* AM_2 they were 3.7×10^7, 2.0×10^7, 2.0×10^8, and 5.0×10^6, respectively.

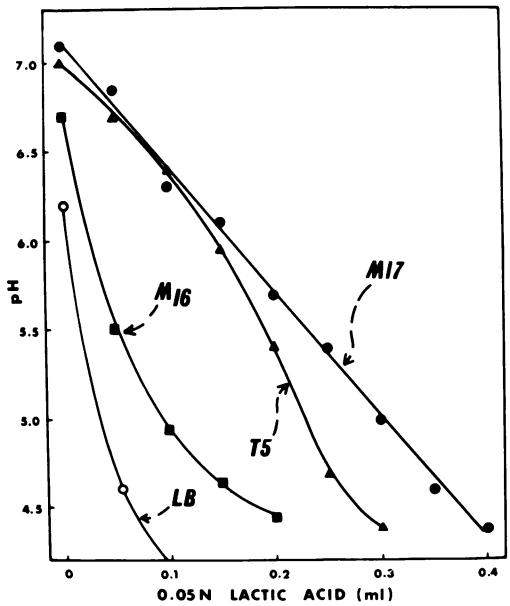

FIG. 1. *Buffering capacity of T_5 (▲), M16 (■), lactic (○), and M17 (●) broth as revealed by pH resulting after addition of various amounts of 20% lactic acid to 10-ml volumes of broth.*

The medium also proved useful in selecting carbohydrate mutants, especially those unable to ferment lactose. For example, colonies of *lac*$^+$ strains measured 3 to 4 mm in diameter at 5 days, whereas *lac*$^-$ mutants, growing only on the small amount of glucose provided by the yeast extract (5) in the medium, reached colony sizes of less than 1.0 mm. The large colony size was typical of all 12 *lac*$^+$ strains tested except *S. cremoris* P_2 (an X-ray derivative of *S. cremoris* HP), which developed more slowly because of a requirement for carbon dioxide for rapid growth on agar plates (29). It should also be mentioned that colonies of all *lac*$^+$ strains tested other than P_2 were clearly visible for counting within 15 h after plating and incubation at 30 C.

The milk-acidifying ability of lactic strepto-

cocci was best preserved by maintenance of strains in M17 medium as compared to M16 or LB. Data for two strains typical for all strains tested appear in Table 1. Furthermore, the M17-derived *S. cremoris* cultures coagulated milk almost every time 24-h-old broth cultures were inoculated into SM and incubated at 22 C for 15 h, whereas this occurred only rarely for strains derived from M16 or lactic broth cultures after the second transfer. *S. lactis* strains, however, did coagulate milk even though the 6-h test showed them to be impaired in acid-producing activity. The pH values attained for strains AM_1, AM_2, ML_1, ML_8, and C_2 in SM by 15 h at 22 C after maintenence for 10 days in the three broth media appear in Table 2.

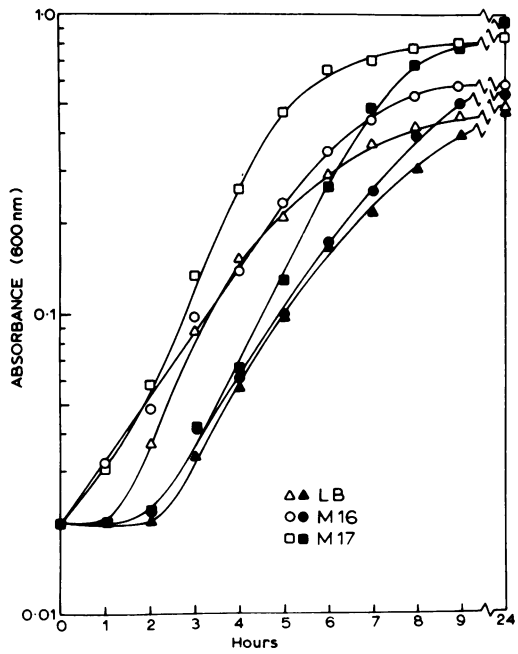

FIG. 2. *Growth of S. cremoris AM_1 in M16 (○, ●), M17 (□, ■), or both lactic broths (△, ▲) when 15-h M16 (closed symbols) or M17 (open symbols) broth cultures were inoculated (2%) into the various media and incubated at 30 C.*

Maintenance of strains in SM containing GP also preserved their rapid-acid-producing abilities in milk, especially early in the growth period. Data for *S. cremoris* ML_1, typical for three other strains tested, appear in Fig. 3. It may be seen that for at least 5 h the SM culture was 1 h slower in achieving the same degree of acidity as compared to the culture grown in the GP-SM, although approximately the same final acidity was achieved by each culture by 15 h.

Bacteriophage development. The M17 medium was superior to the three other media for observing bacteriophages. This is illustrated in Fig. 4, where an M17 broth-derived stock of phage 799 replicating on *S. cremoris* 368 was assayed on M17 and M16 agars. The more clearly defined plaques on the M17 agar are evident. The same was true for whey-derived phage preparations when plated on M17 and

TABLE 1. *Acid-producing activity of S. cremoris AM_1 and S. lactis ML_8 as revealed by pH attained after 6 h at 30 C and 15 h at 22 C in SM inoculated (1%) from M16, M17, or lactic broth (LB) culture lines maintained by daily subculture (1%) and incubation at 30 C for 24 h*

Days in broth	*S. cremoris* AM_1						*S. lactis* ML_8					
	M16		M17		LB		M16		M17		LB	
	pH[a]	Coagulation[b]	pH	Coagulation	pH	Coagulation	pH	Coagulation	pH	Coagulation	pH	Coagulation
1[c]	6.25	1+	5.45	2+	6.30	—	5.65	2+	5.00	2+	5.31	2+
2	6.20	—	6.00	1+	6.05	—	5.90	1+	5.00	2+	5.35	2+
3	6.20	—	5.92	1+	6.23	—	6.00	—	4.99	2+	5.54	2+
4[d]	6.25	—	5.90	1+	6.20	—	5.85	1+	5.80	2+	5.75	2+
5	6.10	—	5.50	1+	6.22	—	5.30	1+	5.08	2+	5.56	1+
6	5.94	—	5.42	1+	6.09	—	5.27	1+	4.99	2+	5.47	1+
7	5.92	—	5.52	2+	6.02	—	5.32	1+	5.00	2+	5.48	1+
8	5.90	—	5.42	2+	6.10	—	5.35	1+	5.10	2+	5.58	1+
9	5.91	—	5.35	2+	6.09	—	5.30	2+	5.01	2+	5.47	1+
10	5.83	—	5.32	1+	6.17	—	5.31	2+	5.03	2+	5.57	2+

[a] pH values taken after incubation at 30 C for 6 h.

[b] Coagulation (at least pH 4.7) observed after incubation at 22 C for 15 h; —, no coagulation; 1+, weak but definite coagulation; 2+, firm coagulation.

[c] pH values resulting when 15-h SM cultures were inoculated (1%) into SM and tested for pH after 6 h at 30 C were 5.3 for both AM_1 and ML_8.

[d] pH values on day 4 were higher due to use for sterile SM that had been held at room temperature (20 to 25 C) for several weeks after autoclaving.

TABLE 2. *Acid-producing activity of lactic streptococcal strains as revealed by pH attained after 15 h at 22 C in SM inoculated (1%) from M16, M17, or lactic broth (LB) culture cell lines maintained 10 days by daily subculture (1%) and incubation at 30 C for 24 h*

Strain	pH		
	M16	M17	LB
AM_1	5.30	4.67	5.59
AM_2	5.22	4.67	5.31
ML_1	5.00	4.77	5.49
ML_8	4.73	4.70	4.74
C_2	4.68	4.62	4.62

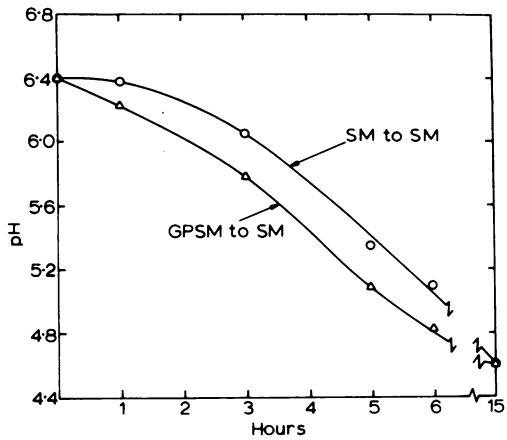

FIG. 3. *Influence of culturing S. cremoris ML_1 in SM or GP-SM on acid production in SM as determined by pH measurements during incubation at 30 C. Cultures (15 h at 22 C) were transferred from GP-SM to SM (Δ) or SM to SM (O).*

M16 agars, as well as for all other virus preparations assayed, although the titers on both media were similar.

During this investigation into the efficiency of plating of lactic streptococcal phages on various hosts in M17 agar, it became clear that the medium, because it supported better host growth, allowed the demonstration of phenomena commonly associated with other bacterial virus systems but not previously reported for lactic streptococcal phages. Three examples appear in Fig. 5, where extremely large clear plaques, turbid plaques, and plaques exhibiting diffusion of phage lysin to surrounding uninfected cells are evident.

DISCUSSION

It is clear from the data presented that the growth of lactic streptococci in M17 medium is improved over that attained in two other commonly used media, M16 and LB (Fig. 1). The buffering action of GP, as evidenced by the higher final pH in mature M17 broth cultures, apparently allows more total growth and reduced cell death and injury caused by the lower pH reached in other media. Maintenance of lactic streptococci in M17 with daily subculture

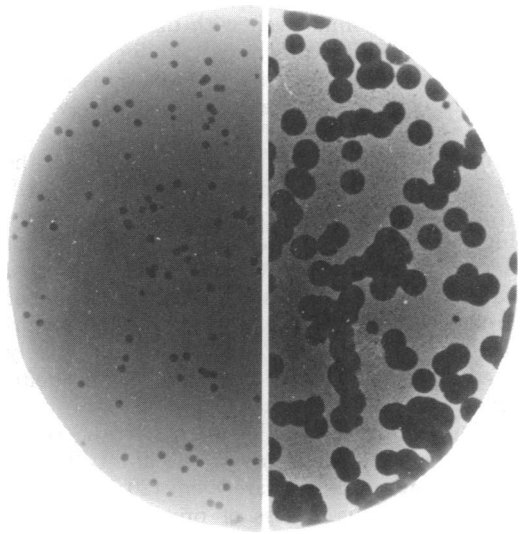

FIG. 4. *Plaques of M17 broth-derived phage 799 developing on S. cremoris 368 when assayed on M16 (left) and M17 (right) agar plates incubated for 18 h at 30 C.*

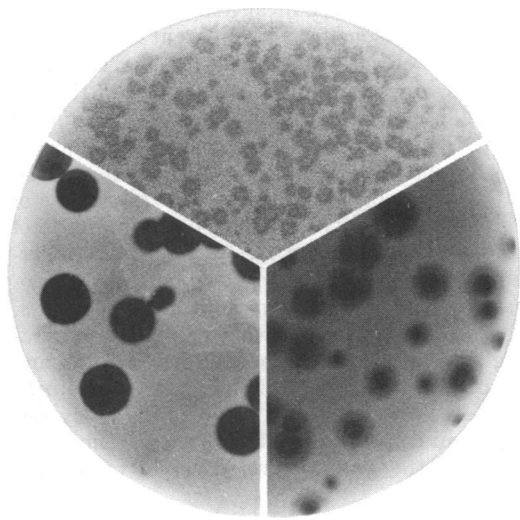

FIG. 5. *Petri plate sections from phage platings demonstrating large clear plaques (left, phage 690 on S. cremoris 368), turbid plaques (top, phage 690 on S. cremoris AM_1), and phage lysin (right, phage 799 on S. cremoris 368) when plates were incubated 18 to 48 h at 30 C.*

and incubation even for 24 h at 30 C before inoculation had little deleterious effect on their subsequent acid-producing ability in milk at either 30 or 22 C (Tables 1 and 2). Maintenance in M16 or LB, however, yielded cells with impaired acid-producing ability, no doubt due to cell death and injury caused by the lower pH attained in these poorly buffered media. These findings suggested that maintenance of the organisms in milk even with daily subculture might cause impaired acid production when cells were reintroduced into milk. This apparently was the case since cells of four widely used starter strains showed improved acid-producing properties when initiating growth in milk if the cells originated from milk containing 1.9% GP (Fig. 3). Since early rapid acid production is highly desirable in such products as cheddar and short-set cottage cheese, future practical value may be found in buffering bulk starter milk with GP. This may prove to be an economical step, since GP is inexpensive and widely used in foods and as a carrier in certain medicines.

Slow acid production by lactic streptococci in milk may be due to loss of the ability to use lactose, presumably a rare event (20, 23, 26), or more frequently to loss of proteolysis, which limits the ability of the organism to obtain nitrogen from milk protein at sufficient rate to allow rapid cell growth (6). Recent data suggest that the genetic determinants for both of these cellular activities (*lac* and *prt*) are carried on plasmids (23, 25, 26), although direct proof is lacking. Reasons for the apparent difference in stability of *lac* and *prt* also have not been shown. Since proteinase is localized in the cell wall (30), it is likely that prolonged exposure to acid, which lactic streptococci experience in both milk and nonmilk media other than M17, alters cell wall integrity and encourages loss of proteinase activity. Studies on the influence of different pH levels on the frequency of appearance of *prt*$^-$ types would be revealing; incorporation of GP in media may minimize the loss, especially when, as shown herein, acid-producing activity of cells is improved by minimizing their exposure to low-pH conditions. Since *prt*$^-$ appears to be a stable state inherited by descendant cells (6), the effect of the acid environment at the genetic level deserves consideration.

Preliminary data obtained in our laboratory indicate that GP addition to milk protects cells from freezing damage. Frozen concentrates of lactic starter cultures are now widely used in the United States, especially for direct inoculation of milk for buttermilk manufacture and to inoculate bulk starter milk intended for use in manufacture of cheddar and cottage cheese. Direct inoculation of vat milk with frozen concentrates, however, has not yet materialized, since at least 10^7 cells per ml is required to initiate acid production in the milk at a rate to ensure cheese manufacture in the accustomed time (18). The large volume of concentrate presently required to achieve such a cell density makes their use for this purpose impractical. Lyophilized cell concentrates may be applied in this manner in the future (28), and use of GP as a growth medium-neutralizing agent will no doubt prove useful.

The usefulness of M17 medium in selecting carbohydrate-requiring mutants also deserves mention. Since wild-type colonies grow to a large size, differences between mutants and parent cells are maximized. The medium, therefore, is finding extensive use in our laboratories to isolate and study such mutants and no doubt will be of value to others for the same purpose. It also is likely that media for other acid-producing bacteria, especially lactobacilli, will be improved by incorporation of GP. In this regard, we have found that *S. thermophilus* and *Lactobacillus bulgaricus* strains grow well in the medium, especially if the pH is adjusted to about 6.8 prior to inoculation; comparative growth studies in other media have not yet been made.

Few meaningful studies on plaque morphology and lysogeny in lactic streptococci have been reported (13, 15, 17), presumably because the media usually used allow little differentiation of plaque types because of poor buffering capacity. Nyiendo et al. (24) found that buffering medium was necessary to achieve high titers of lactic phages, and we have found that whey phage stocks at 10^9 to 10^{11} plaque-forming units/ml can be prepared from SM containing GP.

It is noteworthy that the pH of M17 medium does not fall below 5.7 even upon incubation of lactic streptococcal cultures for 24 h at 30 C. Thus, not only are differences in plaque size, as determined by the phage host interaction, distinguishable, but other phage phenomena such as lysogeny as visualized by turbid plaques (Fig. 5) became demonstrable. In a subsequent publication, we will report on use of M17 medium to demonstrate widespread lysogeny in the lactic streptococci.

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