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Title: LACTIC STREPTOCOCCI: GROWTH CHARACTERISTICS AND PLASMID PROFILES OF DRUG- AND BACTERIOPHAGE - INSENSITIVE MUTANTS

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Fast acid-producing streptomycin-resistant mutants for several strains of lactic streptococci currently used in commercial Cheddar cheese manufacture were isolated. Their isolation involved use of a recently developed differential plating medium that allows identification of fast acid-producing colonies. Both parents and mutants were characterized with regard to growth, acid production, proteolytic abilities, optimum temperatures, and phage sensitivities, and comparisons were made. The mutants were found to be very similar to the parent strains and could conceivably be used to overcome streptomycin in the milk supply in the manufacture of Cheddar cheese.

These streptomycin-resistant mutants were used to study strain interaction in a commercially used mixed-strain starter culture composed of two Streptococcus lactis and four Streptococcus cremoris strains. Mutants were available for each of the strains in the starter and were grown in various combinations with the parent strains in nonfat milk. Resistance to streptomycin served as a marker to identify a particular strain when grown in mixed culture and was identified by plating onto media containing

streptomycin. Both S. lactis strains were found to significantly inhibit two of the four S. cremoris strains and one instance of S. cremoris - S. cremoris inhibition was found. Speculation regarding a dominance pattern in this starter was made.

The plasmid profiles of four strains of S. lactis and their phage-insensitive mutants were examined and compared. The mutants of three of the four strains were found to have very similar plasmid patterns to that of their parents. However, the phage-insensitive mutants of the fourth strain which were examined contained one more plasmid band than their parent strain. Removal of this band by curing with ethidium bromide did not result in loss of phage insensitivity. The plasmid profiles of streptomycin-resistant isolates of two of the four strains were also examined and proved to be similar to that of the parent strains.

A study was conducted to determine if various types of storage abuse influenced the incidence of slow acid-producing variants in three selected strains of lactic streptococci. This also involved use of the new differential plating medium mentioned previously. No increase in slow variants was detected when cultures were stored in either the ungrown or mature states in nonfat milk at -20° and -80°C and subjected to occasional thawing and refreezing. Considerable injury and cell death were detected when cultures were stored in the mature state at 4°C. The presence of injured colony types hindered accurate enumeration of slow acid-producing colonies in this instance.

Lactic Streptococci: Growth Characteristics and Plasmid Profiles of Drug- and Bacteriophage-Insensitive Mutants

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Typed by Carlene Ballew for John J. Wulf

To Cheryl, whose love, patience,
and understanding, have been very
dear to me.

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CHAPTER I

ISOLATION AND CHARACTERIZATION OF ANTIBIOTIC-RESISTANT MUTANTS OF LACTIC STREPTOCOCCI

ABSTRACT

Single-step streptomycin-resistant mutants of nine strains of lactic streptococci used in commercial Cheddar cheese manufacture were isolated. In each case a 24-hr broth culture was concentrated by centrifugation and pour plated in medium containing 600 μg/ml of streptomycin. Mutant colonies were selected and subcultured in broth and then streaked onto antibiotic-containing Fast-Slow Differential Agar which allowed isolation of fast acid-producing organisms. A fast rifampin-resistant mutant was also obtained using this technique. Characterization involved comparing parent and mutant strains with regard to growth, acid production, proteolytic abilities, optimum temperatures, and phage sensitivities. The use of this technique to obtain isolates which are resistant to other antibiotics and their possible use in commercial Cheddar cheese manufacture to overcome antibiotics in the milk supply is discussed.

INTRODUCTION

The problems associated with the presence of antibiotics in the milk supply, due to treatment of mastitis, is well documented from both a public health viewpoint and with regard to inhibition of cheese starter culture activity (1,5,12,15,25). Several suggestions have been made in the literature to facilitate overcoming antibiotics in milk in the manufacture of cheese. These are (as reviewed by Marth and Ellickson): the addition of an enzyme to inactivate the antibiotic such as penicillinase in the case of penicillin; the use of a heavy inoculum of starter bacteria; or the use of antibiotic-resistant starter bacteria (5,14). The third suggestion is very intriguing and is considered in this paper.

Lactic streptococci which are antibiotic resistant have been isolated in the past (7,12,20,21). However, these have been slow acid-producers and hence unsuitable for use in Cheddar cheese manufacture. Strains of lactic streptococci are considered fast acid-producing if they coagulate nonfat milk (NFM) within 16 h at 21°C from a 1% inoculum.

With the use of Fast-Slow Differential Agar (FSDA) (10), fast streptomycin-resistant mutants of nine strains of lactic streptococci have been isolated. This paper describes how they were isolated and their characterization relative to the parent strains. Because of their overall similarity to the parent strains, these isolates were used to study strain dominance and suppression in a commercially used mixed-strain starter culture (Chapter 2).

MATERIALS AND METHODS

Media

Instant Peake nonfat milk (Galloway West Co., Fond du Lac, WI) reconstituted at 11% solids was used throughout this study. Test tubes containing ten ml of nonfat milk (NFM) were autoclaved at 121°C for 12 min for use in culture maintenance. Flasks containing 500 ml of NFM were steamed for 35 min and cooled to the desired temperature before inoculation for use in growth studies. If not used immediately these were stored for up to five days at 4°C before discarding. Tubes containing ten ml of NFM pasteurized at 62°C for 30 min were used for the Pearce activity tests.

Plate counts were on M17 agar (24) in at least quintuplicate replications using a micro-droplet technique. All plates were incubated at 30°C for 24-48 h. Dilutions were made in 0.1% peptone (Bacto) dissolved in distilled water. A concentration of 600 µg/ml of streptomycin was used throughout except in the Pearce activity tests. Streptomycin sulfate, kanamycin, gentamycin, neomycin, penicillin G, erthromycin, (all from Sigma), and rifampin B (Cal Biochem) were dissolved in distilled water and sterilized by filtration through a 0.45 µ Millipore filter and added to all media after autoclaving and subsequent cooling to room temperature. In the case of media containing agar, the antibiotic was added after cooling to 50°C.

Bacterial Strains

The nine strains used were obtained from the culture collection at Oregon State University and included: *Streptococcus cremoris* 224,

SK11-G, 108, 290P, R6, 378, C13; and Streptococcus lactis BA-1 and BA-2. All have recently been used in commercial Cheddar cheesemaking at the Tillamook County Creamery Association (Tillamook, OR) and Olympia Cheese (Olympia, WA) cheese plants. Cultures were maintained in tubes containing ten ml of NFM and stored in the unincubated condition (2% inoculum) at 4°C. These were incubated at 21°C and transferred to fresh NFM weekly. All inocula, unless otherwise noted, were from a freshly-coagulated NFM culture grown at 21°C. Each culture was purified by streaking onto FSDA and subsequent selection and cultivation of a fast colony type before use in mutant selection.

Mutant Selection

For each strain a 1% inoculum was made into 100-300 ml of M17 broth (24) and incubated for 24 hours at 30°C. The turbid broth was then centrifuged at 5000 x g for 20 min and the resulting pellet suspended in 10-15 ml of sterile diluent. Approximately 0.5-ml portions were aseptically transferred to sterile petri dishes which were then poured with 15-20 ml of sterile M17 agar (cooled to 46°C) containing streptomycin. The plates were swirled several times to assure even dispersal of the cells. Incubation was at 30°C until growth appeared (usually 2-4 days). Mutant colonies were selected and subcultured in broth; the broth cultures were then streaked onto FSDA plates containing streptomycin and these were incubated anaerobically (BBL Gas Pak) at 30°C for 48 h. Fast acid-producing colonies were selected and transferred to tubes of NFM. To verify that fast isolates were indeed obtained, tubes of NFM were inoculated with a 1% inoculum and incubated at 21°C for 16 h.

In this manner fast streptomycin-resistant isolates were obtained for the nine strains previously described. A phage-insensitive mutant of S. lactis BA-2 was used in place of the parent strain and will be designated BA-22. Also, a fast rifampin B-resistant mutant of S. cremoris 108 was isolated using the same procedure with 50 µg/ml rifampin B. Only six of the nine strains for which streptomycin-resistant mutants were obtained were extensively characterized. Each streptomycin-resistant mutant will be designated by R (e.g. 108R), for resistant, and each parent by S (e.g. 108S), for sensitive.

Minimum Inhibitory Concentration Studies

Freshly-coagulated cultures in NFM were diluted in sterile diluent by a factor of 1:100 (ca. 10^7 cfu/ml). Loopfuls were streaked onto prepoured plates of M17 agar containing increasing concentrations of streptomycin and incubated at 30°C for 48 h. The presence or absence of growth was noted. The lowest concentration of antibiotic at which no growth occurred was considered the minimum inhibitory concentration (MIC). This method was also employed to test the streptomycin-resistant isolates against other antibiotics to determine if cross resistance exists. Control plates (to assure viability) were inoculated in each case.

Effect of Streptomycin on Growth

Growth of streptomycin-resistant mutants of Escherichia coli is sometimes enhanced in the presence of streptomycin (17). A study was conducted to determine if this phenomenon exists among these isolates. Tubes containing ten ml of autoclaved NFM with and without

added streptomycin were inoculated with a 1% inoculum and incubated at 21°C for 16 h. Samples were taken and appropriately diluted in sterile diluent and plated onto M17 agar.

Comparison of Growth and Acid Production at 27°C

Flasks containing 500 ml of steamed NFM were inoculated at a rate of 1% and incubated at 27°C for ten h. Samples were taken every h for pH measurement, using a pH meter (Corning), and every two h for cell count determination.

Comparison of Growth and Acid Production at 21°C

Flasks containing 500 ml of steamed NFM were inoculated at a rate of 1% and incubated at 21°C for 15 h. Samples were taken at 0, 8, 12, and 15 h for titratable acidity measurement and cell count determination. Titratable acidity measurements were conducted by taking nine ml samples (in duplicate) and titrating to the phenolthalein endpoint using 0.1 N NaOH standardized with potassium acid phthalate. Results were reported as percent titratable acidity (%TA) calculated as lactic acid.

Activity Tests

Tubes containing ten ml of pasteurized NFM were inoculated with a 2% inoculum and incubated according to the Cheddar cheesemaking profile described by Pearce (19) in a water bath. Rennet was not added. Trials were run in duplicate and pH readings made at the end of the five-h run. This was repeated with the addition of 100 µg/ml of streptomycin to each tube.

Growth versus Temperature Characteristics

L-shaped tubes containing 15 ml of M17 broth (autoclaved 15 min at 121°C) were inoculated with a 1% inoculum of M17 broth culture and incubated at various temperatures using a temperature gradient incubator (Scientific Industries Inc.). Optical density (OD) readings were made periodically using a Bausch and Lomb Spectronic 20 spectrophotometer set at 600 nm. Generation times were calculated using the equation g = .693/k. Specific growth rate (k) values were determined from plots of OD against time using the formula:
$k = 2.303 (\log_{10} X_2 - \log_{10} X_1)/(t_2-t_1)$ (13). Tubes were placed in the incubator approximately two hours previous to inoculation to allow a temperature gradient to form and were staggered approximately 0.4° to 0.8°C apart by adjusting the maximum and minimum temperatures of the gradient. Plate counts were made after five or six h of incubation on tubes incubated in the 38° to 40°C range to determine if die-off occurred at temperatures which approximate cooking temperatures used in Cheddar cheese manufacture.

Proteolytic Abilities

Proteolytic abilities were determined according to the method described by Hull as modified by Citti, et al. (4,11). Thirty ml batches of NFM pasteurized 30 min at 62°C were inoculated with 1% inoculum. Incubation was at 21°C for 15 h at which time five-ml samples were taken (in triplicate) and treated according to Hull. OD readings were made using the Spectronic 20 set at 650 nm. These

were read against a standard tyrosine (Sigma) curve and degree of proteolysis was expressed as mg/ml of tyrosine.

Phage Sensitivities

Tubes containing three ml of melted M17 overlay agar (24) with 0.15% bromcresol purple were cooled to 46°C and inoculated with 0.1 ml of coagulated culture. Also, one drop of sterile $CaCl_2$ was added to each. These were poured onto the surface of prepoured M17 agar plates, allowed to harden, and spotted with one drop (ca. 0.05 ml) of bacteriophage preparation. Triplicate platings were made for each phage-host combination and one of each incubated at 25°, 30°, and 37°C. These were examined for plaque formation at 24 and 48 h.

RESULTS

Minimum Inhibitory Concentrations and Cross Resistance

As seen in Table 1 the MIC's varied from strain to strain and also from species to species. The two S. lactis strains displayed much greater resistance to streptomycin than the S. cremoris strains. The streptomycin-resistant mutants of each of the six strains in Table 1 were tested against: neomycin, 10 µg/ml; kanamycin, 10 µg/ml; erythromycin, 10 µg/ml; and penicillin G, 2.0 µg/ml (concentrations based on previous MIC studies). Cross resistance to any of these antibiotics, at these concentrations, was not observed.

Effect of Streptomycin on Growth

The results in Table 2 demonstrate that five of the six resistant isolates grew to similar cell numbers in NFM whether exposed to streptomycin or not. However, SK11-GR reached a number of approximately one log less when grown in the presence of antibiotic. None of the mutants were dependent on streptomycin for growth.

Comparison of Growth and Acid Production at 27°C

Figures 1 - 6 indicate that both sensitive and resistant strains performed similarly when grown at 27°C in NFM with regard to acid production (change in pH) and growth. In several cases the mutant actually produced acid faster than the parent. However, strain 224R showed somewhat less activity than 224S. This temperature (27°C) was chosen to simulate bulk-starter incubation at Tillamook where bulk tanks are incubated at 80°F (26.7°C).

Comparison of Growth and Acid Production at 21°C

Figures 7 - 12 illustrate that both sensitive and resistant strains also performed similarly when grown at 21°C in NFM with regard to acid production (change in %TA) and growth.

Activity Tests

Table 3 illustrates that three of the six resistant isolates were considerably slower (108R, BA-1R, and BA-22R) than their parent strains with regard to final Pearce test pH in NFM without streptomycin. Some of the resistant strains appeared to have improved performance (108R and BA-1R) and others reduced performance (SK11-GR and BA-22R) in the presence of antibiotic. This concentration of streptomycin completely inhibited acid production by the parent strains.

Growth versus Temperature Characteristics

As can be seen in Figures 13-15, the growth curves for all of the resistant strains appear at least fairly similar to those of the parents. However, SK11-GR, 224R, 290PR, and BA-1R were consistently slower growing than the sensitive strains throughout the temperature spectrum. In contrast, the performance of 108R and BA-22R was almost identical to that of their parents.

Optimum temperatures for all *S. cremoris* strains ranged from 27.3° to 31.2°C and for the *S. lactis* strains between 31.3° and 33.6° C as determined from the graphs (Figures 13-15). Except for BA-1R, all resistant strains had optimum temperatures within one degree of their parent strains.

Plate counts made on tubes incubated in the 38° - 40°C range (simulating cooking temperatures) revealed that all four resistant and sensitive S. cremoris strains died off. Some strains (SK11-G and 290P) appeared to die off more rapidly than the others but parents and mutants responded similarly in each case. However, both S. lactis strains and their resistant mutants showed considerable growth at these temperatures.

Proteolytic Abilities

The results in Table 4 illustrate that five of the six mutants were similar to their parents with respect to proteolytic abilities. Strain 224R actually appeared to have improved capacity for proteolysis relative to 224S, and BA-22R a reduced capacity compared to BA-22S.

Phage Sensitivities

As seen in Table 5, none of the mutants have gained insensitivity to phage, and strain BA-22R has maintained the phage insensitivity of its parent. This confirms that mutations for antibiotic resistance and phage insensitivity can co-exist in a strain without loss of acid producing capacity.

DISCUSSION

Laboratory-derived antibiotic-resistant strains such as these are most likely due to a chromosomal mutation rather than plasmid mediated. They exist as a result of a spontaneous mutation which has been selected for by exposure to a high concentration of antibiotic (single-step mutants) which inhibits the growth of the parent organisms. To obtain resistance to other antibiotics used in the treatment of mastitis, repeated exposure to antibiotic may be required (step-wise mutants) as the development of resistance is dependent upon exposure to a critical concentration (26).

In chromosomal mutants, as such, there is a change in the phenotype which serves to prevent an antibiotic from reaching or reacting with its site of action. Contrastingly, plasmid-mediated resistance is associated with a chemical modification and inactivation of the antibiotic. This form of resistance is usually found in clinical or environmental isolates and plasmid DNA in these mutants may code for resistance to more than one drug (2).

Streptomycin is known to inhibit protein synthesis by binding to the ribosomes and interfering with translation. In chromosomal mutants of E. coli, resistance is due to a mutation in the str A gene (3,16) which codes for a protein found in the 30s ribosome (6). The resistant ribosomes are sufficiently altered so that they do not bind streptomycin and protein synthesis can proceed. In some isolates streptomycin is required for growth. This is called streptomycin

dependence and in E. coli is due to a mutation in another allele of the str A gene (9,16).

It is conceivable that these streptomycin-resistant isolates could be used in Cheddar cheese manufacture to overcome streptomycin in the milk supply. The average level of resistance on M17 agar of the four parent S. cremoris strains was 5.75 µg/ml. For the S. lactis strains this value was 24 µg/ml. These represent the concentrations necessary to completely inhibit growth. Much lower levels, of course, will partially inhibit growth and thereby slow acid production in the cheese vat. The resistant strains, however, will grow and produce acid even in the presence of 100 µg/ml of streptomycin as demonstrated by the Pearce activity test. This concentration represents a much higher level than would be allowed by health authorities to exist in the milk supply (15,26). As far as stability is concerned, it should be noted that the mutants were maintained as described in Materials and Methods (in the absence of antibiotic) for a period of over one year without any detectable loss of resistance.

The basic question behind this proposal is whether or not cheese made from milk containing streptomycin (or any other antibiotic) is safe for human consumption. Consumption of foods containing antibiotics may cause allergic reactions and may select for antibiotic-resistant pathogens in the intestine (26). Aminoglycoside antibiotics, such as streptomycin, have traditionally been regarded as poor sensitizers when taken through oral consumption and were of little concern as a cause of allergy (25). More recently, however, reports

of hypersensitivity have appeared that have been very severe in some instances (26).

The W.H.O./F.A.O. expert committee (26) recommended that the level of streptomycin in milk not exceed 0.0 - 0.2 ppm (approximately 0.2 µg/ml) calculated as base. In the United States it is established that no detectable antibiotics should be present in milk (15), which of course, depends on the accuracy of the assay being used. Perhaps the resistant isolates described in this study could be used to overcome low levels of streptomycin in the milk supply (as deemed safe for human consumption) which would otherwise cause some degree of inhibition of starter activity.

Reports in the literature are very inconsistent with regard to the minimum concentrations of streptomycin in milk which are necessary for partial and complete inhibition of starter activity. One report stated that as little as 0.4 µg per 100 ml (0.0004 µg/ml)* retarded the development of a mixed-strain starter culture and 10.0 µg per 100 ml (0.1 µg/ml) completely inhibited acid production (18). Another indicated that 0.1 mg/ml (100 µg/ml) resulted in poor starter activity (23). A third report showed that 0.01 mg/ml (10 µg/ml) completely inhibited acid production in a starter composed of S. lactis and L. dextranicum (8).

*This original paper was not seen but a review was noted in Dairy Science Abstracts. The original was cited by another author (14) who reported that the concentration which caused partial inhibition was 0.04 µg/ml, not 0.0004 µg/ml.

The amount of streptomycin in milk obtained from a cow being treated for mastitis by infusion of antibiotic is dependent on: the dosages employed; the time interval between infusion and sampling; and the milk production of the individual quarters. This concentration may be as high as 15 µg/ml as long as 72 h after infusion with 200,000 µg as determined by Smith et al. (22). As expected, these values can vary considerably from animal to animal.

Streptomycin is only one of several antibiotics used in mastitis treatment. Others used are: penicillin, bacitracin, neomycin, polymyxin, and oxytetracycline (25). It is hoped that this technique could be used to isolate fast acid-producing lactic streptococci which are resistant to some of these other antibiotics and perhaps banks of resistant starter cultures could be established.

The streptomycin-resistant mutants described in this paper could also be used as a research tool to facilitate study of interaction between strains when grown in mixed culture. Resistance to streptomycin would serve as a marker to identify a particular strain when grown under these conditions. A resistant strain could be grown in combination with a streptomycin-sensitive strain(s) and growth of the mutant could be followed by plating on media containing added streptomycin. In this manner, interactive effects such as strain dominance and suppression could be investigated.

LITERATURE CITED

1. Bell, W. B., C. C. Flora, P. M. Reaves, and C. W. Holdaway. 1951. Aureomycin concentration in milk following intramammary infusion and its effect on starter activity. J. Dairy Sci. 34:675-679.

2. Benveniste, R., and J. Davies. 1973. Mechanisms of antibiotic resistance in bacteria. Annu. Rev. Biochem. 42:471-506.

3. Breckenridge, L., and L. Gorini. 1970. Genetic analysis of streptomycin resistance in Escherichia coli. Genetics 65:9-25.

4. Citti, J. E., W. E. Sandine, and P. R. Elliker. 1963. Technical notes: some observations on the Hull method for measurement of proteolysis in milk. J. Dairy Sci. 46:337.

5. Claybaugh, G. A., and F. E. Nelson. 1951. The effect of antibiotics in milk: A review. J. Milk and Food Tech. 14:166-160.

6. Fanustu, G., and H. G. Wittman. 1972. Ribosomal proteins XXXIII. Location of amino-acid replacements in protein S12 isolated from Escherichia coli mutants resistant to streptomycin. J. Mol. Biol. 68:547-550.

7. Hargrove, R. E., H. E. Walter, J. P. Malkames, Jr., and K. T. Maskell. 1950. The effect of penicillin and streptomycin on Swiss cheese starters. J. Dairy Sci. 33:401 (M21).

8. Harmon, L. G. 1951. Antibiotics in milk. Texas J. Sci. 3:176-179.

9. Hashimoto, K. 1960. Streptomycin resistance in Escherichia coli analyzed by transduction. Genetics 45:49-62.

10. Huggins, A. R., and W. E. Sandine. 1979. Differentiation of fast and slow milk-coagulating isolates of lactic streptococci. Proceedings, 74th Annual Meeting. Am. Dairy Science Association, p. 70-71.

11. Hull, M. E. 1947. Studies on milk proteins. II. Colorimetric determination of the partial hydrolysis of the proteins in milk. J. Dairy Sci. 30:881-884.

12. Katznelson, H., and E. G. Hood. 1949. Influence of penicillin and other antibiotics on lactic streptococci in starter cultures used in Cheddar cheesemaking. J. Dairy Sci. 32:961-968.

13. Lee, D. A., and E. B. Collins. 1976. Influences of temperature on growth of Streptococcus cremoris and Streptococcus lactis. J. Dairy Sci. 59:405-409.

14. Marth, E. H., and B. E. Ellickson. 1959. Problems created by the presence of antibiotics in milk and milk products: A review. J. Food Milk Technol. 22:266-272.

15. Mol, H. 1975. Antibiotics and milk. A. A. Balkema, Rotterdam.

16. Momose, H., and L. Gorini. 1971. Genetic analysis of streptomycin dependence in Escherichia coli. Genetics 67:19-38.

17. Newcombe, H. B., and M. H. Nyholm. 1950. The inheritance of streptomycin resistance and dependence in crosses of Escherichia coli. Genetics 45:49-62.

18. Overby, A. J. 1952. The effect of various antibiotics in milk following intramammary infusion. Nord. Vet. Med. 4:993-1004. (Abs. in Dairy Sci. Abstr. 15, 1:63. Original not seen).

19. Pearce, L. E. 1969. Activity tests for Cheddar starter cultures. N.Z. J. Dairy Sci. 4:246-247.

20. Shahani, K. M., and W. J. Harper. 1958. Differences in the acid production and phosphorus metabolism of oxytetracycline sensitive and resistant organisms. Appl. Microbiol. 6:9-14.

21. Shahani, K. M., and W. J. Harper. 1958. The development of antibiotic resistance in cheese starter cultures. Milk Prod. J. 149:15-16, 53-54.

22. Smith, C. R., W. E. Petersen, and R. W. Brown. 1948. Effect of infused streptomycin in the mammary gland. Proc. Soc. Exptl. Biol. Med., 68:216-219.

23. Stoltz, E. I., and D. J. Hankison. 1953. Antibiotics and lactic starter cultures. Appl. Microbiol. 1:24-29.

24. Terzaghi, B. E., and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29:897-813.

25. Welch, H. 1957. Problems of antibiotics in foods as the Food and Drug Administration sees them. Am. J. Pub. Health. 47:701-705.

26. W.H.O./F.A.O. Expert Committee on Food Additives. 1969. Specifications for the identity and purity of food additives and their toxicological evaluation : Some antibiotics. W.H.O. Techn. Report Series No. 430, Geneva.

Table 1. A comparison of MIC values for each of the parent lactic streptococcal strains studied.

Strain	MIC for streptomycin on M17 agar (µg/ml)
290PS	4
108S	7
SK11-GS	4
224S	8
BA-1S	27
BA-22S	21

Table 2. Cell numbers achieved in NFM with and without added streptomycin as determined by plate count. Incubation was at 21°C for 16 h.

Strain	Log_{10} cfu/ml Grown in absence of streptomycin	Log_{10} cfu/ml Grown in the presence of streptomycin
290PR	9.04	8.92
108R	9.20	9.20
SK11-GR	9.15	8.18
224R	9.30	9.15
BA-1R	9.15	8.96
BA-22R	9.30	9.04

Table 3. Final pH values achieved in NFM after five h of incubation according to the Cheddar cheesemaking profile described by Pearce (2% inoculum).

	Without added streptomycin		With added streptomycin (100 µg/ml)	
Strain	Sensitive	Resistant	Sensitive	Resistant
290P	5.56	5.54	6.50	5.56
108	5.20	5.60	6.46	5.47
SK11-G	5.50	5.62	6.51	5.90
224	5.49	5.60	6.48	5.58
BA-1	5.12	5.52	6.41	5.32
BA-22	5.06	5.49	6.48	5.67

Control (uninoculated) - 6.60

Table 4. A comparison of proteolytic abilities in NFM expressed as mg of tyrosine per ml of NFM. These represent net values as compared to a control.

Strain	Sensitive	Resistant
290P	0.026	0.029
108	0.062	0.062
SK11-G	0.061	0.060
224	0.043	0.067
BA-1	0.037	0.035
BA-22	0.029	0.018

Table 5. A comparison of phage sensitivities at 25°, 30°, and 37°C of streptomycin-sensitive and -resistant strains.

Strain	Phage	25°C		30°C		37°C	
		Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
290P	8	S	S	S	S	NG	NG
	9	S	S	S	S	NG	NG
108	11	S	S	S	S	NG	NG
	12	S	S	S	S	NG	NG
SK11-G	23	S	S	S	S	NG	NG
224	24	S	S	S	S	NG	NG
BA-1	1	S	S	S	S	S	S
	2	S	S	S	S	S	S
	3	S	S	S	S	S	S
BA-22	4	I	I	I	I	I	I
	5	I	I	I	I	I	I

S = Sensitive, I = Insensitive, NG = No Growth.

Figure 1. A comparison of change in pH when grown at 27° C in NFM.

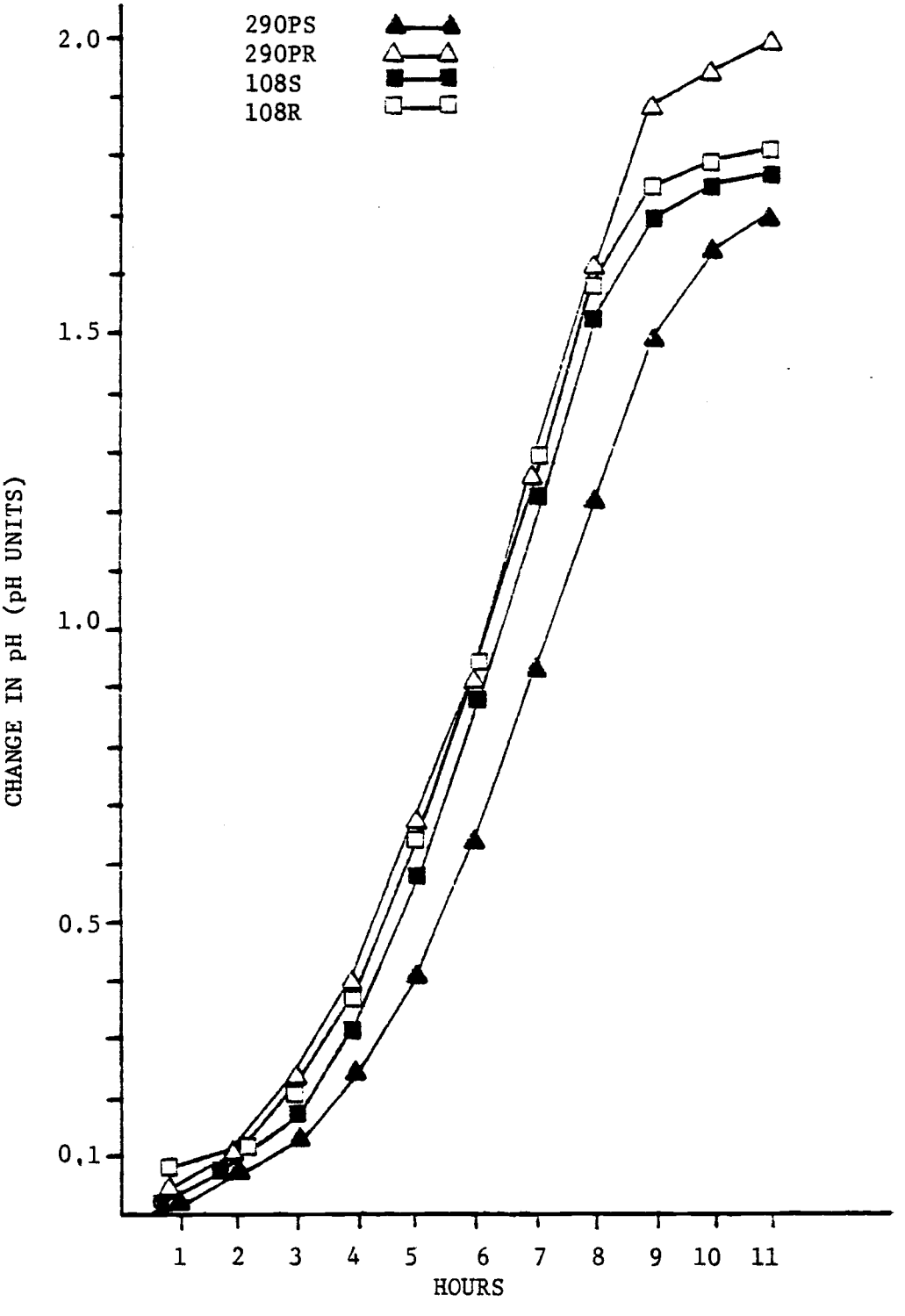


Figure 2. A comparison of change in pH when grown at 27° C in NFM.

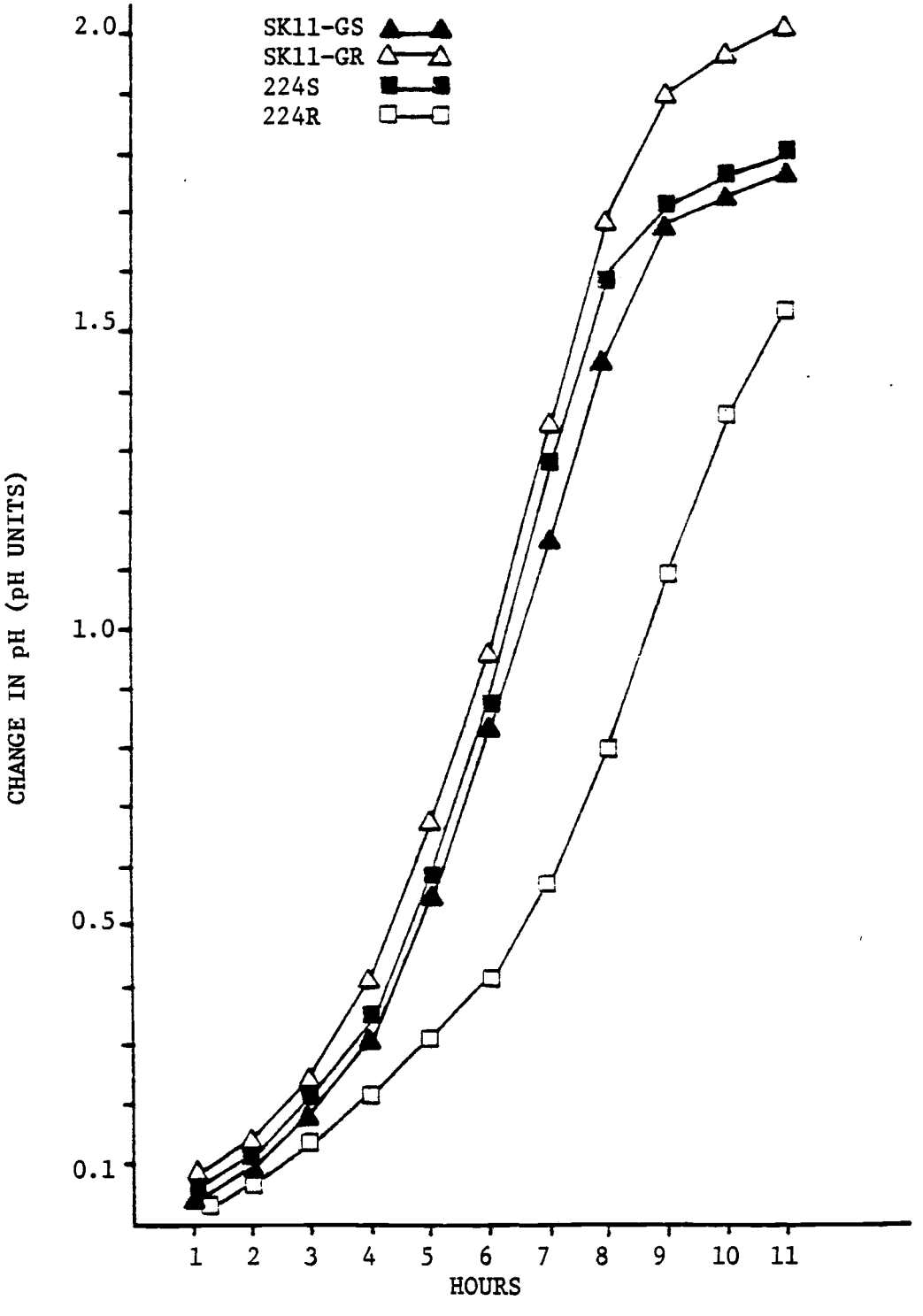


Figure 3. A comparison of change in pH when grown at 27° C in NFM.

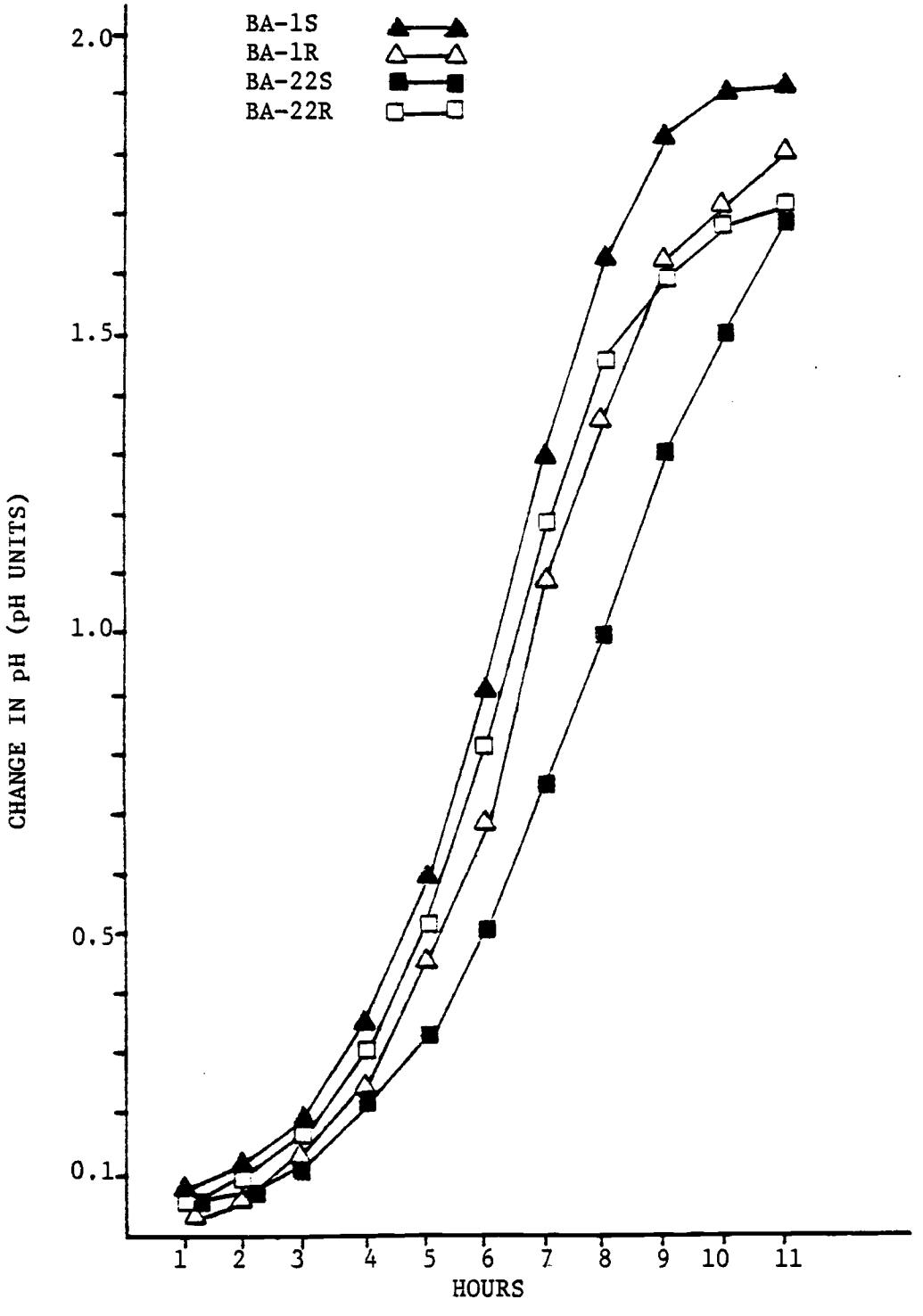


Figure 4. A comparison of cell numbers achieved when grown at 27° C in NFM.

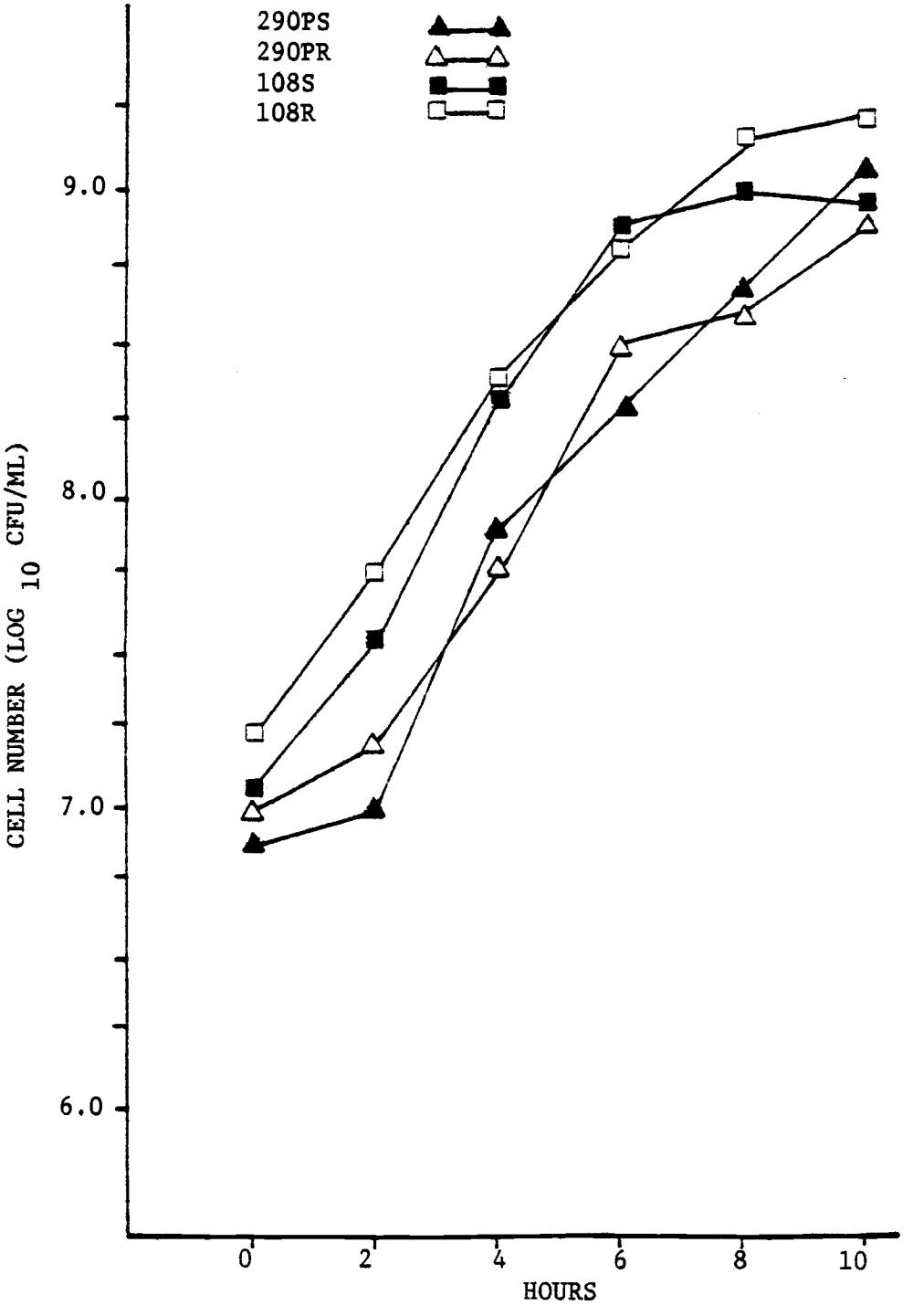


Figure 5. A comparison of cell numbers achieved when grown at 27° C in NFM.

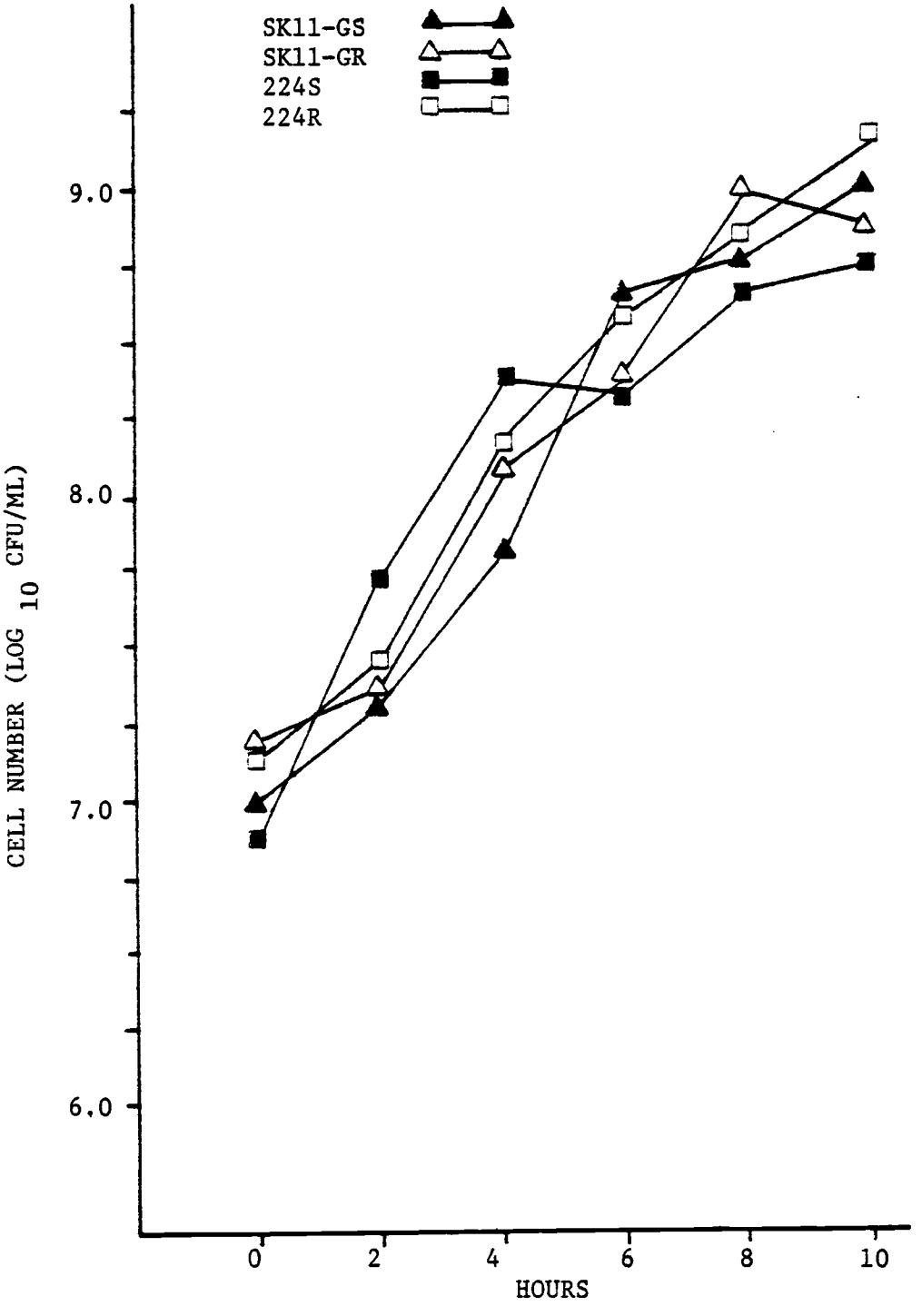


Figure 6. A comparison of cell numbers achieved when grown at 27° C in NFM.

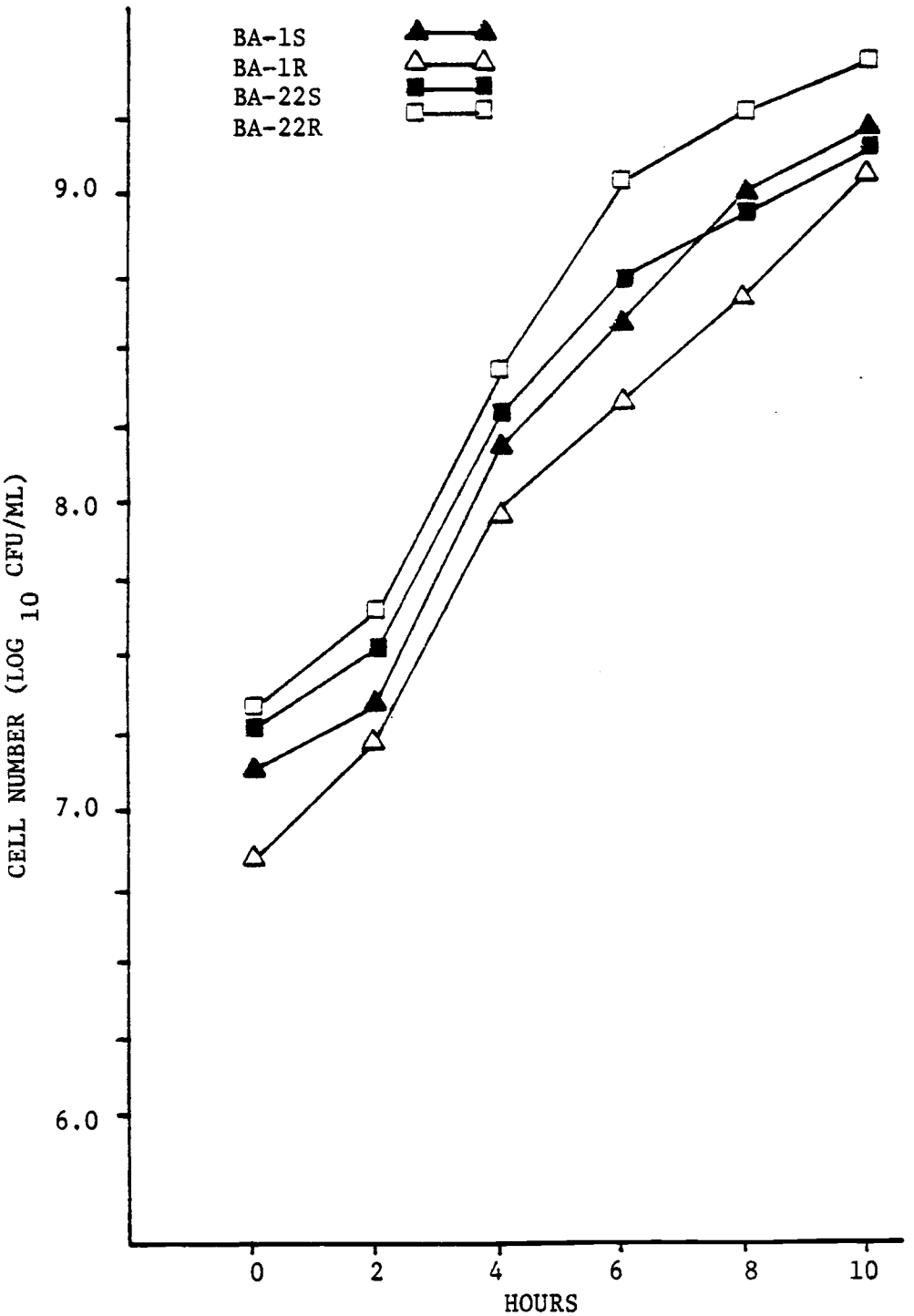


Figure 7. A comparison of acid production (%TA) when grown at 21° C in NFM.

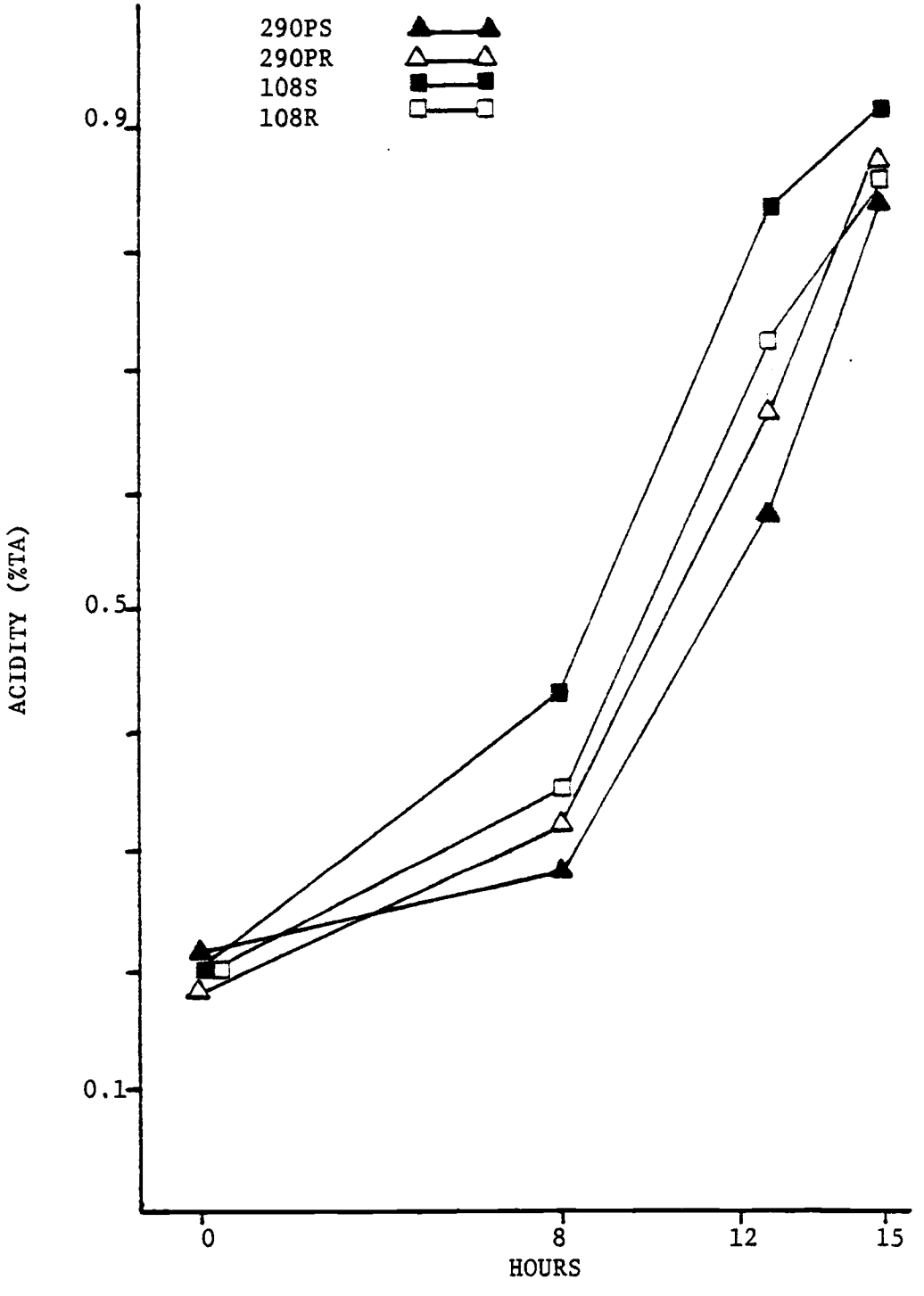


Figure 8. A comparison of acid production (%TA) when grown at 21° C in NFM.

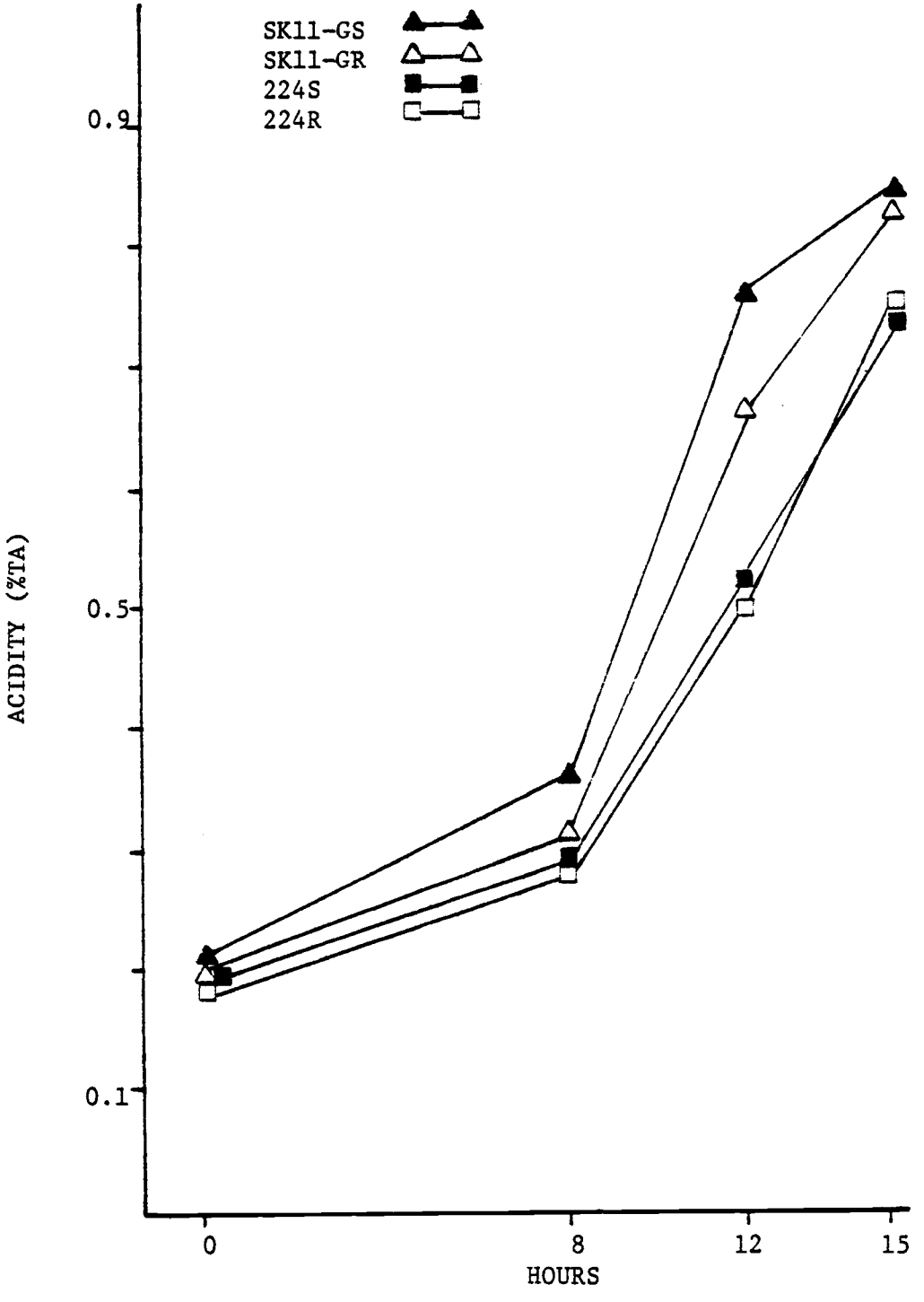


Figure 9. A comparison of acid production (%TA) when grown at 21° C in NFM.

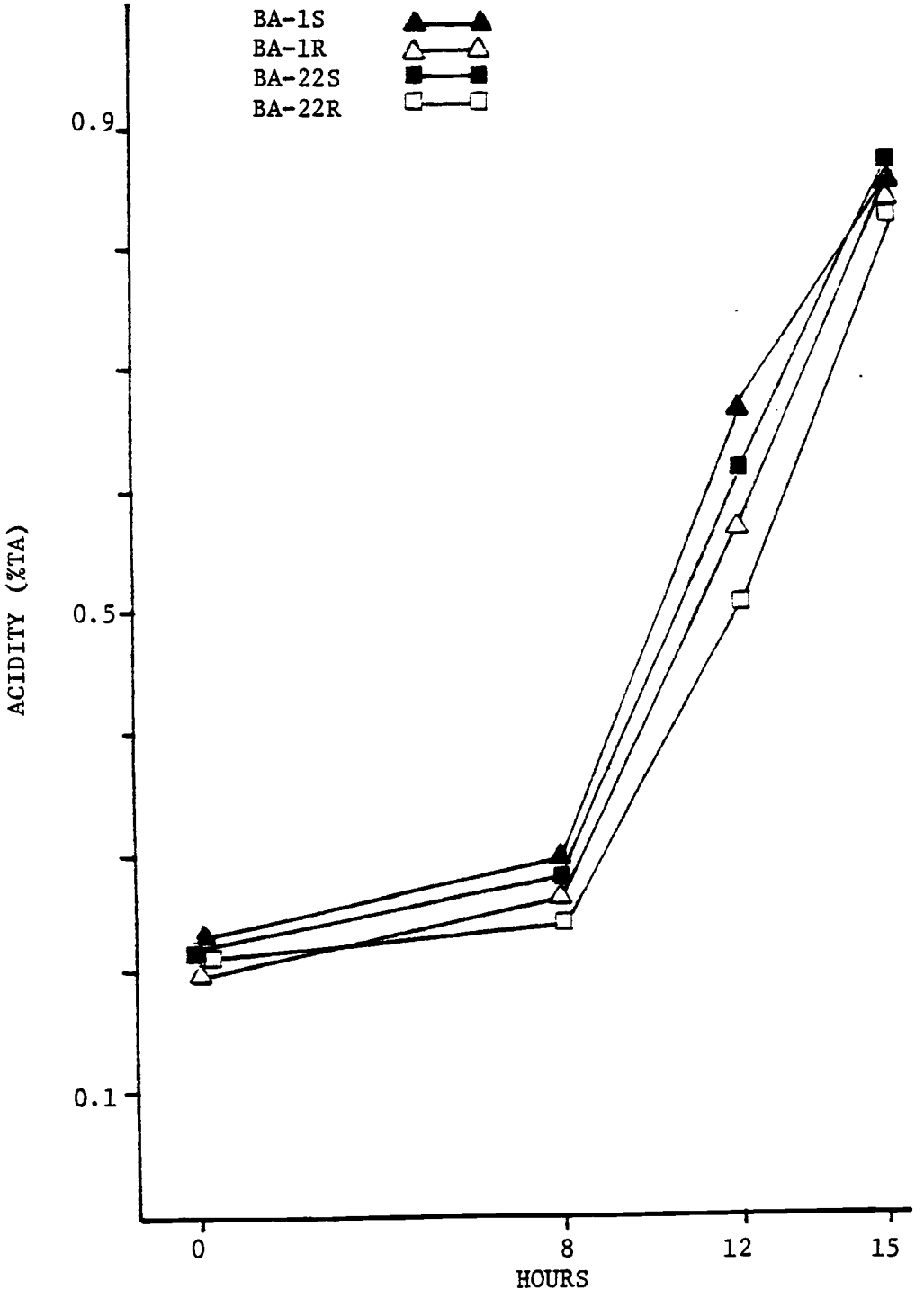


Figure 10. A comparison of cell numbers achieved when grown at 21° C in NFM.

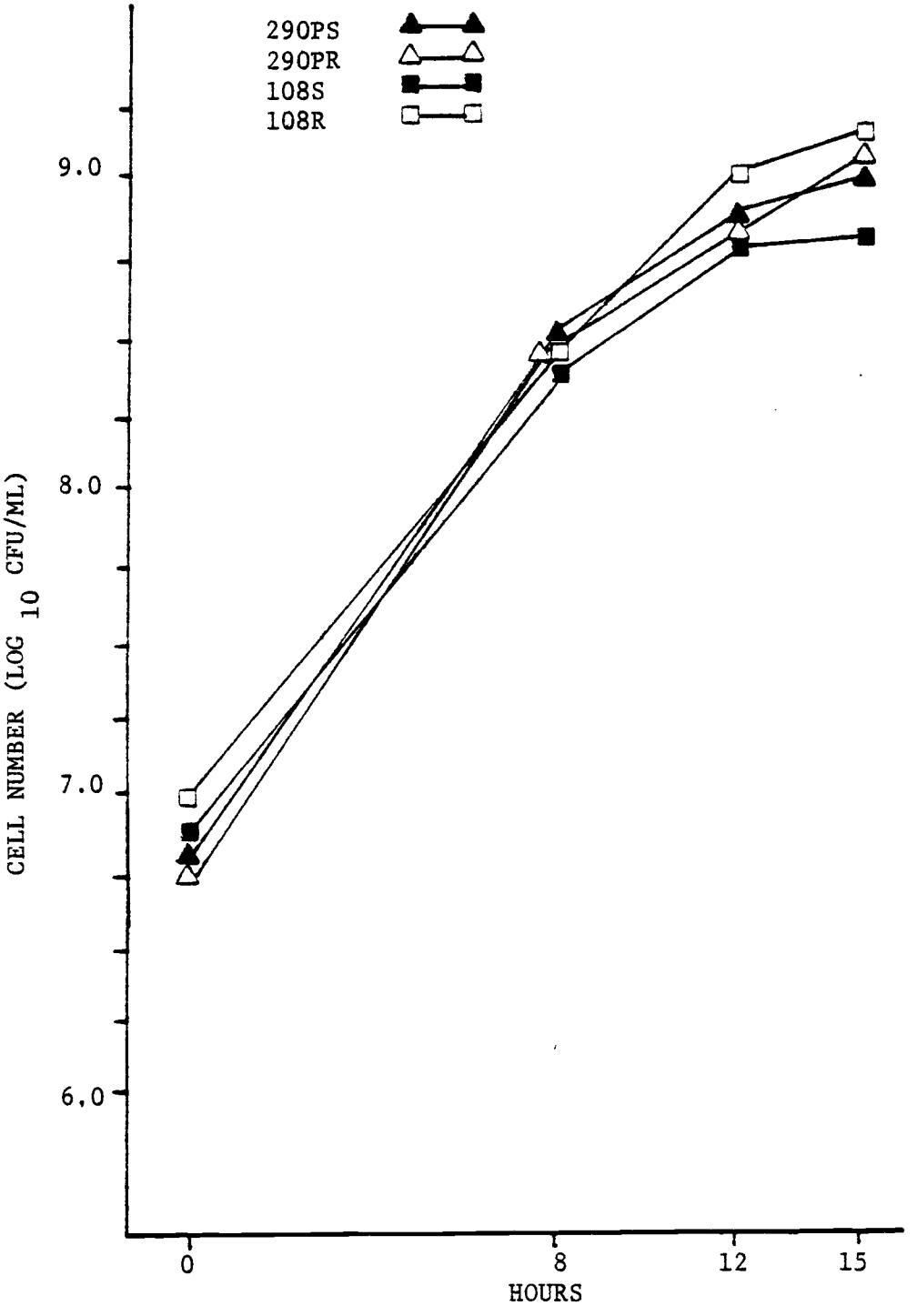


Figure 11. A comparison of cell numbers achieved when grown at 21° C in NFM.

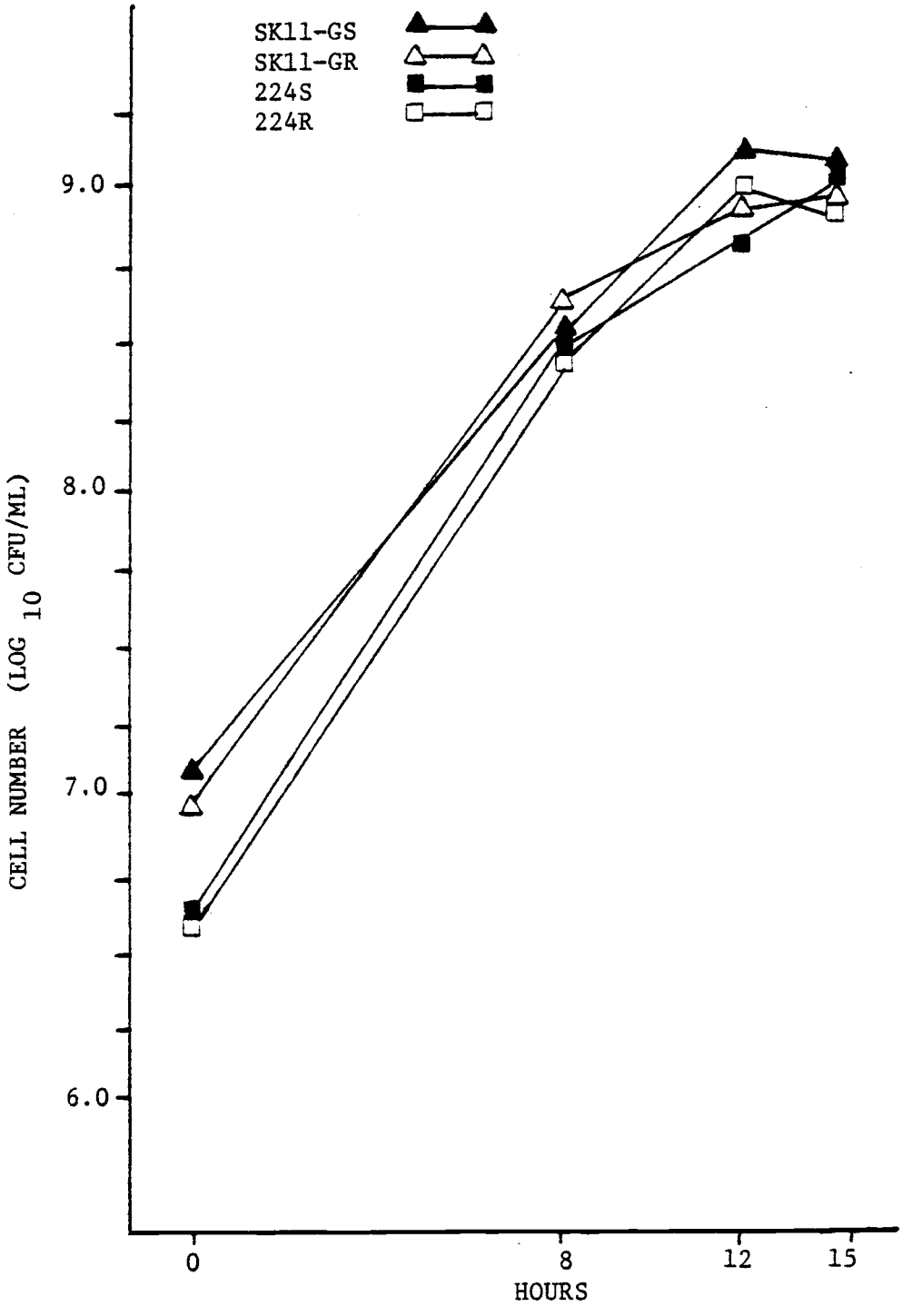


Figure 12. A comparison of cell numbers achieved when grown at 21° C in NFM.

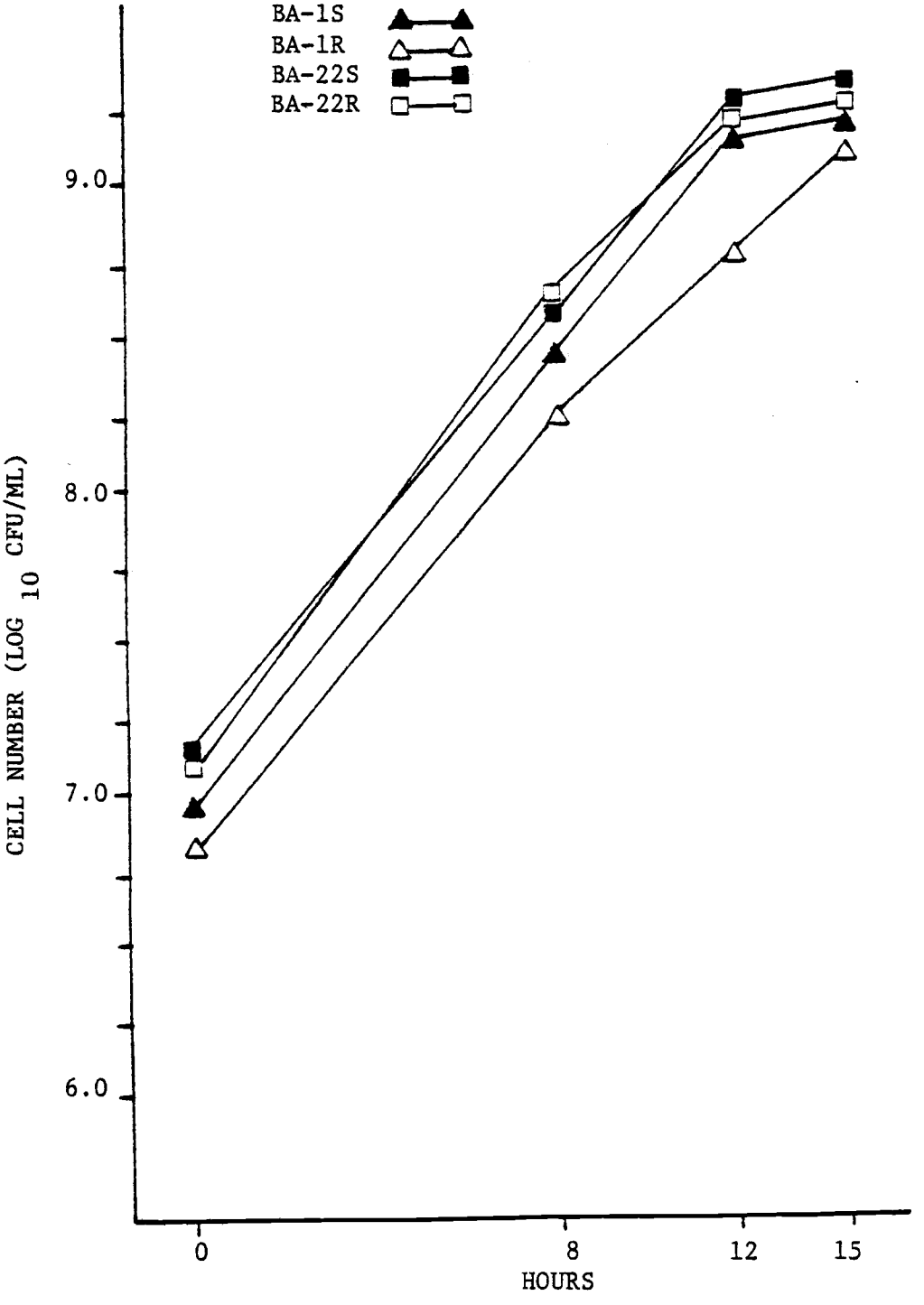


Figure 13. A comparison of the influence of temperature on generation time when grown in M17 broth.

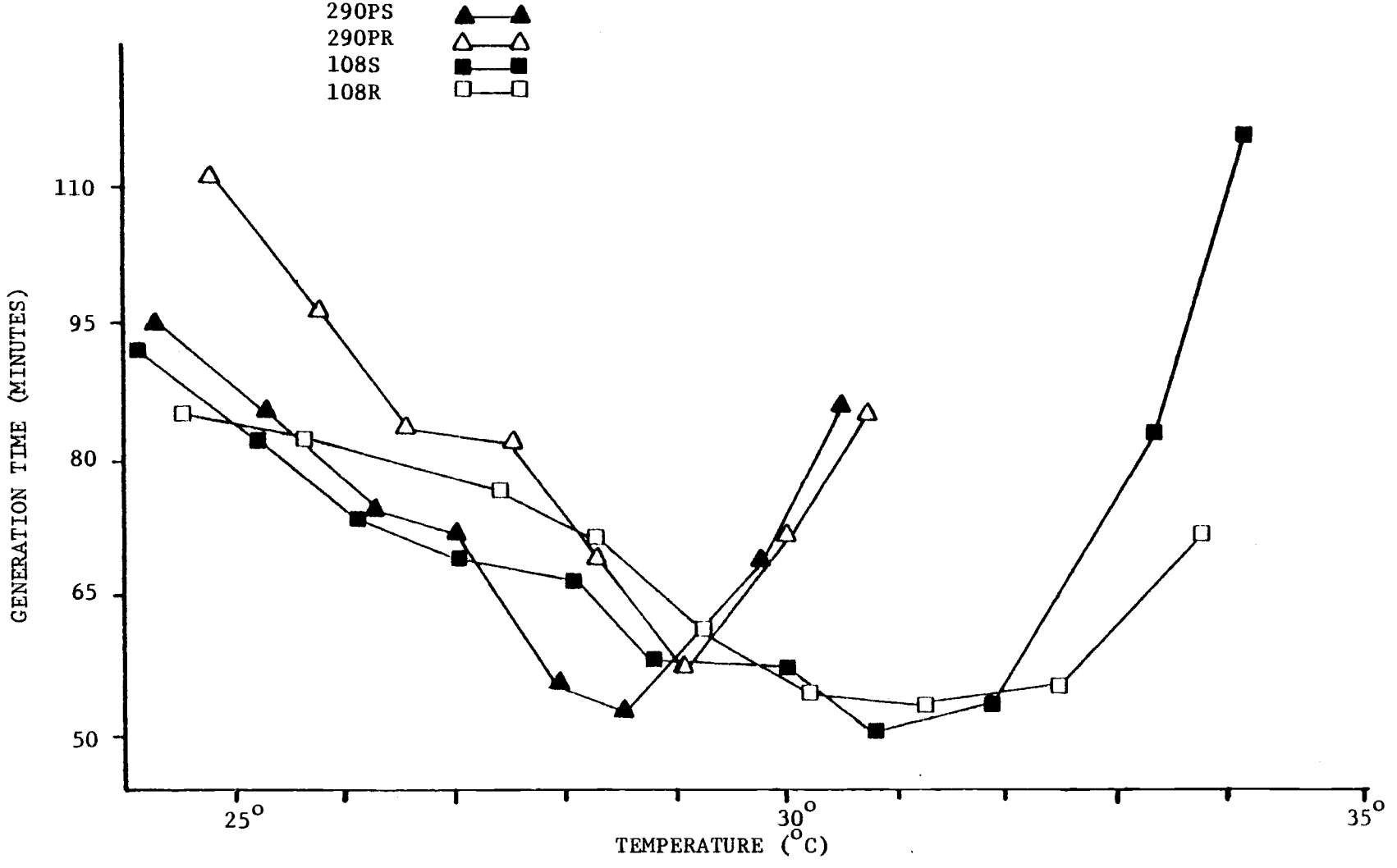


Figure 14. A comparison of the influence of temperature on generation time when grown in M17 broth.

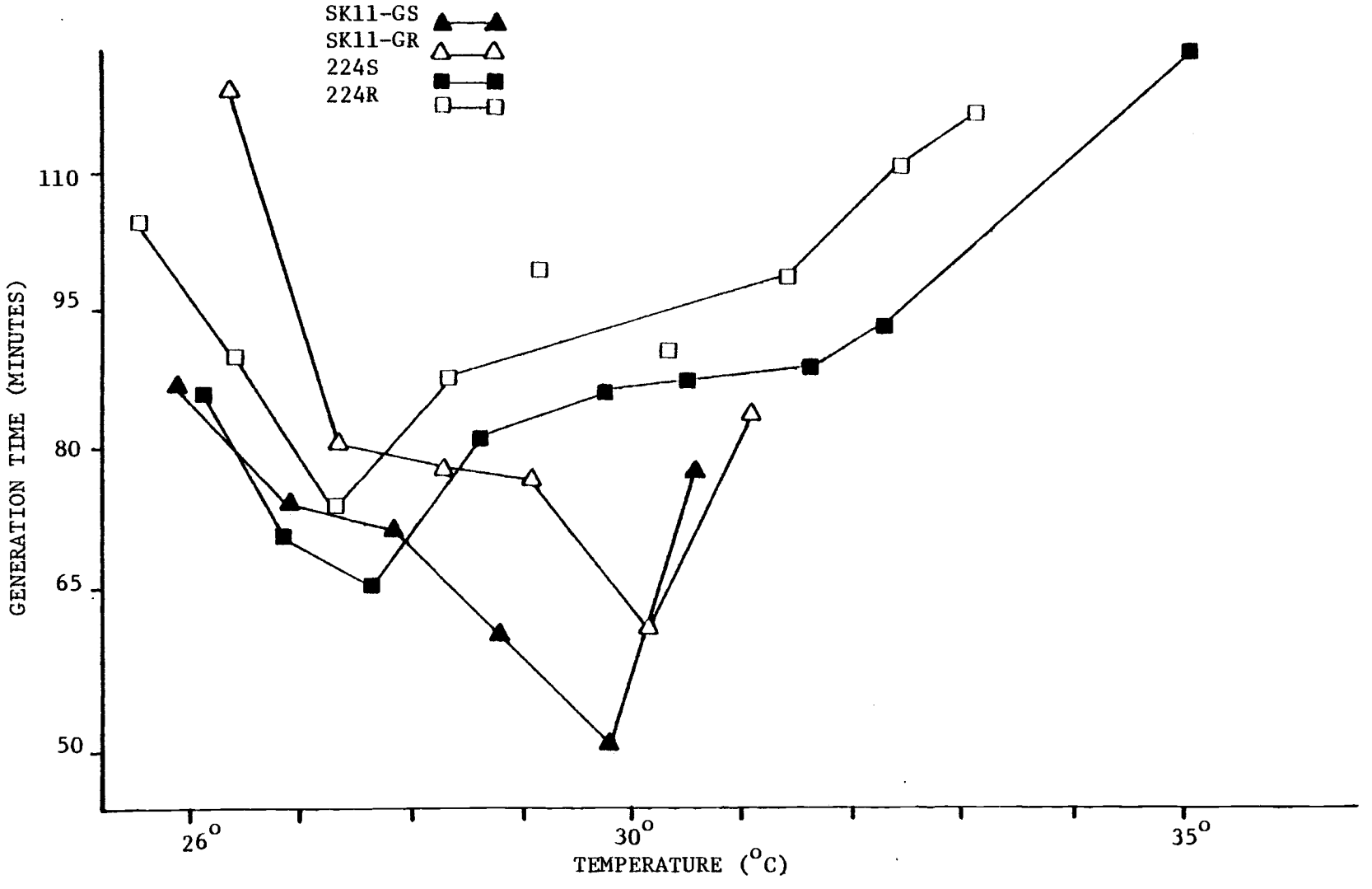
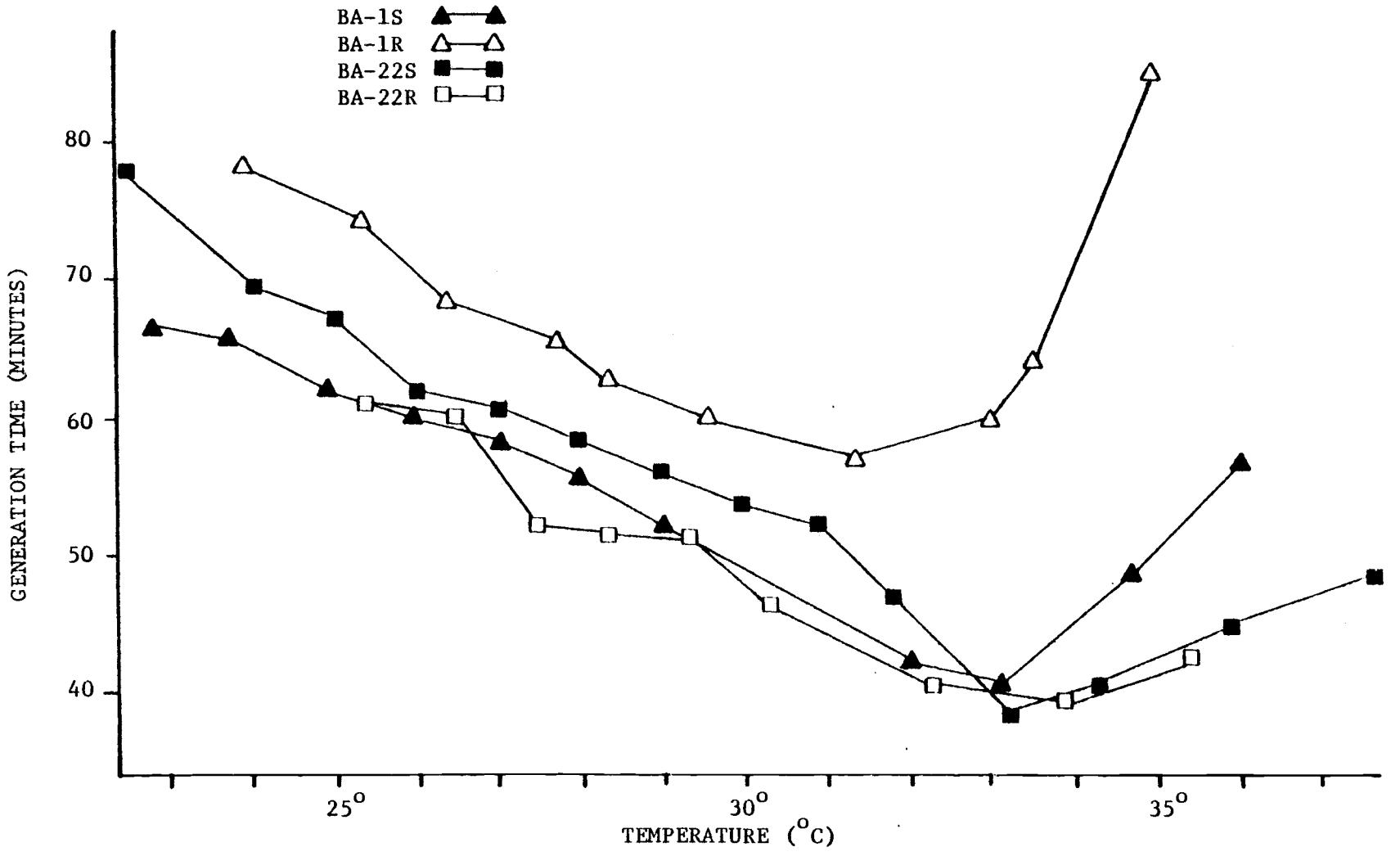


Figure 15. A comparison of the influence of temperature on generation time when grown in M17 broth.



CHAPTER II

EXAMINATION OF STRAIN INTERACTION IN A MIXED-STRAIN LACTIC STARTER CULTURE USING STREPTOMYCIN-RESISTANT MUTANTS OF LACTIC STREPTOCOCCI

ABSTRACT

Streptomycin-resistant mutants of lactic streptococci were used in a study to determine if strain dominance and suppression existed in a mixed-strain Cheddar cheese starter culture composed of two Streptococcus lactis and four Streptococcus cremoris strains. This was accomplished by growing the six strains together in six separate batches of nonfat milk and substituting a different streptomycin resistant mutant in each experiment. Samples were taken periodically and plated onto media with and without added streptomycin. In this manner, the resistant strain in each batch could be identified during growth in mixed culture. Two-strain combinations were studied similarly. Both S. lactis strains were found to significantly inhibit two of the four S. cremoris strains and one instance of S. cremoris-S. cremoris inhibition was found. Speculation regarding a dominance pattern in this starter was made.

INTRODUCTION

Strains of lactic streptococci are selected for use in cheese manufacture based on their ability to rapidly and consistently produce acid. The most common cause of failure in this regard is a result of bacteriophage infection which causes lysis of starter bacteria and subsequent reduction of acid production in the cheese vat. To minimize the possibility of this occurring, many starter cultures are composed of two or more strains with different bacteriophage sensitivities. So if one strain becomes infected and lysed by phage, another unaffected strain(s) will be present to carry out the fermentation.

When starter cultures are composed of more than one strain, the possibility exists that one strain may dominate the others (1,2,3,5,13,14). If this occurs, the starter will eventually consist of primarily that one strain after subsequent subculturing. One strain can become dominate : by producing an antibiotic (9,13); by being more acid tolerant than other strains (2); or by simply outgrowing the others (6). Compatability with regard to antibiotic production can be easily tested for in the laboratory (3). However, attempting to predict whether strains are compatable with regard to other causes of dominance is much more difficult.

Dominance has been studied in the past primarily with the use of bacteriophage infection (1,2,4,7,11,13,14). In these studies, batches of milk were inoculated with a multiple-strain starter culture and each batch infected with a different phage specific for one of the strains present. The dominant strain could then be identified by the batch

which showed the greatest inhibition of acid production. This method adequately identifies the dominate strain, but does not provide a quantitative account of the relative bacterial numbers involved. Also, with the recent introduction of phage-insensitive mutants into starter cultures, this method becomes obsolete.

This paper describes an attempt to look at dominance and strain interaction with the use of streptomycin-resistant mutants. It provides a distinct advantage in that it allows quantification of what is occurring when different strains are grown together. The streptomycin resistance serves as a marker to identify a particular strain which can be enumerated by plating on media containing streptomycin.

The primary objective of this study was to determine if dominance or suppression exists among the six strains used as starter at the Tillamook County Creamery Association (Tillamook, OR) in Cheddar cheese manufacture (Table 1). A streptomycin-resistant mutant has been isolated and characterized (see Chapter 1) for each of the six strains. The study involved growing the six strains together in six separate batches of nonfat milk (NFM) and substituting a different streptomycin-resistant mutant into each batch. Samples were then taken at various intervals and plated onto media with and without added streptomycin. In this manner we obtained a total cell count and also enumeration of each mutant. From the results we could speculate as to whether a dominance pattern or suppression existed. The basic premise is that each of the resistant strains is very similar to its parent and will behave as such in mixed culture.

The starter culture used at Tillamook is considered a mixed-strain culture in that strains are cultivated separately and not mixed until inoculation of the bulk tank occurs. So if domination takes place, it will initiate in the bulk tank. In contrast, strains in a multiple-strain starter culture are mixed before inoculation of the bulk tank which provides a greater opportunity for one strain to become dominate even when in frozen concentrate form (11).

MATERIALS AND METHODS

Media

Instant Peake nonfat milk (Galloway West Co., Fond du Lac, WI) reconstituted at 11% solids was used throughout this study. Test tubes containing ten ml of nonfat milk (NFM) were autoclaved for 12 min for use in culture maintenance. Flasks containing 500 ml of NFM were steamed for 35 min and cooled to the desired temperature before inoculation for use in strain-interaction studies. These were stored for up to five days at 4°C before discarding.

Plate counts were on M17 agar (16) in at least quintuplicate replications using a micro-droplet technique. All plates were incubated at 30°C for 24 - 48 h. Dilutions were made in 0.1% peptone (Bacto) dissolved in distilled water. A concentration of 600 µg/ml of streptomycin was used throughout. Streptomycin sulfate (Sigma) was dissolved in distilled water and sterilized by filtration through a 0.45 µ Millipore filter and was added to M17 agar after autoclaving and subsequent cooling to 50°C.

Bacterial Strains

The six strains used were obtained from the culture collection at Oregon State University and included: *S. cremoris* 224, SK11-G, 108, 290P; and *S. lactis* BA-1, and BA-22 (a bacteriophage-insensitive mutant of BA-2). All have recently been used in commercial Cheddar cheese-making at the Tillamook County Creamery Association (Tillamook, OR) and Olympia Cheese (Olympia, WA) cheese plants (Table 1). A streptomycin-resistant mutant for each strain as described in Chapter 1 was

also used. Cultures were maintained in tubes containing ten ml of NFM and stored in the unincubated condition (2% inoculum) at 4°C. These were incubated at 21°C and transferred to fresh NFM weekly. Each culture was purified by streaking onto Fast-Slow Differential Agar (10) and subsequent selection of a fast colony type before use in strain-interaction studies. All inocula used in these studies were from a freshly coagulated culture grown at 21°C. Each streptomycin-resistant mutant is designated by R (e.g. 108R), for resistant, and each parent by S (e.g. 108S), for sensitive.

Plating Comparison

A plating comparison of the mutant strains in the absence and presence of streptomycin was made. Tubes containing ten ml of autoclaved NFM were inoculated with a 1% inoculum and incubated for 16 h at 21°C. Samples were taken, appropriately diluted in sterile diluent, and plated onto M17 agar both with and without added streptomycin.

Strains SK11-GR and 224R were subjected to a more elaborate plating comparison. This involved use of the procedure described under strain-interaction studies and inoculum size was 1%.

Strain-Interaction Studies

Flasks containing 500 ml of steamed NFM were inoculated and incubated at 27°C. This temperature was chosen to simulate bulk-tank conditions at Tillamook where bulk starter is incubated at 80°F (26.7°C). Also, Lee and Collins (12) suggest that difference in generation time would be less of a factor in strain domination at this temperature. Cell counts were made at two-h intervals from time of

inoculation until ten h. Plates of M17 agar with and without added streptomycin were used to obtain a total count and also enumeration of the resistant mutant. Acidity readings were made with a pH meter (Corning) on a hourly basis to monitor progress of the fermentation.

For studies dealing with the six strain combination, a 0.2% (1 ml) inoculum of each strain was used giving a total inoculum of 1.2% (6 ml). Six flasks were inoculated, each with a different streptomycin-resistant mutant and the remaining five parent strains. A seventh flask was inoculated with all six parent strains to serve as a control. In this manner, the growth characteristics of each individual strain when grown in mixed culture could be observed.

For studies dealing with two-strain combinations, a 1% (5 ml) inoculum of each strain was used giving a total inoculum of 2% (10 ml).

RESULTS

Plating Comparison

Table 2 contains the results of the plating comparison. There was no significant difference with any of the six resistant strains regarding plate counts on media with and without added streptomycin. It is important that added streptomycin did not increase or decrease the number of countable colonies so that accurate results are obtained when the resistant strains are enumerated in strain-interaction studies.

Strain-Interaction Studies

Table 3 shows the total cell numbers achieved when six-strain combinations were grown in NFM. Each of the seven batches was highly similar with regard to total cell count and change in pH. This suggests that conditions were very similar from batch to batch even though each batch contained a different streptomycin-resistant mutant.

Figure 1 portrays the growth of the streptomycin-resistant mutant in each of the six batches. Each of the resistant strains followed a similar pattern of growth except for SK11-GR and 224R. With the exception of these two, this data is consistent with that in Chapter 1 which describes the growth of these organisms under similar conditions in single-strain cultures.

Strains SK11-GR and 224R are different in that they showed a significant drop-off in countable colonies at the eight-h reading. A drop-off was also apparent with 108R but was much less significant. These cells could not have died as there was a two-three log recovery by the

ten-h reading. Identical results were obtained when this experiment was repeated.

In the characterization of these mutants (Chapter 1) a drop-off as such was not observed when SK11-GR and 224R were grown in NFM by themselves. However, these cells were plated onto M17 agar without streptomycin. It was thought that perhaps exposure to a low pH somehow reduced their ability to form colonies on media containing streptomycin. To disprove this, these strains were subjected to a more elaborate plating comparison as described in Materials and Methods. Figure 2 shows the subsequent results and indicates that no such effect existed.

It was then obvious that this drop-off was due to strain interaction. Both SK11-GR and 224R were then grown in two-strain combinations with each of the other parent strains to find out which strains were responsible for this inhibition. Tables 4 and 5 display the total count data for this experiment and show that the fermentations were fairly similar with regard to growth and acid production. However, Figures 3 and 4 illustrate that the two S. lactis strains (BA-1S and BA-22S) were responsible for inhibition of both SK11-GR and 224R. When grown in two-strain combinations as such, this phenomenon was more pronounced at the four-and six-h readings than in the six-strain combination. Also, strain 290PS appeared to inhibit 224R at the eight- and ten-h readings. This was not observed in the six-strain combination study as it was masked by the inhibition due to BA-1S and BA-22S. Table 6 and Figure 5 show the results of a similar study conducted with 108R. In this case only BA-1S caused inhibition whereas BA-22S did

not, although this suppression was very insignificant by comparison. All of these experiments were repeated and good correlation was found.

DISCUSSION

The cause of this drop-off phenomenon observed with strains SK11-GR, 224R, and to a lesser extent 108R, has not been positively identified. Only possibilities can be speculated. However, it was a result of strain interaction as it occurred only when certain strains were grown together. The most likely explanation is that the two S. lactis strains produced a metabolite (antibiotic) which somehow injured the SK11-GR and 224R strains so that they could not grow and produce colonies on the plating medium. They were, however, not killed as rapid recovery followed. Perhaps this period of low plating efficiency reflected the time needed for activation of an inducible enzyme system which could breakdown the metabolite and destroy its inhibitory effect. If this was the case, the enzyme was more rapidly induced in strain 108R as recovery occurred much more quickly. Or perhaps the metabolite was eventually inactivated by the gradual reduction in pH of the milk culture which allowed normal growth of the inhibited strains to resume. Collins (3) showed that both pH and age of culture had an effect on antibiotic production and/or susceptibility by lactic streptococci. At any rate, it is not known whether this same susceptibility exists with strains SK11-GS, 224S, and 108S. Strain 290PR was not affected by the inhibitory factor(s) produced by the two S. lactis strains. Whereas, 108R was only affected when grown with BA-1S and not BA-22S. This indicates that either BA-1S produced more of the substance than BA-22S, or each produced a distinctly different substance.

Strain 290PS also seemed to produce a substance which inhibited 224R but not SK11-GR. In this case the drop-off occurred much later during the course of the fermentation and it cannot be determined from the data (Figure 4) whether or not recovery ever occurred. This inhibition was more gradual and not as dramatic as that caused by the two S. lactis strains. It should be noted that none of these inhibitions were observed when the involved strains were crossed streaked onto M17 agar plates and incubated at 30°C for 24 h, a technique which is commonly used to determine if strains are compatable for use in cheesemaking.

If these phenomena do indeed occur in the actual Tillamook starter culture, strain 224S will be very rapidly dominated by BA-1S, BA-22S, and 290PS. When BA-1S was grown in a two-strain combination with 224R, the ratio of viable BA-1S cells to 224R cells at the end of the fermentation was 19:1. Similarly, the ratio of BA-22S to 224R was 27:1, and the ratio of 290PS to 224R was 56:1. If a bulk-starter tank were inoculated with any of these three pairs and a similar imbalance occurred, what was an inoculum of two strains is reduced essentially to one strain at the time of cheese vat inoculation. Although SK11-GR was similarly inhibited, its recovery by the end of the fermentation was greater than that of 224R. The final ratios of BA-1S and BA-22S to SK11-GR were 3:1 and 6:1 respectively.

Past studies (3,13) have reported that neither S. lactis nor S. cremoris strains have a tendency to dominate over the other species when grown in mixed culture. Another study (7) reported that blends of

S. lactis and S. cremoris were frequently more stable than those consisting of S. cremoris strains alone. This study obviously provides evidence to the contrary. Both S. lactis strains caused significant inhibition of two out of the four S. cremoris strains tested when grown in mixed culture.

Observation of Figure 1 reveals that three basic echelons of growth existed at the ten-h sampling. Strains 290PR and 108R achieved the highest cell numbers at this time. Strains BA-22R and BA-1R were in the second echelon somewhat lower, and strains SK11-GR and 224R achieved the lowest numbers with 224R being the absolute lowest. With respect to 224R, the ratios of each of the other strains were : 290PR, 13.3 : 1; 108R, 12.7 : 1; BA-22R, 4.5 : 1; BA-1R, 4.1 : 1; and SK11-GR, 1.9 : 1. If these values are an accurate reflection of the dominance pattern in this starter culture, several subcultures would probably result in elimination of all strains except 290PS and 108S. Hence, in a long term sense these would have to be considered the dominate strains. Since these strains are not cultured together until inoculation of the bulk tank, though, all strains would be present in the bulk inoculum used to inoculate the cheese vat. However, the predominate strains would be 290PS, 108S, BA-1S, and BA-22S.

The relative percentages of each strain at the time of cheese vat inoculation were estimated by dividing the final cell number for each strain (Figure 1) by the summation of all six final cell numbers and multiplying by 100. These values were : 290PR, 35.5%; 108R, 33.7%; BA-22R, 11.9%; BA-1R, 11.0%; SK11-GR, 2.6%; and 224R, 1.2%. So essentially, a six-strain starter has been reduced to four

strains. It should be noted that this represents a hypothetical situation since NFM is not used for a growth medium in the bulk tank. A completely different dominance pattern could exist in other media (9) such as neutralized whey, Phase 4, etc...

The dominance pattern developed in this study can be explained in terms of the three common causes mentioned in the introduction. Again referring to Figure 1, it appears that 290PR, 108R, and BA-22R grew rapidly and consistently, and were not significantly inhibited by metabolites of other strains. However, the cell number of BA-22R tailed off at the end of the fermentation which indicates that it might be less acid tolerant (2). Strain BA-1R appeared to grow slower than the others and could potentially be dominated on this basis (13). And as discussed before, SK11-GR and 224R were susceptible to metabolites produced by other strains in the combination (13).

Inhibition and stimulation of acid production by the associative growth of two or more strains of lactic streptococci and detection of antibiotic production have been reported in the literature many times (3,5,6,8,9,14,15). However, a quantitative monitoring of fluctuation in cell concentration of a particular strain during associative growth, as such, has not previously been reported. Any speculation with regard to a dominance pattern made based on the results of this study assumes that the parent strains perform similarly to the mutants in mixed culture. The data in Chapter 1 indicates that parents and mutants perform very similarly when grown singly, but they are nevertheless

not identical. Results of this study should be substantiated with the traditional method of assessing dominance (by bacteriophage infection) once a specific, lytic phage for each strain is obtained. If good correlation is found, perhaps this technique could be used to construct mixed-strain combinations for use in commercial Cheddar cheese manufacture to minimize interactive effects such as strain dominance and suppression.

LITERATURE CITED

1. Collins, E. B. 1955. Action of bacteriophage on mixed strain cultures. III. Strain dominance due to the action of bacteriophage and variations in the acid production of secondary growth bacteria. Appl. Microbiol. 3:137-140.

2. Collins, E. B. 1955. Action of bacteriophage on mixed strain cultures. IV. Domination among strains of lactic streptococci. Appl. Microbiol. 3:141-144.

3. Collins, E. B. 1961. Domination among strains of lactic streptococci with attention to antibiotic production. Appl. Microbiol. 9:200-205.

4. Czulak, J., and L. A. Hammond. 1954. Compatibility amongst strains of lactic streptococci. Aust. J. Dairy Technol. 9:15-18.

5. Dahiya, R. S., and M. L. Speck. 1962. Symbiosis among lactic streptococci. J. Dairy Sci. 45:607-612.

6. Dahiya, R. S., and M. L. Speck. 1963. Identification of stimulatory factor involved in symbiotic growth of Streptococcus lactis and in Streptococcus cremoris. J. Bacteriol. 85:858-589.

7. Gilliland, S. E. 1971. Strain balance of multiple strain lactic streptococcus concentrated cultures. J. Dairy Sci. 54:1129-1133.

8. Greene, V. W. 1959. Interaction among pure strains of lactic streptococci isolated from multiple strain cheese starters. J. Dairy Sci. 42:906. M50.

9. Hoyle, M. and A. A. Nichols. 1948. Inhibitory strains of lactic streptococci and their significance in the selection of cultures for starter. J. Dairy Res. 15:398-408.

10. Huggins, A. R., and W. E. Sandine. 1979. Differentiation of fast and slow milk-coagulating isolates of lactic streptococci. Proceedings, 74th Annual Meeting. Am. Dairy Science Association, p. 70-71.

11. Leach, R. D., and W. E. Sandine. 1976. Numerical relationship between strains in frozen concentrates of lactic streptococcal starter cultures. J. Dairy Sci. 59:1392-1397.

12. Lee, D. A., and E. B. Collins. 1976. Influences of temperature on growth of Streptococcus cremoris and Streptococcus lactis. J. Dairy Sci. 59:405-409.

13. Lightbody, L. G., and L. J. Meanwell. 1955. The growth of lactic acid streptococci in mixed starter cultures. J. Appl. Bacteriol. 18:53-65.

14. Nichols, A. A., and P. J. Ineson. 1948. Cheese starter 'recovery' after attack by bacteriophage. (Strain dominance in multiple strain starters). J. Dairy Res. 15:99-111.

15. Olson, H. C. 1960. Symbiosis in lactic cultures. J. Dairy Sci. 43:439.

16. Terzaghi, B. E., and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29:807-813.

Table 1. Strains of lactic streptococci used in mixed culture at the Tillamook County Creamery Association (Tillamook, OR) and Olympia Cheese (Olympia, WA) cheese plants.

Tillamook	Olympia
S. lactis BA-1	S. cremoris 290P
S. lactis BA-2	S. cremoris 108
S. cremoris 108	S. cremoris C13
S. cremoris 290P	S. cremoris 224
S. cremoris 224	S. cremoris SK11-G
S. cremoris SK11-G	S. cremoris R6

Table 2. A comparison of plating the streptomycin-resistant mutants on media with and without added streptomycin.

Strain	Log_{10} cfu/ml: Plate count on media without streptomycin	Log_{10} cfu/ml: Plate count on media containing streptomycin
290PR	8.89	8.79
108R	9.23	9.20
SK11-GR	9.32	9.32
224R	9.15	9.20
BA-1R	9.08	9.11
BA-22R	9.26	9.34

Table 3. A comparison of cell numbers achieved and acid production in various batches of NFM each containing a different streptomycin-resistant mutant in addition to the other five parent strains. Batch No. 7 contained all of the parent strains to serve as a control. These values represent a total count as plated on media without streptomycin.

Log_{10} cfu/ml

Time (h)	#1 290PR*	#2 108R*	#3 SK11-GR*	#4 224R*	#5 BA-1R*	#6 BA-22R*	#7 Control
0	7.11	7.23	7.08	7.04	7.08	7.08	7.08
2	7.42	7.40	7.45	7.40	7.43	7.38	7.42
4	8.20	8.08	8.26	8.00	8.11	8.20	8.18
6	8.38	8.40	8.42	8.36	8.40	8.42	8.36
8	8.82	8.72	8.83	8.86	8.89	8.86	8.87
10	9.04	8.98	9.04	9.11	8.98	9.00	8.96
Initial pH	6.59	6.59	6.59	6.59	6.59	6.59	6.59
Final pH	2.02	1.99	2.03	2.02	2.03	2.03	2.01
Δ pH	2.02	1.99	2.03	2.02	2.03	2.03	2.01

*Denotes which streptomycin-resistant mutant used

Table 4. A comparison of cell numbers achieved and acid production in NFM when SK11-GR was grown in combination with each of the other strains. These values represent a total count of both strains in the combination as plated on media without streptomycin.

	Log_{10} cfu/ml				
Time (h)	290PS*	108S*	224S*	BA-1S*	BA-22S*
0	7.20	7.08	7.08	7.30	7.26
2	7.60	7.51	7.87	7.60	7.82
4	8.26	8.08	8.34	8.11	8.20
6	8.69	8.53	8.62	8.32	8.95
8	8.97	8.84	8.79	8.89	9.04
10	9.08	8.82	9.20	8.91	9.00
Initial pH	6.44	6.44	6.43	6.49	6.49
Final pH	4.49	4.48	4.54	4.60	4.62
Δ pH	1.95	1.96	1.89	1.89	1.89

* Denotes which strain was paired with SK11-GR.

Table 5. A comparison of cell numbers achieved and acid production in NFM when 224R was grown in combination with each of the other strains. These values represent a total count of both strains in the combination as plated on media without streptomycin.

	Log_{10} cfu/ml				
Time (h)	290P*	108S*	SK11-GS*	BA-1S*	BA-22S*
0	6.99	7.04	7.18	6.98	7.11
2	7.65	7.97	7.97	7.89	7.87
4	8.32	8.26	8.11	7.96	8.15
6	8.63	8.62	8.75	8.28	8.49
8	8.99	9.04	9.04	8.63	9.00
10	8.87	8.92	9.04	9.04	9.11
Initial pH	6.50	6.45	6.46	6.49	6.49
Final pH	4.56	4.35	4.39	4.68	4.75
Δ pH	1.94	2.10	2.07	1.81	1.74

*Denotes which strain was paired with 224R.

Table 6. A comparison of cell numbers achieved and acid production in NFM when 108R was grown in combination with each of the other strains. These values represent a total count of both strains in the combination as plated on media without streptomycin.

Time (h)	Log_{10} cfu/ml 290PS*	SK11-GS*	224S*	BA-1S*	BA-22S*
0	7.36	7.26	7.23	7.23	7.26
2	7.85	7.78	7.79	7.86	7.72
4	8.34	8.26	8.32	8.18	8.18
6	8.89	8.54	8.73	8.76	8.59
8	9.08	8.84	9.04	8.94	8.70
10	9.08	8.86	9.18	9.14	8.83
Initial pH	6.64	6.64	6.64	6.63	6.64
Final pH	4.56	4.56	4.68	4.59	4.59
Δ pH	2.08	2.08	1.96	2.04	2.05

*Denotes which strain was paired with 108R.

Figure 1. A comparison of cell numbers achieved when grown at 27° C in NFM using six-strain combinations. Each line represents enumeration of a different streptomycin-resistant mutant grown in combination with five parent strains.

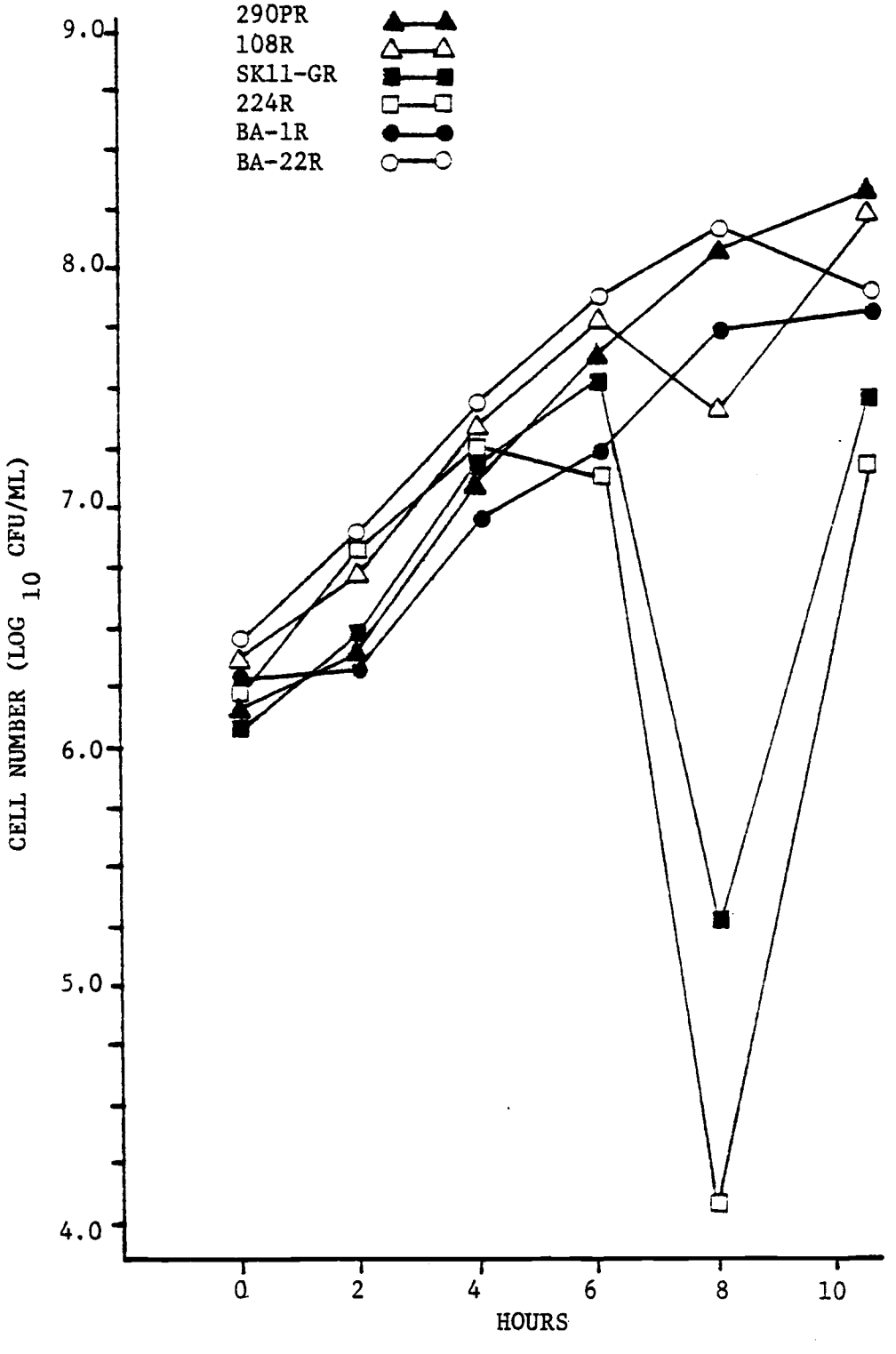


Figure 2. A comparison of plating on media with and without added streptomycin.

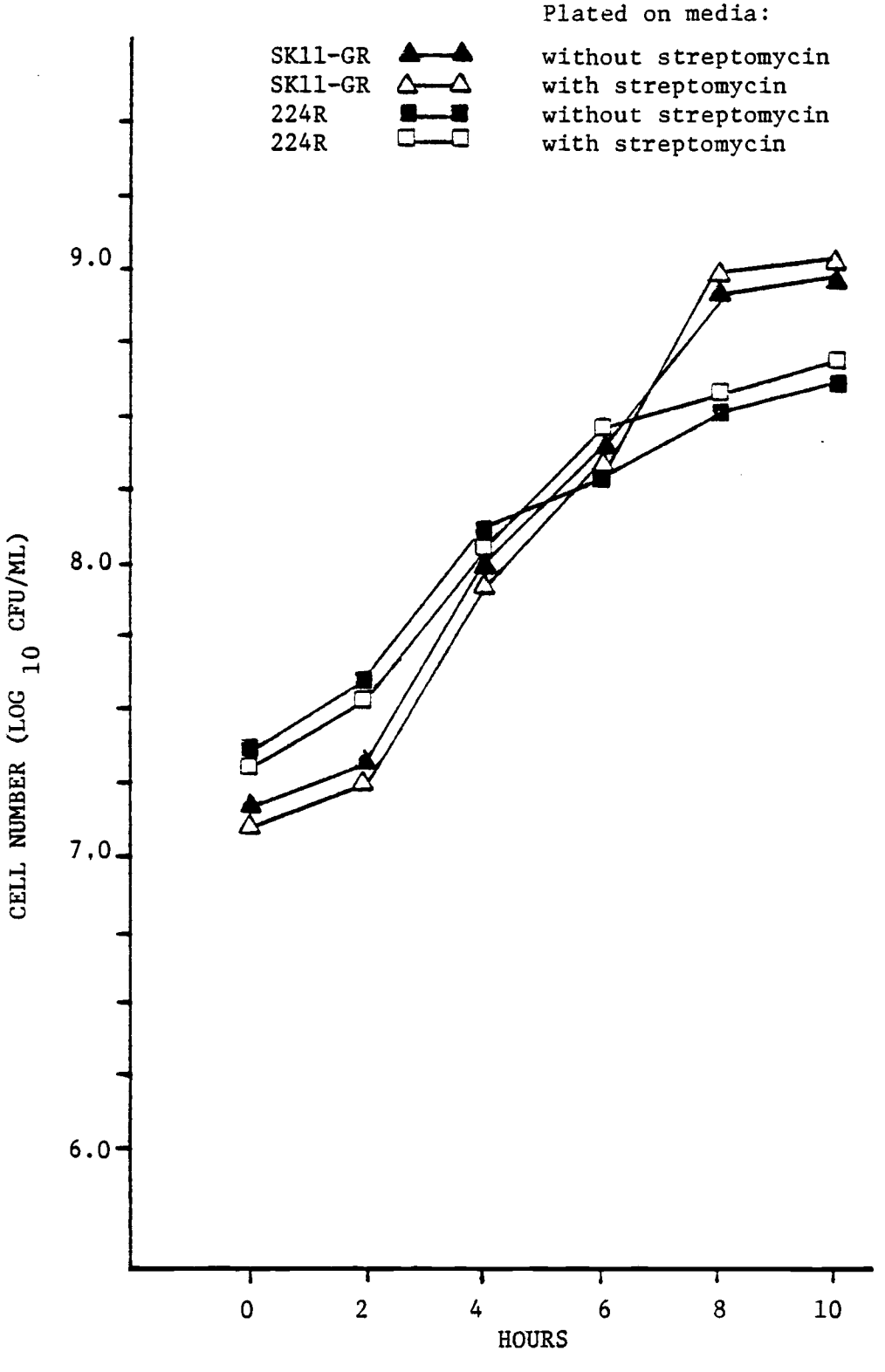


Figure 3. A comparison of cell numbers achieved when grown at 27° C in NFM using two-strain combinations. Each line represents enumeration of SK11-GR when grown in combination with:

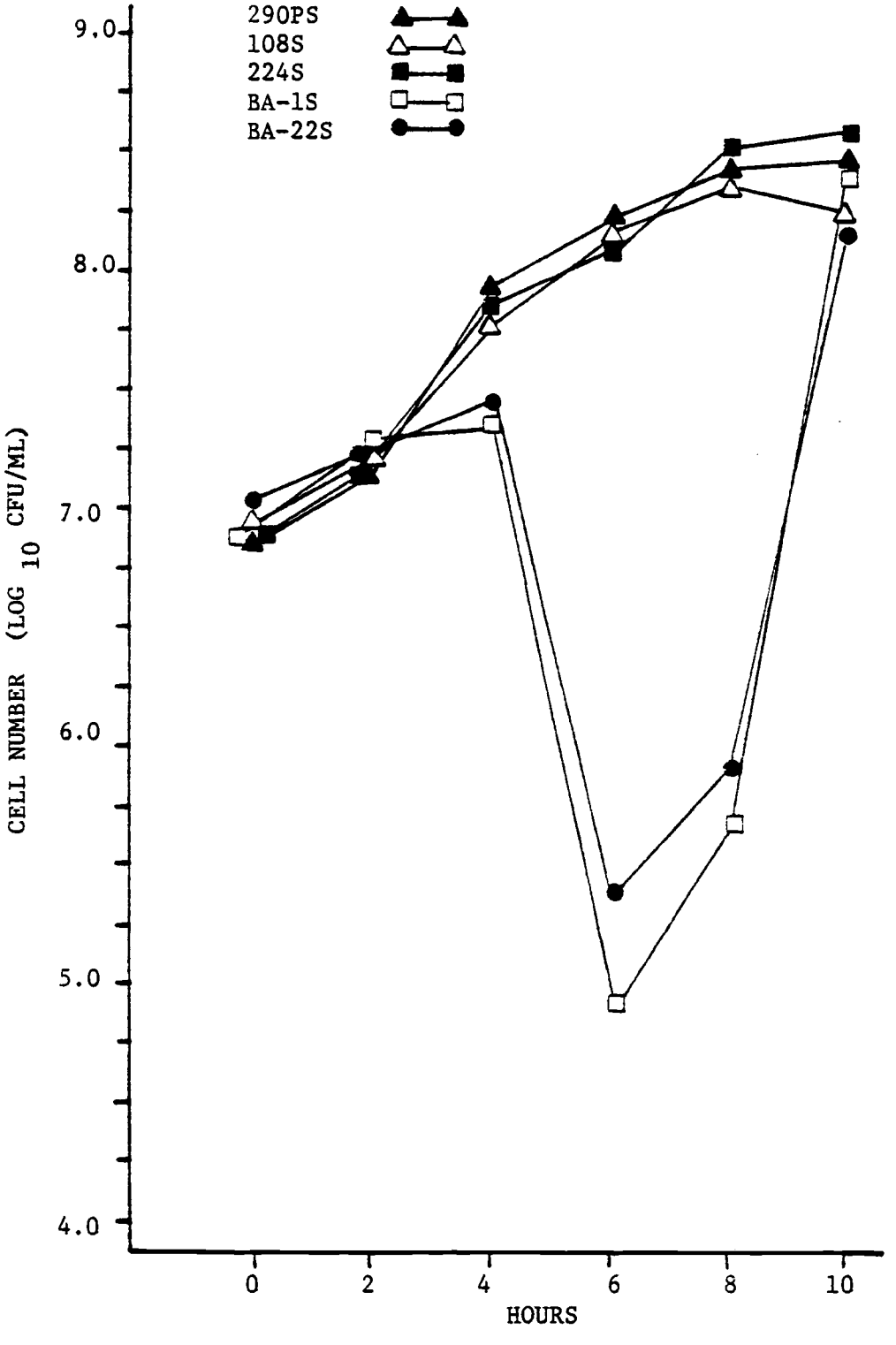


Figure 4. A comparison of cell numbers achieved when grown at 27° C in NFM using two-strain combinations. Each line represents enumeration of 224R when grown in combination with:

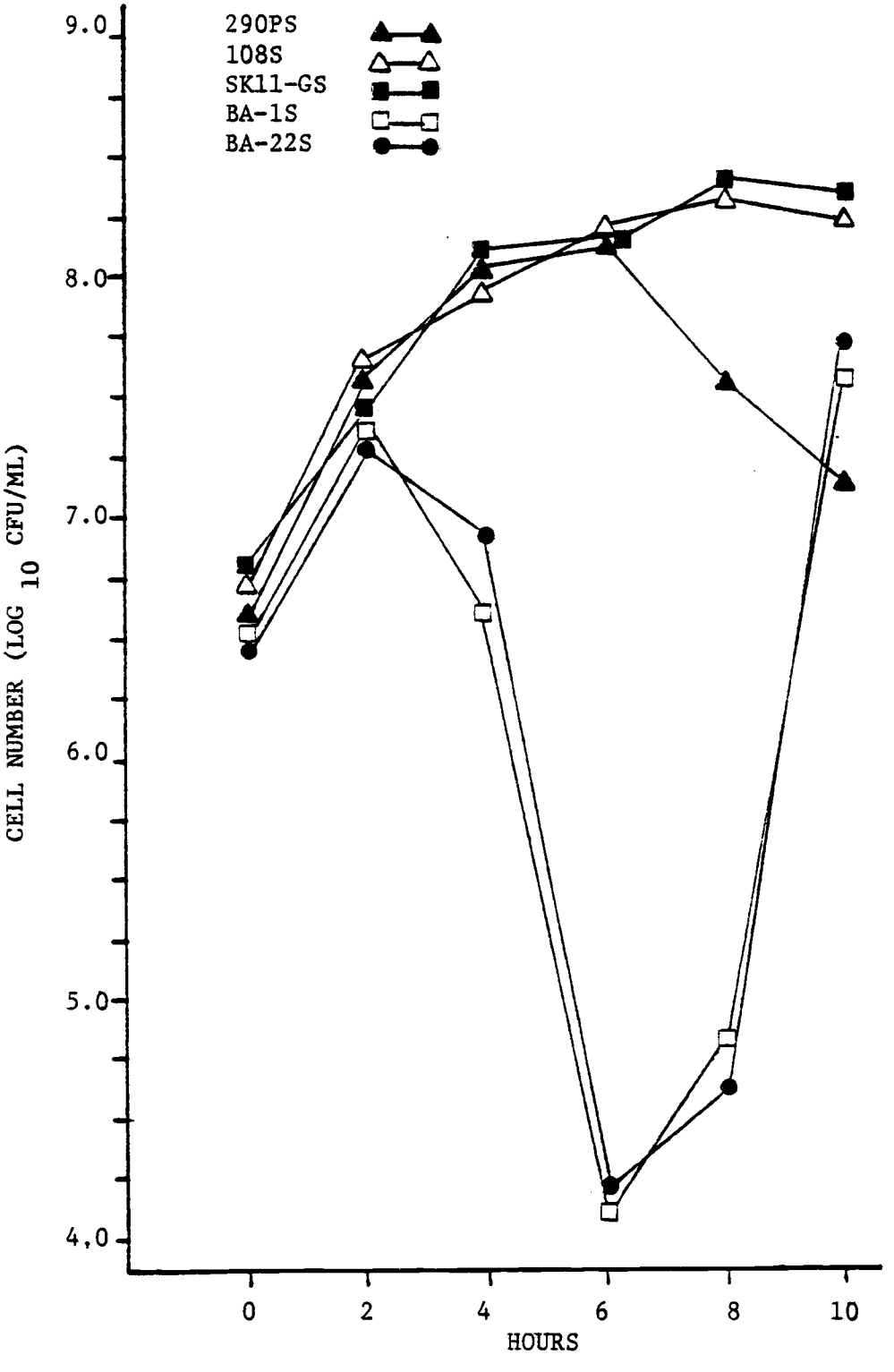
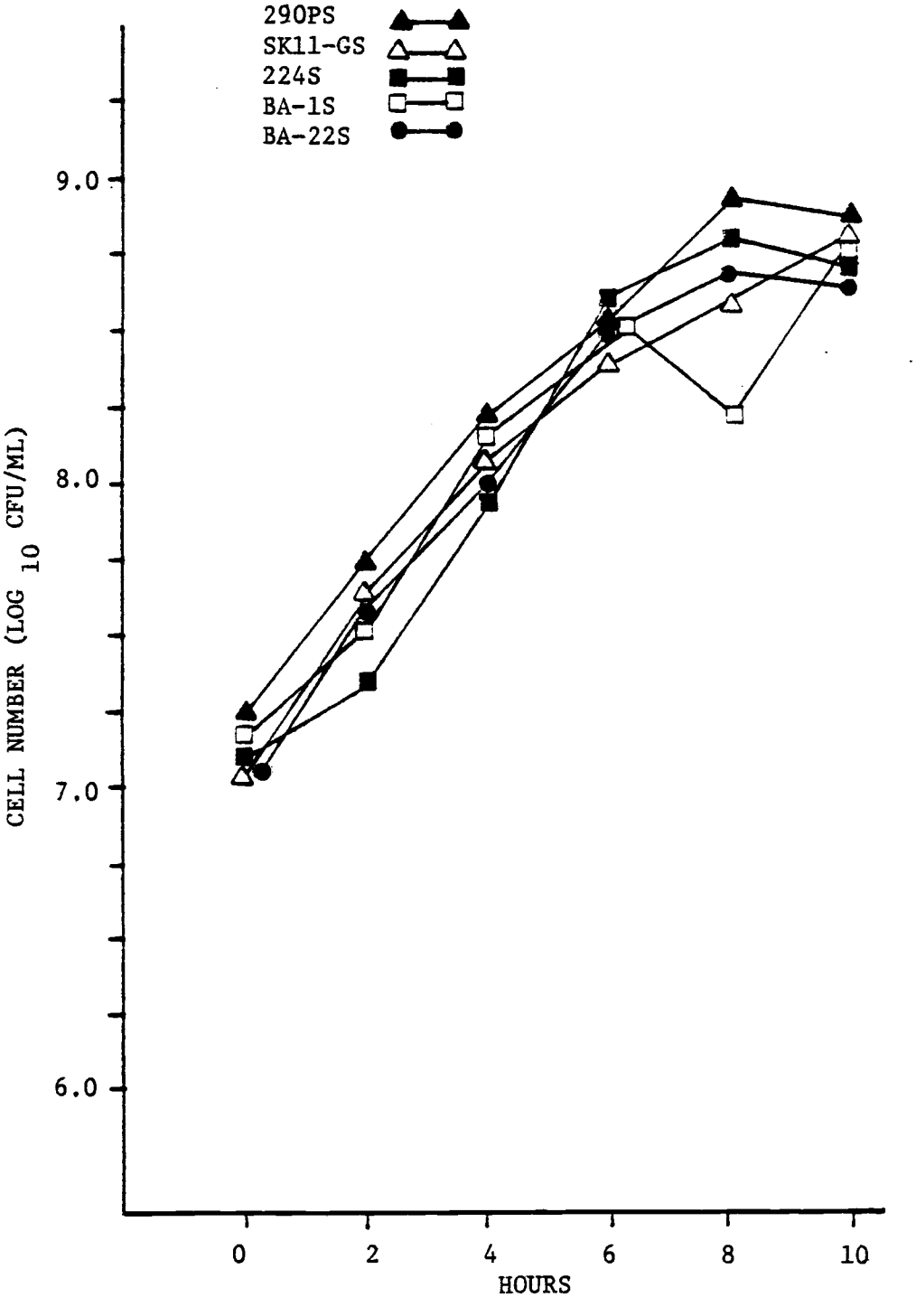


Figure 5. A comparison of cell numbers achieved when grown at 27° C in NFM using two-strain combinations. Each line represents enumeration of 108R when grown in combination with:



CHAPTER III

PLASMID PROFILES OF PHAGE- AND STREPTOMYCIN-INSENSITIVE MUTANTS OF *STREPTOCOCCUS LACTIS*

ABSTRACT

The plasmid profiles of Streptococcus lactis strains BA-1, BA-2, S1E, and ML8 and phage-insensitive mutants of each were compared. The profiles of parents and mutants for strains BA-1, BA-2, and S1E proved to be similar. However, two ML8 mutants examined possessed one more band than their parent strain. Removal of this plasmid by curing with ethidium bromide did not result in a loss of phage insensitivity. Also, the plasmid profiles of streptomycin-resistant mutants of strains BA-1 and BA-2 were examined and were found to be similar to that of their parents.

INTRODUCTION

Extrachromosomal DNA has been shown to play an important role in the lactic streptococci. Recent evidence illustrates that plasmid DNA in Streptococcus lactis is associated with such metabolic functions as lactose (1,3,14) and galactose metabolism (16), proteinase activity (3,14), and citrate utilization in S. lactis subsp. diacetylactis (12). Proteinase activity has also been correlated with plasmid DNA in S. cremoris (15). Other reports indicate that plasmids may be responsible for glucose and mannose metabolism (17), production of the antibiotic nisin (7), and resistance to inorganic ions in S. lactis (4).

A recent study (6) reported that a plasmid is responsible for resistance to a particular bacteriophage in a strain of S. faecalis subsp. zymogenes. This is very interesting in view of the fact that phage-insensitive mutants are currently being used in the commercial manufacture of Cheddar cheese. Although these are laboratory derived which suggests that they are chromosomal mutants (18), perhaps plasmid DNA could be responsible for insensitivity in some isolates.

It was the purpose of this investigation to compare plasmid profiles of several phage-insensitive mutants of S. lactis with that of their parent strains. With a similar objective in mind, the plasmid profiles of laboratory-derived streptomycin-resistant mutants of S. lactis (see Chapter 1) were also examined. Due to the poor lysing quality of S. cremoris strains, profiles of S. cremoris mutants were not conducted.

MATERIALS AND METHODS

Bacterial Strains

The four parent strains used were obtained from the culture collection at Oregon State University and included S. lactis strains S1E, BA-1, BA-2, and ML8. The latter three have recently been used in commercial Cheddar cheesemaking. Phage-insensitive mutants were obtained for all strains by either whey adaption or direct selection and were isolated by various personnel in our laboratory. The streptomycin-resistant mutants were obtained by single-step exposure to streptomycin at a level of 600 µg/ml (Chapter 1). A total of eight different phage-insensitive mutants were examined and included three derived from strain BA-1, two each derived from strains BA-2 and ML8, and one derived from strain S1E. These were designated BA-11, BA-12, BA-13, BA-21, BA-22, ML81, ML82, and S1E-1, respectively. Also, strain ML82 was treated with ethidium bromide (EB) and two isolates were obtained and studied. These were designated ML82EB1 and ML82EB2. Two streptomycin-resistant mutants were examined; these were derived from strain BA-1, BA-22 (a phage-insensitive mutant of BA-2) and were designated BA-1R and BA-22R, respectively. All cultures were maintained in tubes containing ten ml of M17 broth (22) which were stored in the unincubated condition (2% inoculum) at 4°C. Cultures were incubated at 30°C for 16 h and transferred to fresh broth weekly. Each culture was purified by streaking onto Fast-Slow Differential Agar (FSDA) (10) and subsequent selection and culturing of a fast colony type before use in this investigation.

Escherichia coli K-12 substrains J5, J52, and C600, containing plasmids RP4, Sa, and RSF 1010, respectively, were provided by L. L. McKay (Dept. of Food Science and Nutrition, Univ. of Minnesota, St. Paul, MN). These plasmids, having molecular weights (MW) of 34, 23, and 5.5 megadaltons (Mdal), respectively, served as mobility reference standards during agarose gel electrophoresis. Each has been characterized previously (2,5,9). All substrains were maintained in M17 broth (5% inoculum) and frozen at -80°C.

Isolation of Plasmid DNA

Plasmid DNA was isolated according to the method described by Klaenhammer et al. (13). (The composition of all buffers and a detailed description of the entire procedure are contained in this reference). A 1% inoculum of overnight M17 broth culture was made into tubes containing 40 ml of lysis broth (13) and incubated at 30°-32°C for four h (to allow cells to reach late log phase). The cells were harvested by centrifugation and washed in ten ml of TES buffer, then recentrifuged and suspended in one ml of sucrose buffer. This suspension was vortexed and treated with lysozyme (Sigma) at a rate of 2 mg/ml, followed by incubation at 37°C for six min. After addition of 0.4 ml of 0.25 M ethylenediaminetetraacetate (EDTA) (Sigma), lysis was effected by addition of 0.4 ml of 5% sodium dodecyl sulfate (Fisher) and gentile mixing. Subsequently, the lysate was treated with 0.7 ml of 5 M NaCl and cooled to 4°C in an ice bath, followed by centrifugation at 27,000 x g for 30 min at 4°C. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the cleared lysate and gently agitated. After cooling in an

ice bath for five min, the chloroform-treated lysate was centrifuged at 15,000 x g for ten min and the aqueous phase carefully removed. This was treated with one-tenth volume of 3 M sodium acetate solution and two volumes of cold 95% ethanol, then frozen overnight at -20°C. The following morning the ethanol precipitated DNA was centrifuged at 12,000 x g for 20 min at -10°C, resuspended in 0.1 ml of TES buffer, and treated with ten µl of a 1 mg/ml ribonuclease solution (Ribonuclease A, Sigma), followed by incubation at 37°C for one h. The DNA sample was analyzed immediately by gel electrophoresis or frozen at -20°C until examined.

For the E. coli K-12 substrains, tubes containing 40 ml of brain heart infusion broth (Difco) were inoculated with a 1% inoculum of overnight broth culture and incubated at 37°C for 10-12 h. These were lysed according to the procedure described by Meyers et al. (20) as modified by Klaenhammer (13) for use in electrophoresis.

Agarose Gel Electrophoresis

Electrophoresis was carried out as described by Meyers (20) as modified by Klaenhammer (13) on a vertical slab gel (13.8 x 16.2 x 0.3 cm) at 40 mA (constant current) for three h. Ten to 20 µl samples of prepared DNA were loaded onto the gel along with ten µl of indicator dye (20). Agarose (SeaKem, Marine Colloids Inc.) was dissolved in Tris-borate buffer (89 mM Tris, 2.5 mM disodium EDTA, and 89 mM boric acid, pH 8.0) at a concentration of 0.65%. Following electrophoresis, each gel was stained in 5 µg/ml solution of EB (Sigma) for one h and destained in distilled water overnight before being photographed. Gels were illuminated by placing them on an ultraviolet light box (Ultra-

violet Products Inc.) and photographed with a stationary Polaroid camera (model MP-3) fitted with an orange filter (Tiffen, #16). The camera settings were f/4.7 and 0.25 sec using Polaroid Type 47 film.

Curing of Plasmid DNA with EB

Strain ML82 was grown in the presence of EB to cure it of plasmid DNA. EB was dissolved in distilled water and sterilized by filtering through a 0.45 μ Millipore filter and added to tubes containing ten ml of M17 broth to obtain a final concentration of 2×10^{-5} M. These were inoculated with a 2% inoculum of overnight broth culture and incubated at 30°C for 24 h. Samples were removed, appropriately diluted in 0.1% peptone (Bacto) dissolved in distilled water, spread plated onto FSDA plates, and incubated anaerobically (BBL Gas Pak) for 48 h. Several injured colony types (see Chapter 4) were selected, subcultured, lysed, and examined by electrophoresis to determine if loss of plasmid DNA occurred.

Phage Sensitivity

Tubes containing three ml of melted M17 overlay agar (22) with 0.15% bromcresol purple were cooled to 46°C and inoculated with 0.1 ml of overnight broth culture. Also, one drop of sterile $CaCl_2$ was added to each. These were poured onto the surface of prepoured M17 agar plates (22), allowed to harden, and spotted with one drop (ca. 0.05 ml) of bacteriophage preparation. The plates were incubated at 30°C and examined for plaque formation at 24 and 48 h.

RESULTS

Molecular Weight Estimations

The MW's of the plasmid bands isolated were estimated by preparing a standard curve as described by Meyers (20) based on the migration distances of the *E. coli* reference plasmids which were included in each gel. These plasmids have been shown to be very sturdy and remain in their original covalently closed circular (CCC) configuration during extraction from the host cells and also during storage at -20°C in purified form. Plasmid DNA in the CCC configuration will migrate through an agarose gel of this concentration at a rate inversely related to its MW. The standard curve was prepared by plotting the logarithm of the migration distance versus the logarithm of MW for each reference plasmid. The MW's of uncharacterized bands could then be estimated by reading their migration distances against the standard curve.

This estimation technique assumes that all bands are in the CCC configuration. If a niche occurs in a plasmid (as a result of extraction, freezing, etc...) it will convert from the original CCC form to an open circle (OC) configuration and its migration distance will no longer be inversely proportional to its MW. OC forms of plasmids which are less than ten Mdal will migrate at a slower rate than the original CCC form, contrastingly, OC forms of those greater than ten Mdal will migrate faster than the original (L. L. McKay, personal communication). To assure that plasmid DNA is in the CCC configuration, an additional step in preparing it for electrophoresis is necessary which was not included

in this study. Purification by cesium chloride-ethidium bromide density gradient centrifugation (CCEBDGC) will remove any DNA which is not in the CCC form. This purification technique not only removes OC forms but will also remove residual chromosomal DNA as it exists in a linear configuration. In spite of possible inaccuracies due to the presence of OC forms, all plasmid bands in Figure 1 were assigned a MW by this estimation technique for convenience of discussion.

As can be seen in Figure 1 and Table 1 the plasmid profiles of both parents and mutants for strains BA-2, S1E, and BA-1 are fairly similar. All have the same number of plasmid bands, however, some differences do exist in the intensity of the bands which relates to the quantity (copy number) of the plasmid DNA present. The two most notable differences with this regard are found in strains S1E-1 and BA-11. Strain S1E-1 has a much higher copy number of the 4-Mdal band than does its parent. In the parent strain this band is very faint and only visible in the original photograph. Similarly, BA-11 has a higher copy number of the 19-Mdal band than does it parent or any of the other BA-1 mutants examined.

Two other limitations exist because the DNA samples were not purified by CCEBDGC. The first is that the residual chromosomal DNA was not removed and its presence in these plasmid profiles may be preventing the observation of additional plasmid DNA which bands in the same region. This is exemplified in the profiles of strain BA-2 and its derivatives as it appears that two plasmid bands may be partially

obscured by the presence of chromosomal DNA. The second problem is that some of the bands observed may actually be replicate forms of other bands that exist in a different configuration and thus migrate at a different rate. With strain BA-1 and its derivatives, the fact that so many bands are present (17 for each) strongly suggests that some may be OC forms. However, even though these limitations exist comparisons can still be made.

Both strains ML81 and ML82 have one more band than their parent; this being at 19 Mdal. In an effort to determine if this band is responsible for phage insensitivity, strain ML82 was grown in the presence of EB as described in Materials and Methods to cure it of plasmid DNA. Two colonial isolates were obtained, out of six which were selected and examined by electrophoresis, which had been cured of the 19-Mdal band (Figure 1, tracks S and T). These cured isolates were then tested with two different phages (to which ML82 was insensitive) to determine if insensitivity had been lost. The results in Table 2 show that phage insensitivity remained in both isolates in spite of the fact that they lacked the 19-Mdal band.

Although ML8EB1 and ML8EB2 were not extensively characterized to determine if loss of the 19-Mdal band could be associated with loss of some metabolic function, they showed no reduction in their ability to rapidly coagulate nonfat milk. This indicated that their genetic capacity to produce the enzymes for lactose metabolism and proteolysis was still intact.

The fact that phage insensitivity was not lost does not entirely eliminate the possibility that the 19-Mdal band is responsible for phage

insensitivity as it may have entered into the chromosome (became an episome) as a result of EB treatment. However, a particular phenotype can only be attributed to a certain plasmid when curing of the plasmid results in alteration of the phenotype which did not occur in this instance. The possibility also exists that this band was an OC form (and not a separate plasmid entity) which was eliminated due to exposure to EB.

DISCUSSION

The phage-insensitive mutants examined in this investigation are uncharacterized as to their mechanisms of immunity. Immunity as such can exist as a result of four possible mechanisms. These are (as reviewed by Limsowtin and Terzaghi): non-adsorption of phage due to an altered receptor site - "phage resistance"; restriction-modification of phage DNA; failure to inject DNA following adsorption; or immunity created by lysogeny (19). The mechanisms of antibiotic resistance are discussed in Chapter 1.

Laboratory-derived mutants such as these, whether they are phage or antibiotic insensitive, are most likely the product of a chromosomal mutation (18). They exist as a result of a spontaneous mutation that has been selected for by exposure to phage or antibiotic which kills or inhibits growth of the parent organisms. Phage insensitivity, however, can also be a result of lysogeny.

In spite of the previous discussion, plasmid involvement cannot be ruled out unless proved otherwise. Although the results of this study are not definitive as plasmid DNA samples were not purified by CCEBDGC, they do provide evidence that plasmid DNA is not associated with phage or streptomycin insensitivity in those mutants examined.

In the mutant selection procedure described by Huggins and Sandine (Alan R. Huggins, Ph.D. Thesis, Oregon State University, Corvallis, OR 1980) which was used to isolate these phage-insensitive mutants, each strain is initially purified by single-colony isolation on FSDA of a

fast acid-producing isolate and subsequently cultured before exposure to phage. In this respect, the culture exposed to phage has recently been derived from a single colony-forming unit. If insensitivity to phage is coded for by a plasmid in these mutants (and not a result of a chromosomal mutation), as has recently been shown to exist in a strain of S. faecalis subsp. zymogenes (6), the plasmid in question must have been present in the colony-forming unit. If so, many of the cells in the colony selected would have the plasmid and the culture exposed to phage would contain many insensitive cells. This scheme is improbable as our experience in the laboratory has been that only a very small percentage of the population exposed to phage is insensitive.

The possibility of sensitivity to phage being coded for by a plasmid, as sensitivity to a bacteriocin has been reported to exist in a strain of S. faecalis (21), would better fit our laboratory observations. If the colony-forming unit possessed the plasmid coding for sensitivity, its maturation into a colony and subsequent subculturing (due to errors in replication, etc...). Exposure to phage would then select for those cells which have lost the plasmid. This type of scenario describes a selection mechanism very similar to that of a chromosomal mutation and would explain the fact that only a low percentage of the population is found to be insensitive upon exposure to phage.

Environmental isolates of S. lactis would probably be a more likely source of plasmid-mediated resistance to antibiotics (23). It is conceivable that isolates obtained from the milk of an animal being

treated for mastitis by antibiotic therapy may contain plasmid-mediated resistance. A plasmid coding for multiple-antibiotic resistance (including resistance to streptomycin) in S. faecalis subsp. zymogenes has been reported (11). Another report (8) describes the transfer of a plasmid which codes for drug resistance in S. faecalis to a strain of S. lactis and subsequent transfer between species of lactic streptococci. This suggests that perhaps plasmids coding for multiple resistance could be found in S. lactis also. Once resistant isolates are found, they could be treated with a curing agent and subsequently tested for loss of resistance. In this manner, a correlation could be made associating loss of a particular plasmid with loss in resistance.

The potential of genetic research dealing with lactic streptococci and its application to industry are tremendous. However, much work needs to be done to reach the present day state of the art of genetic engineering with E. coli. Further work regarding the transfer of DNA from strain to strain by conjugation, transformation, and transduction is important in laying the foundation for future genetic engineering efforts. As further research is conducted in this area and the function of cryptic plasmids and segments of chromosomal DNA are identified, it is conceivable that "super strains" of these bacteria could be constructed by transfer of DNA from one strain to another and maybe even from other species of bacteria to produce better cheese flavor, greater resistance to environmental stress, greater activity, etc... Such manipulations could be of great importance to the cheese industry by producing more reliable and better starter cultures.

LITERATURE CITED

1. Cords, B. R., L. L. McKay, and P. R. Guerry. 1974. Extrachromosomal elements in group N streptococci. J. Bacteriol. 117:1149-1152.

2. Crosa, J., L. K. Luttropp, and S. Falkow. 1975. Nature of R-factor replication in the presence of chloramphenicol. Proc. Natl. Acad. Sci. U.S.A. 72:654-658.

3. Efstathiou, J. D., and L. L. McKay. 1976. Plasmids in Streptococcus lactis: evidence that lactose metabolism and proteinase activity are plasmid linked. Appl. Environ. Microbiol. 32:38-52.

4. Efstathiou, J. D., and L. L. McKay. 1977. Inorganic salts resistance associated with a lactose-fermenting plasmid in Streptococcus lactis. J. Bacteriol. 130:257-267.

5. Falkow, S., P. Guerry, R. W. Hedges, and N. Dalta. 1975. Polynucleotide sequence relationships among plasmids of the I compatability complex. J. Gen. Microbiol. 85:65-76.

6. Frazier, M. L., and L. N. Zimmerman. 1980. Plasmid-borne resistance to ultraviolet light and phage in Streptococcus faecalis ssp. zymogenes. Can. J. Microbiol. 26:1253-1255.

7. Fuchs, P. G., J. Zajdel, and W. T. Dobrzanski. 1975. Possible plasmid nature of the determinant for production of the antibiotic nisin in some strains of Streptococcus lactis. J. Gen. Microbiol. 88:189-192.

8. Gasson, M. J., and F. L. Davies. 1980. Conjugal transfer of the drug resistance plasmid pAMß in the lactic streptococci. FEMS Microbiology Letters 7:51-53.

9. Hiffron, F., C. Ruebens, and S. Falkow. 1975. Transduction of a plasmid DNA sequence which mediates ampicillin resistance: molecular nature and specificity of insertion. Proc. Natl. Acad. Sci. U.S.A. 72:3623-3627.

10. Huggins, A. R., and Sandine, W. E. 1979. Differentiation of fast and slow milk-coagulating isolates of lactic streptococci. Proceedings 74th Annual Meeting. Am. Dairy Science Association, p. 70-71.

11. Jacob, A. E., and S. J. Hobbs. 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in Streptococcus faecalis var. zymogenes. J. Bacteriol. 117:360-372.

12. Kempler, G. M., and L. L. McKay. 1979. Characterization of plasmid deoxyribonucleic acid in Streptococcus lactis subsp. diacetylactis. Evidence for plasmid-linked citrate utilization. Appl. Environ. Microbiol. 37:316-323.

13. Klaenhammer, T. R., L. L. McKay, and K. A. Baldwin. 1978. Improved lysis of group N streptococci for isolation and rapid characterization of plasmid deoxyribonucleic acid. Appl. Environ. Microbiol. 35:592-600.

14. Kuhl, S. A., L. D. Larsen, and L. L. McKay. 1979. Plasmid profiles of lactose-negative and proteinase-deficient mutants of Streptococcus lactis C10, ML3, and M18. Appl. Environ. Microbiol. 37:1193-1195.

15. Larsen, L. D., and L. L. McKay. 1978. Isolation and characterization of plasmid DNA in Streptococcus cremoris. Appl. Environ. Microbiol. 36:944-952.

16. LeBlanc, D. J., V. L. Crow, L. N. Lee, and F. C. Garon. 1979. Influence of the lactose plasmid on the metabolism of galactose by Streptococcus lactis. J. Bacteriol. 137:878-884.

17. LeBlanc, D. J., V. L. Crow, and L. N. Lee. 1980. Plasmid mediated carbohydrate catabolic enzymes among strains of Streptococcus lactis, p. 31-41. In C. Stuttard, and K. R. Rozee (ed.), Plasmids and transposons: environmental effects and maintenance mechanisms, Academic Press, Inc., New York.

18. Lewin, B. 1977. Gene expression, Vol. 3. John Wiley and Sons, Inc., New York.

19. Limsowtin, C. K. Y., and Terzaghi, B. E. 1976. Phage resistant mutants: their selection and use in cheese factories. N. Z. J. Dairy Sci. Technol. 11:251-256.

20. Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127:1529-1537.

21. Oliver, D. R., B. L. Brown, and D. B. Clewell. 1977. Analysis of plasmid deoxyribonucleic acid in a cariogenic strain of Streptococcus faecalis: an approach to identifying genetic determinants on cryptic plasmids. J. Bacteriol. 130:759-765.

22. Terzaghi, B. E., and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29:807-813.

23. W.H.O./F.A.O. Expert committee on food additives. 1969. Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics. W.H.O. Techn. Report Series No. 430, Geneva.

Table 1. Enumeration and descriptions of the plasmid bands in Figure 1.

Track[a]	Strain	No. of distinct plasmid bands	MW[b] (Mdal) of each plasmid band[c]	Comment
B	BA-2 (parent)	7	22.5, chrm[d], 6.4, 5, 3.6, 2.3, 1.7, 1.5	
C	BA-22	7	same	
D	BA-22R	7	same	
E	BA-21	7	same	
G	S1E (parent)	6	23, 18.5, chrm, 4, 2.3, 1.9, 1.4	23- and 4-Mdal bands very faint[e]
H	S1E-1	6	same	23-Mdal band very faint[e]
J	BA-1R	17	28.5, 24, 19, 14, chrm, 6.7, 6, 5.2, 4, 3.4, 2.6, 2.3, 2.2, 1.9, 1.8, 1.6, 1.4, 1.3	many bands very faint[e]
K	BA-1 (parent)	17	same	"
L	BA-11	17	same	"
M	BA-12	17	same	"
N	BA-13	17	same	"
P	M1.81	5	26, 19, chrm, 4.9, 2.0, 1.4	19-Mdal band not present in parent
Q	M1.8 (parent)	4	26, chrm, 4.9, 2.0, 1.4	
R	M1.82	5	26, 19, chrm, 4.9, 2.0, 1.4	19-Mdal band not present in parent
S	M1.82EB1	4	26, chrm, 4.9, 2.0, 1.4	19-Mdal band removed by EB treatment
T	M1.82EB2	4	same	"

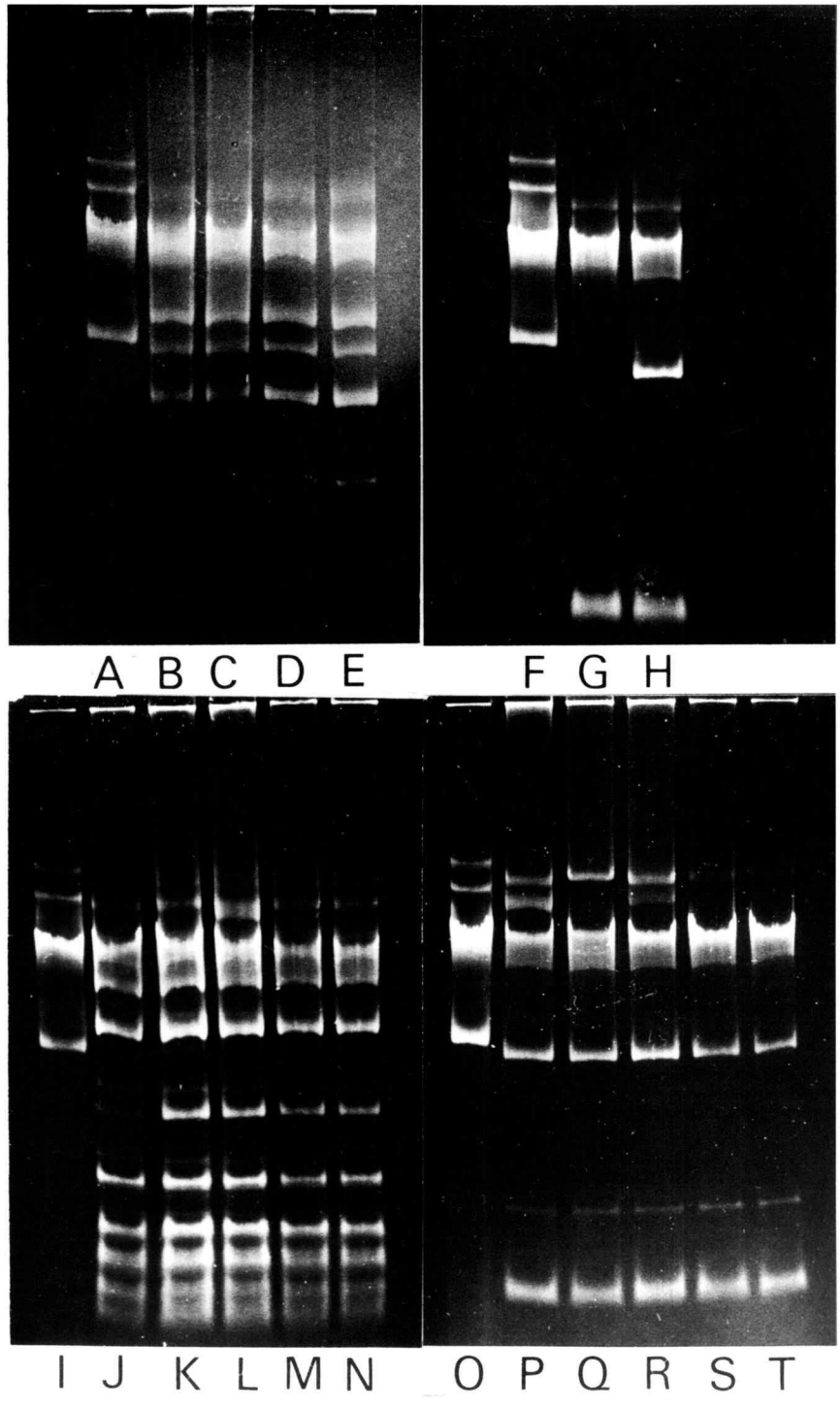
[a] Tracks A,F,I, and O contain E. coli reference plasmids.
[b] See text for limitations of the method used to calculate MW.
[c] Bands are listed in order of appearance from top of gel to bottom.
[d] chrm = chromosomal DNA
[e] Bands are very faint but nevertheless present in the original photograph.

Table 2. A comparison of phage susceptibilities of parent, phage -insensitive, and EB-treated phage -insensitive strains.

Strain	Phage	Reaction
ML8 (parent)	776	S
	876	S
M182	776	I
	876	I
ML82EB1	776	I
	876	I
ML82EB2	776	I
	876	I

S = Sensitive, I = Insensitive

Figure 1. Plasmid profiles of four strains of S. lactis and their phage- and streptomycin-insensitive mutants. Tracks A, F, I, and O contain the three E. coli reference plasmids. These are (from top to bottom, identifying letter represents bottom of gel) RP4 (34 Mdal), Sa (23 Mdal), chromosomal DNA, and RSF1010 (5.5 Mdal). Tracks B, C, D, and E contain BA-2 (parent strain), BA-22, BA-2R, and BA-21, respectively. Tracks G and H contain S1E (parent strain), and S1E-1, respectively. Tracks J, K, L, M, and N contain BA-1R, BA-1 (parent strain), BA-11, BA-12, and BA-13, respectively. Tracks P, Q, R, S, and T contain ML81, ML8 (parent strain), ML82, ML82EB1, and ML82EB2, respectively. The very wide band in each of the tracks represents chromosomal DNA.


A B C D E
F G H
I J K L M N
O P Q R S T

CHAPTER IV

EFFECTS OF STORAGE ABUSE ON LACTIC STREPTOCOCCAL CULTURES

ABSTRACT

Selected strains of lactic streptococci were incubated at various temperatures and then refrigerated at 4°C as mature cultures. Samples were taken periodically and plated onto Fast-Slow Differential Agar to determine whether this type of abuse influenced the number of slow acid-producing variants given off. Problems were encountered as the presence of injured colony types prevented accurate enumeration of slow types. A similar study was conducted to determine if short term storage of cultures at -20° and -80°C and occasional thawing and refreezing influenced the occurrence of slow variants.

INTRODUCTION

Studies have shown (5,15) that when a fast acid-producing culture of Streptococcus lactis is plated, 1-2% of the colonies are slow acid-producing when subsequently grown in milk. By definition, a fast culture will coagulate nonfat milk in 16 h from a 1% inoculum when grown at 21°C. Slow acid-producing variants of S. cremoris have also been similarly isolated and identified (3,4). This phenomenon has been shown to be a result of loss in proteinase activity and/or the ability to ferment lactose (1,10,11). Recent evidence suggests that the genetic information coding for these characteristics is plasmid linked (9,12,13).

Plasmids are unstable entities and are frequently lost from the cell which explains why slow variants spontaneously occur at a high rate. Incoporation of this genetic material into the chromosome would result in much greater stability and more reliable cheese starter cultures (14). It is not clear what role environmental factors (e.g. growth temperature, pH, freezing, etc...) play in influencing the loss of plasmid DNA. However, it is known that cultures of lactic streptococci which are maintained in the laboratory sometimes lose their ability to rapidly produce acid.

Fast-Slow Differential Agar (FSDA) (8) allows differentiation of fast and slow milk-coagulating colonies on the basis of morphology alone, and eliminates the need for painstaking selection and transfer of hundreds of colonies to milk for characterization. Slow colony types, which represent colonies that are proteinase-deficient and/or lactose-negative, can be distinguished from fast types which are

composed of parental cells. With the use of FSDA, it was hoped that the role of various environmental factors involved in the occurrence of slow variants could be clarified. It was the purpose of this investigation to determine if growth at various temperatures and subsequent storage at 4°C, and/or frozen storage (at two temperatures) and freezing and thawing have any effect on the incidence of slow variants in three selected strains of lactic streptococci.

MATERIALS AND METHODS

Media

Instant Peake nonfat milk (Galloway West Co., Fond du Lac, WI) reconstituted at 11% solids was used throughout this study. Tubes containing ten ml of nonfat milk (NFM) were autoclaved at 121°C for 12 min for use in culture maintenance. French-square dilution bottles (160 ml capacity, Kimax) containing 50 ml of NFM, autoclaved as above, were used in shelf-life studies at 4° and -20°C. Polypropylene centrifuge bottles (250 ml capacity, Nalgene) were used for storage at -80°C so that breakage would not occur.

Plate counts were on FSDA in duplicate replications using the spread-plate technique and incubation was at 30°C for 72 h under anaerobic conditions. Anaerobiosis was obtained by evacuation of a large bell jar (ca. five gallon) fitted with a two-valved screw cap and a pressure gauge, and subsequent filling with a speciality gas containing 90% N_2, 5% H_2, and 5% CO_2 (Airco Co., Vancouver, WA), or by use of BBL Gas Pak Systems. Dilutions were made in 0.1% peptone (Bacto) dissolved in distilled water. All bottles containing stored cultures were shaken vigorously for one min before samples were removed and diluted.

Bacterial Strains

The three strains used were obtained from the culture collection at Oregon State University and included: *S. lactis* strains C2 and BA-1 and *S. cremoris* strain 205. Strains C2 and 205 were found to contain slow variants when originally plated by Huggins and Sandine (8).

It was hoped, based on this information, that these strains would be high producers of slow variants. Cultures were maintained in tubes containing ten ml of NFM and stored in the unincubated conditions (2% inoculum) at 4°C. Tubes were incubated at 21°C and cultures transferred to fresh NFM weekly. Each culture was purified by streaking onto FSDA and subsequent selection of a fast colony type before use in these experiments. All inocula used were from a freshly-coagulated culture grown at 21°C.

Characterization of Colony Types on FSDA

This experiment was conducted to prepare a key containing a detailed description of the different colony types of these three strains on FSDA, so as to eliminate any doubt that may be encountered in colony differentiation. Fast cultures of C2, BA-1, and 205 which were known to contain slow variants were appropriately diluted and plated onto FSDA. Colonies were observed and characterized according to size, shape, optical properties, and presence or absence of acid halo and red fringe. The characterized colonies were then picked with sterile wooden-applicator sticks, transferred to tubes of NFM, and incubated at 21°C until coagulation occurred. A 1% inoculum was then made into fresh NFM and subsequently incubated at 21°C for 16 h. All tubes which coagulated in this time period were considered to contain fast isolates, and those which took longer were considered to contain slow isolates. If the original tube in which a colony was subcultured did not coagulate after extended incubation, a methylene-blue stain was made to assure that

cells were indeed present. Once this was established, it was considered to contain a slow isolate.

After this investigation was begun, a third colony type was observed when cultures were abused by storage at a low pH at 4°C for extended periods of time. These were classified as injured types and were characterized in the same manner as described previously. Although these appeared differently than fast types, they were identical in their ability to rapidly coagulate NFM.

Effect of Growth Temperature and Subsequent Storage at 4°C

For each strain, three bottles containing 50 ml of autoclaved NFM were inoculated at a rate of 1% and incubated (one each) at 21°, 30°, and 37°C for 16 h. A fourth bottle for each was also inoculated as such, but was held unincubated (refrigerated at 4°C) to serve as a control. After incubation, all bottles were refrigerated at 4°C for three weeks at which time this experiment was terminated. Samples were taken at 16 h (prior to refrigeration) and weekly thereafter, and were plated onto FSDA. The pH of each of the incubated cultures was measured using a pH meter (Corning) before storage. Colonies were counted following incubation of the FSDA plates and the various types recorded.

Effect of Freezing and Thawing on Grown and Ungrown Cultures

For each strain, four bottles containing 50 ml of autoclaved NFM were inoculated at a rate of 1%. Two bottles were incubated at 21°C for 16 h, and the remaining two were held unincubated (refrigerated) at 4°C. Afterwards, one of each was frozen at -20° and -80°C. Samples

were taken at 16 h (before freezing), and then periodically during a period of 17 - 21 days and plated onto FSDA. The pH of each incubated culture was also determined before freezing. Cultures were thawed by partial immersion in a water bath held at room temperature (20°C) for 30 - 45 min and were then immediately refrozen after sampling. Colonies were counted following incubation of the FSDA plates and the various types recorded. It was hoped that comparisons could be made regarding storage of lactic cultures in the ungrown and mature states when frozen, as opposed to refrigerated (previous experiment), with respect to occurrence of slow variants.

RESULTS

Characterization of Colony Types on FSDA

For strain BA-1, fast colonies were 1.5-4.5 mm in diameter, convex, shiny, and surrounded by a halo and fringe. Slow colonies were 0.8-1.0 mm, semi-convex to flat, translucent, and lacked halo and fringe. Injured colonies were 0.3-1.5 mm, convex, shiny, and also lacked halo and fringe.

For strain C2, fast colonies were 1.5-4.5 mm in diameter, convex, shiny, and surrounded by a halo and fringe. Slow colonies were 0.75-1.5 mm, slightly convex, translucent, and sometimes had a faint halo but no fringe. Injured colonies were 0.5-1.5 mm, convex, shiny, and lacked halo and fringe.

For strain 205, fast colonies were 1.0-2.0 mm in diameter, convex, shiny, and surrounded by halo and fringe. Slow colonies were 0.4-1.0 mm, slightly convex to flat, translucent, and lacked halo and fringe. Injured colonies were 0.2-1.0 mm, convex, shiny, and also lacked halo and fringe.

Injured colony types were not observed and discussed by Huggins and Sandine (8). These were often intermediate in appearance as compared to fast and slow types, but sometimes were considerably smaller than slow types. Slow colonies could easily be distinguished from fast types based on their smaller size, translucent appearance, relative flatness, and lack of halo and fringe. Injured types could be easily distinguished from fast types based on their lack of halo and fringe, and smaller size. However, the slow and injured types were frequently

very difficult to differentiate (Figure 1). When well isolated on the surface of the agar, differentiation could usually be made on the basis of relative flatness, translucency, and to a lesser degree size. But on a more crowded plate (175-300 colonies), differentiation could not always be made with certainty. Subculturing an injured colony in NFM allowed for sufficient recovery to occur, as only fast types were observed when subsequently plated onto FSDA.

This inability to differentiate slow and injured colonies presented a problem when plates were counted and colony types recorded. Sometimes adequate differentiation could be made and other times not. Although this problem somewhat thwarted the objective of this investigation, it was still carried out to a considerable extent. As a result, colonies were frequently classified into two groups - fast and non-fast. The non-fast classification consisted of both the slow colony types and injured colony types combined. This is somewhat of a misclassification with regard to the injured types since subculturing allows for return of their fast acid-producing capacity, which is not the case with slow types. However, the fact that a period of recovery is needed (6), puts injured cells into the same category as slow variants in the sense that they are (in this condition) unsuitable for inoculation of the cheese vat.

Effect of Growth Temperature and Subsequent Storage at 4°C

The results for this experiment are recorded in Table 1 and indicate that the pH of the milk culture during storage is responsible for the percentage of the non-fast colonies counted, as more were

counted at lower pH values. The majority of these were injured types, although exactly how many were injured and how many were slow (if any) could not be accurately determined without subculturing each into NFM. The effect of temperature was not directly important, but indirectly involved in that it was responsible for the amount of growth and resulting drop in pH. The incubation temperature of 30°C created the greatest degree of cell death and injury because it is nearest to the optimum for lactic streptococci and provided for the most acid production as compared to 21° and 37°C. This was the case for strains BA-1 and 205, as strain C2 showed greater die-off at 21°C than at 30°C in the presence of less acid. However, more injury was observed at 30°C. Obviously, the best form of storage was in the unincubated condition as no non-fast colonies were counted for any of the strains and cell death did not occur.

The percentages of non-fast colony types represent only those values (number of non-fast colonies counted) which were found to be significantly different than zero. This required statistical evaluation for data arranged in two classes (2), and the level of significance was set at 5% with a probability of 90%. This does not necessarily mean that the values were found to be significantly different from each other.

Effect of Freezing and Thawing on Grown and Ungrown Cultures

Tables 2 and 3 contain the results of this experiment. None of the frozen samples showed an increase in percentage of slow colony types during short term storage at -20° or -80°C in either the ungrown or mature state. Drop-off in number of cells was not pronounced

either. All frozen bottles of strain BA-1 were sampled again after two months of storage; none showed an increase in slow colony types or considerable reduction in cell number. Slow colonies were observed in this experiment, but at a low percentage which could not be statistically proven to be greater than zero by the previously described method.

A comparison of the results for each of the strains when grown at 21°C and stored at 4°C (Table 1) with the results in Table 3, indicates that freezing at -20° or -80°C eliminated the appearance of non-fast colony types and drastically reduced the amount of cell death when cultures were stored in the mature condition. In contrast, a similar comparison of the data in Table 1 with that in Table 2 shows that freezing provided no detectable advantage when cultures were stored unincubated.

Although the results of this investigation do not illustrate whether one condition of storage is better or worse than another with regard to incidence of slow variants, they do show that if cultures are to be stored in the mature state they should be frozen as opposed to refrigerated to maintain maximum viability and to prevent injury. However, freezing is not necessary for storage of unincubated cultures over a short period of time. Also, it appears that repeated freezing and thawing as conducted in this study does not influence the occurrence of slow variants.

DISCUSSION

Damage to cells of lactic streptococci occurs when they are grown below pH 5.0, and a certain period of growth (below pH 5. 0) is required before the injury is expressed. Subsequently, a period of growth above pH 5.0 is required for repair of the damage and return to normal capacity. This damage is associated with a reduction in the specific activities of various enzymes (6).

Injury and death occurred with the strains used in this investigation when stored in the mature condition at 4°C. However, when mature cultures were frozen at -20° or -80°C injury was not observed and death was greatly reduced, although freezing at -80°C seems to be slightly superior to -20°C with regard to the latter (Table 3). It appears that detectable injury and death are considerably reduced when all cellular functions (i.e. enzyme activity and metabolism) and growth are completely arrested as was the case in the frozen state. In this manner, freezing serves to minimize the damaging effects of low pH.

It is believed that perhaps the considerable injury and death observed when mature cultures were stored at 4°C was partially due to slight growth and/or enzyme activity (in the presence of high acidity) which may have occurred at this temperature. Some growth was detected with the unincubated cultures held at 4°C as a slight increase in cell counts was observed in all three strains, especially with strains C2 and 205 (Table 1). This suggests that slight growth, or at least enzyme activity, could have also occurred with the mature cultures (in the presence of low pH) and as a result caused injury and death.

According to Bergey's Manual (7), S. lactis and S. cremoris do not grow at 45°F (7.2°C) but will grow at 50°F (10°C). The temperature of the refrigerator used was consistently measured at 4°C, however, continued opening and closing may have allowed enough variation to permit slight growth. Or, perhaps very slight growth does indeed occur with these particular strains at this temperature.

Although this study fell considerably short of its goal, it does provide insights as to what kind of problems must be overcome if further investigations are to be made. After its conclusion, it was found that extended incubation (a total of six to eight days) of FSDA plates containing injured colony types allowed most injured types to recover and transform into fast types. However, it was not clear whether some did not recover or new injured colonies appeared, as injured colonies were still present even after extended incubation. What is needed is a means to allow injured cells to recover before plating onto FSDA, so that problems of colony differentiation will not be encountered. It is conceivable that samples could be taken and subcultured in NFM before plating to allow recovery of injured cells. However, only a short period of incubation could be used (just sufficient to allow recovery and not an increase in cell number) so that the ratio of fast and slow cells is not disrupted. If incubation is too long and growth does occur, fast cells will outgrow those that are slow and results will be inaccurate. Addition of 1.0% glucose and 0.25% milk protein hydrolysate to the NFM used for subculturing would allow slow cells to grow at roughly the same rate as fast cells (11) and may minimize the possibility of disrupting the ratio of fast and slows.

If further investigations regarding the occurrence of slow variants as a result of freezing and thawing are to be made, experiments should be conducted over a longer period of time than in this study and with more frequent thawings. Also, more strains should be examined.

LITERATURE CITED

1. Citti, J. E., W. E. Sandine, and P. R. Elliker. 1965. Comparison of slow and fast acid-producing Streptococcus lactis. J. Dairy Sci. 48:14-18.

2. Cochran, W. G., and Cox, G. M. 1957. Experimental designs, 2nd ed., John Wiley and Sons, Inc., New York.

3. Garvie, E. I., and L. A. Mabbit. 1956. Acid production in milk by starter cultures-the effect of peptone and other stimulatory substances. J. Dairy Res. 23:305-314.

4. Garvie, E. I. 1959. Some observations of slow and fast acid-producing strains of Streptococcus cremoris and Streptococcus lactis used as cheese starters. J. Dairy Res. 26:227-237.

5. Harriman, L. A., and B. W. Hammer. 1931. Variation in the coagulation and proteolysis of milk by Streptococcus lactis. J. Dairy Sci. 14:40-49.

6. Harvey, R. J. 1965. Damage to Streptococcus lactis resulting from growth at low pH. J. Bacteriol. 90:1330-1336.

7. Holt, J. G. (ed.). 1977. The shorter Bergey's manual of determinitive bacteriology, 8th ed. Williams and Wilkins Co., Baltimore.

8. Huggins, A. R., and W. E. Sandine. 1979. Differentiation of fast and slow milk-coagulating isolates in strains of lactic streptococci. Proceedings, 74th Annual Meeting, Am. Dairy Science Association, p. 70-71.

9. Kuhl, S. A., L. D. Larsen, and L. L. McKay. 1979. Plasmid profiles of lactose-negative and proteinase-deficient mutants of Streptococcus lactis C10, ML_3, and M18. Appl. Environ. Microbiol. 37:1193-1195.

10. McKay, L. L., K. A. Baldwin, and E. A. Zottola. 1972. Loss of lactose metabolism in lactic streptococci. Appl. Microbiol. 23: 1090-1096.

11. McKay, L. L., and K. A. Baldwin. 1974. Simultaneous loss of proteinase- and lactose-utilizing enzyme activities in Streptococcus lactis and reversal of loss by transduction. Appl. Microbiol. 28:342-346.

12. McKay, L. L., and K. A. Baldwin. 1975. Plasmid distribution and evidence for a proteinase plasmid in Streptococcus lactis C2. Appl. Microbiol. 29:546-548.

13. McKay, L. L., K. A. Baldwin, and J. D. Efstathiou. 1976. Transductional evidence for plasmid linkage of lactose metabolism in Streptococcus lactis C2. Appl. Environ. Microbiol. 32:45-52.

14. McKay, L. L. 1978. Microorganisms and their instability in milk and milk products. Food Technol. 32(no. 5): 181-185.

15. Westhoff, D. C., R. A. Cowman, and M. L. Speck. 1971. Isolation and partial characterization of a particulate proteinase from a slow acid-producing mutant of Streptococcus lactis. J. Dairy Sci. 54:1253-1264.

Table 1. A comparison of change in cell number and in percentage of non-fast colony types during storage at 4°C for three strains of lactic streptococci. Each strain was stored after incubation at 21°, 30°, and 37°C, and unincubated. The degree of cell death and injury observed is directly related to the pH of the culture during storage. As a result, the preferred form of storage is in the unincubated condition.

Sampling time	BA-1			C2			205		
	pH	Log_{10} cfu/ml	Percent non-fast colonies	pH	Log_{10} cfu/ml	Percent non-fast colonies	pH	Log_{10} cfu/ml	Percent non-fast colonies
Unincubated									
16 h		7.11	---[a]		7.15	---		6.97	---
7 days		6.98	---		7.48	---		7.38	---
14 days		7.20	---		7.60	---		7.54	---
21 days		7.23	---		7.68	---		7.40	---
Grown at 21°C									
16 h	4.63	9.25	---	4.56	9.18	---	4.48	9.15	---
7 days		9.04	---		9.26	---		9.11	6.0
14 days		8.90	3.3		6.60	---		8.63	34.9
21 days		7.43	16.4		5.08	---		7.18	26.5
Grown at 30° C									
16 h	4.41	9.14	---	4.40	9.26	---	4.40	8.83	---
7 days		8.70	---		9.08	---		8.63[b]	---[b]
14 days		8.32	14.3		7.83	14.0		6.40	34.6
21 days		7.20	32.0		6.20	21.6		4.76	55.2
Grown at 37°C									
16 h	4.54	9.00	---	4.71	8.77	---	4.98	8.04	---
7 days		8.69	---		8.60	---		7.44	---
14 days		8.87	---		8.72	---		7.04	2.3
21 days		8.48	10.8		8.72	---		7.20	13.4

[a] --- = 0%.

[b] Values for a sampling made at 18 days.

Table 2. A comparison of change in cell number and in percentage of slow colony types during storage of unincubated cultures at -20° and -80°C for three strains of lactic streptococci. Each sampling (other than that conducted at 16 h) represents a thawing and refreezing. This type of abuse did not influence the occurrence of slow variants. The data in Table 1 for unincubated cultures stored at 4°C served as a control for this experiment.

	BA-1			C2			205		
	Sampling time	Log_{10} cfu/ml	Percent slow colonies	Sampling time	Log_{10} cfu/ml	Percent slow colonies	Sampling time	Log_{10} cfu/ml	Percent slow colonies
-20°C	16 h	7.11	---[a]	16 h	7.18	---	16 h	6.99	---
	7 days	7.11	---	3 days	7.30	---	7 days	7.11	---
	14 days	7.11	---	10 days	7.26	---	14 days	7.08	---
	21 days	6.98	---	17 days	7.34	---	21 days	7.04	---
	2 mos.	7.04	---						
-80°C	16 h	7.11	---	16 h	7.04	---	16 h	7.00	---
	7 days	6.90	---	3 days	7.15	---	7 days	7.08	---
	14 days	7.18	---	10 days	7.20	---	14 days	7.15	---
	21 days	7.18	---	17 days	7.18	---	21 days	6.97	
	2 mos.	7.11	---						

[a] --- = 0%

Table 3. A comparison of change in cell number and in percentage of slow colony types during storage of mature cultures at -20° and -80°C for three strains of lactic streptococci. All cultures were grown at 21°C for 16 h. Each sampling (other than that conducted at 16 h) represents a thawing and refreezing. This type of abuse did not influence the occurrence of slow variants. However, freezing (at either temperature) minimized cell death and injury in mature cultures as compared to refrigerated controls. The data in Table 1 for cultures grown at 21°C and subsequently stored at 4°C served as a control for this experiment.

	BA-1				C2				205			
	Sampling time	pH	Log_{10} cfu/ml	Percent slow colonies	Sampling time	pH	Log_{10} cfu/ml	Percent slow colonies	Sampling time	pH	Log_{10} cfu/ml	Percent slow colonies
-20°C	16 h	4.63	9.11	---[a]	16 h	4.51	9.26	---	16 h	4.49	9.15	---
	7 days		9.04	---	3 days		9.08	---	3 days		8.93	---
	14 days		9.00	---	10 days		9.28	---	10 days		8.57	---
	21 days		8.53	---	17 days		9.28	---	17 days		8.50	---
	2 mos		8.73	---								
-80°C	16 h	4.62	9.11	---	16 h	4.51	9.23	---	16 h	4.52	9.17	---
	7 days		9.04	---	3 days		9.18	---	3 days		9.04	---
	14 days		8.90	---	10 days		9.28	---	10 days		8.89	---
	21 days		8.92	---	17 days		9.30	---	17 days		9.00	---
	2 mos.		9.04	---								

[a]--- = 0%.

Figure 1. A comparison of fast, slow, and injured colony types on FSDA. Top photo contains both fast and slow colony types. Slow colonies are considerably smaller than fast types, lack a surrounding halo and fringe, and appear dull-white in this photograph. Bottom photo contains both fast and injured colony types. Injured types are considerably smaller than fast types and lack a surrounding halo and fringe. Both FSDA plates depicted were incubated for 72 h at 30°C under anaerobic conditions.

Differentation between fast and injured colonies can be made in these photographs. The major distinctions between the two types are that slow colonies are flat and somewhat dull, whereas injured colonies are convex and shiny. However, when slow and injured types are present together on a crowded plate (175-300 colonies) accurate differentiation cannot always be made.