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Title: LACTIC STREPTOCOCCI: THE USE OF DEFINED STRAINS AND BACTERIOPHAGE-INSENSITIVE MUTANTS IN COMMERCIAL MANUFACTURE OF CHEDDAR AND COTTAGE CHEESES

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Phage-insensitive Streptococcus cremoris starter strains were selected by assaying cheese whey against potential starter strains. Six strains were selected and characterized for continual use in cheesemaking. Upon phage-infection, strains were removed from the blend. Cheesemaking continued with remaining strains. A phage-insensitive, fast-acid-producing mutant of the infected strain was isolated and characterized. This mutant, similar to the parent, was returned to the strain mixture. Multiple-blend starters were also used in cottage cheese and cultured buttermilk manufacture.

Individual strains were used as antigens for a rapid detection test for lactic-streptococcal agglutinins in cheese milk. When sedimentation was encountered, agglutinin-sensitive strains were identified and replaced instead of an entire culture blend.

Phage-insensitive mutants were compared to their respective parent strains. Traits examined included acid-producing activity, optimum temperature, generation time, proteolysis, phosphate and NaCl tolerance, phage adsorption, agglutination, morphology, and induction. Mutant strains showed variations in individual characteristics, but no general pattern of variation was observed.

Bulk starters, prepared by growing then freezing individual strains in a commercial internal-pH-control medium (PHASE 4), were stored for 3 mo with and without glycerol. Strains varied in storage survival at -20 C. Glycerol enhanced cell viability and activity at -20 C. Storage in PHASE 4 at -40 C and -80 C preserved activity and viability without glycerol. Unfrozen PHASE 4 cultures retained original activity and viability after 1 mo refrigerated storage. Frozen and refrigerated PHASE 4 starters have been used in Cheddar and cottage cheese manufacture for more than 1 yr.

Exclusive use of defined-strain cultures resulted in significant manufacturing and economic improvements including elimination of culture rotations and starter failure from phage infection, no ripening period, greater cheese uniformity, predictable starter activity, standardized manufacture, and improved cheese quality. Grade-A cheese production was increased by almost 10%. This technology enabled some factories to increase cheese yields by adding whey cream to cheese milk. The combined improvements, based on defined-strain technology, have enabled factories to increase production--some by nearly 50%. To date, more than 150 million lb of Cheddar cheese have been manufactured with defined-strain cultures.

LACTIC STREPTOCOCCI: THE USE OF DEFINED STRAINS AND BACTERIOPHAGE-INSENSITIVE MUTANTS IN COMMERCIAL MANUFACTURE OF CHEDDAR AND COTTAGE CHEESES

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INTRODUCTION

> "On second thought why do we put up with rotations? . . . Furthermore, what other respectable fermentation industry chops and changes, or continuously borrows or buys their 'ferments'? . . . Should we not rather aim for the daily use of the same starter and perhaps a 'bespoke (custom-made)' starter for a particular flavour."
>
> Bruno Reiter, 1973

Since 1936, when Whitehead and Cox first indicted bacteriophages as the main cause of cheese starter failure, phage control measures have relied on cleaning and sanitation programs, phage-inhibitory media, and culture rotations. With the discovery that starter bacteria themselves represent the greatest source of phage, much emphasis has been put on the careful selection of starter strains. However, the majority of the world's cheese continues to be manufactured with rotations of mixed-strain starter cultures of unknown composition. Such unknown compositions make it difficult to accurately monitor phage infection, since samples containing phage must be assayed against individual strains.

The concept of defined single strains for cheesemaking originated in New Zealand in 1934 with Dr. H. R. Whitehead. Since then, defined strains have been used as single-strain, paired-strain, and multiple-strain starters.

When starter strains become phage-infected, replacement strains are introduced into an environment where a range of lytic phages is already present. Thus, replacement strains must be insensitive to phages already in a cheese plant. The most obvious way of overcoming phage infection is the use of phage-insensitive strains. These strains can be selected either from commercial mixed-strain starters, or by growing sensitive cultures in the presence of lytic phage to develop phage-insensitive mutants.

The intent of this research has been to introduce defined-strain technology to the U.S. as an improvement upon current mixed-strain starter technology, and to show the feasibility for selection of phage-insensitive mutants as replacement strains.

CHAPTER 1

A PHAGE-INSENSITIVE, MULTIPLE-STRAIN STARTER APPROACH TO CHEDDAR CHEESEMAKING[a]

ABSTRACT

Bacteriophage-insensitive Streptococcus cremoris starter strains were selected by plaquing cheese whey against a bank of potential starter strains. From those found phage insensitive, six strains were selected and characterized for use in cheesemaking. This six-strain mixture was used exclusively. As phage appeared, the infected strain was removed from the mixture and cheesemaking continued with five strains. A phage-insensitive, fast-acid mutant of the infected strain was isolated and characterized. This mutant, possessing the same parent-strain qualities, was returned to the strain mixture. Make times (3.5-4.0 h), milling acidities (.47%), and cheese pH (5.1) were essentially identical for each vat of cheese produced. Plant A has used the six-strain mixture for over 23 months to produce more than 9,400 consecutive vats of cheese. Plant B has used the culture blend for 500 days to produce 10,000 consecutive vats. Since adopting this culture system, no plants have experienced starter failure due to phage attack. Total cheese production for four cheese plants using the multiple blend exclusively, was over 69 million lbs (31.4 million kg). These data emphasize the technological and economic advantages of this culture methodology.

[a]Technical Paper No. 5698, Oregon Agricultural Experiment Station.

INTRODUCTION

During the past several decades numerous research efforts have been made to improve starter culture technology for Cheddar cheesemaking. Some studies have emphasized improvement of body, texture and flavor of cheese; others have sought to overcome or minimize problems related to bacteriophage infection. The use of characterized single strain starters has resulted in greater control over cheese flavor and phage infections. These strains have been successfully used: singly, in paired rotations and in multiple-strain blends (1,2,7,8,9,11,14). Advantages reported for multiple-strain blends include continual use in every vat, lower inoculum levels, consistent starter activity in spite of individual strain variations or seasonal milk changes, flavor uniformity, and known composition for easy identification of affected strains (9,11). As an added advantage, the rate of acid production of individual strains is often increased when the strains are grown in association (7,9).

Several investigators have used bacteriophage-insensitive mutants (BIM) in cheese starters in an effort to control phage infection (1,5,6,7,10,13,21). Czulak et al. (1) reported on their use in Australia for a 3-year period without any loss of cheese due to complete starter failure ("dead vats"). The concept of applying continual phage stress on a starter to select for BIM strains has been successfully used to prolong starter life in casein manufacture (21). Attempts have not been made to characterize mutants as immune or resistant--terms that have specific meanings in the field of microbial genetics (9,10). They are called BIM herein to broadly indicate their inability to support phage replication.

While bacteriophage-insensitivity is not a permanent property of these strains (1,5,7), it is possible to select insensitive mutants that will remain stable for periods of time sufficient to make them useful in cheese manufacture (1,5,7,10,13). The use of

BIM obviates the need to replace infected strains with new ones that could acquire phages capable of attacking other strains in a multiple-strain starter.

Combining phage-insensitive strains of S. cremoris into a multiple-strain starter has the advantage of greater control over phage infection as well as more consistent starter performance which results in a more uniform product. This paper will review the successful use of such six-strain, phage-insensitive starters in four Oregon and Washington cheese plants.

METHODS AND PROCEDURES

Selection of strains

Starter strains of Streptococcus cremoris and Streptococcus lactis were selected by spotting filter-sterilized whey on M17 agar (20) against a bank of potential starter strains from the Oregon State University collection. Inoculated plates were incubated at 25, 30, and 37 C to account for differences in phage growth-temperature (16). From those found phage-insensitive, six strains, 290-P, 108, SK11G, 224, 134, and C13, were selected for use in cheesemaking. Initially, S. lactis strains BA-1 and BA-2 were selected for use in plants B and D, but were later replaced with S. cremoris strains 134 and C13. These six strains were tested against incoming plant whey samples in order to monitor continued phage insensitivity. Compatibility was checked by cross-streaking the strains against each other on M17 agar, and by spotting supernatants of milk coagulated by individual strains on lawns of other strains in the blend. All strains, at a 1% inoculum level, were capable of coagulating sterile reconstituted skim milk (RSM) in 15-18 h at 21 C. Once introduced into a plant, the multiple-strain starter was used exclusively.

Isolation of mutants

Composited wheys from each plant were regularly spotted against strains in use to monitor phage presence or appearance. As phage appeared for a strain, increased to over 10^5 plaque-forming units per ml (pfu/ml) and persisted, the infected strain was removed from the multiple blend and subjected to whey-adaptation (1,5,6) to produce a phage-insensitive mutant (Figure 1.1). Fast-acid producing clones of the mutants were selected on fast-slow differential agar (A. R. Huggins, 1979. J. Dairy Sci. 62:70 - abstract); a patent for this medium has been applied for. Milk-citrate agar (19) has also been successfully used for fast-acid mutant selection (18,19).

Multiple-strain Culture Scheme

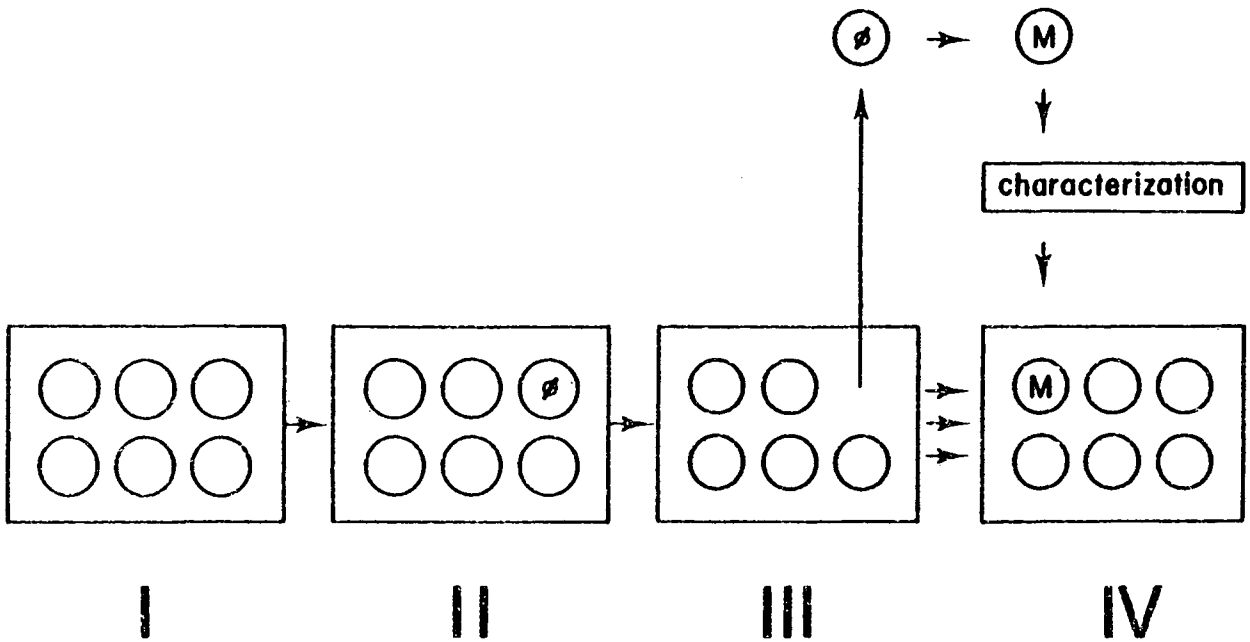

Figure 1.1. Schematic diagram illustrating strain replacement and mutant selection procedures for the culture program.

Mutant characterization

Phage-insensitivity was determined by plaquing whey used for adapting against the BIM. In-coming whey samples from each plant were plaqued against the BIM to insure insensitivity against other possible in-plant phages. BIM stability against phage was determined using the method of Heap and Lawrence (3).

The activity of new mutants was measured by two methods: (1) the ability of the mutant at a 1% inoculum to coagulate RSM in 18 h at 21 C and (2) ability to perform like the parent strain through a simulated cheesemaking test similar to that described by Pearce (15) except that pH was measured after 5 h and the rennet was omitted.

Whole milk cultures of the mutants incubated overnight were evaluated to assess the development of any possible off-flavor.

Mother cultures

Starter cultures were supplied to the four cheese plants as individual strains in screw-capped tubes of sterile RSM (18 ml/tube). Tubes were inoculated and iced for transport and frozen upon arrival at the plant. Tubes of each strain were thawed daily, incubated, and used to inoculate individual RSM intermediate cultures. The six, coagulated intermediates were then combined in equal volumes in the bulk starter tank to provide 0.5 to 0.8% inoculum. Bulk starter tanks for all four cheese plants were equipped with pH-control instrumentation and whey-base starter medium (Biolac, Logan, Utah) was used for the bulk starter.

Commercial cheesemaking

All four commercial cheese plants refilled vats with milk three to five times daily. Three plants used conventional vats; the fourth was equipped with enclosed vertical vats. All plants used open Cheddaring tables. The vats were cleaned and sanitized between fills and milk ripening periods were eliminated. Cooking temperatures were 37.2 to 37.8 C.

In addition to whey monitoring in our laboratory, all plants monitored whey daily for phage by means of a bromcresol purple milk test (BCP test) (17). Tubes containing 10 ml of RSM and bromcresol dye (0.15 g/liter) were inoculated with 0.2 ml of an individual strain and serial dilutions (10^1 to 10^5) of filter-sterilized whey. Tubes were incubated 1 h at 37 C followed by 5 h at 30 C. Inhibition of activity was determined by comparing the color of each dilution tube against the control tube containing the individual strain only (no whey). Any milk coagulation was ignored due to residual milk-clotting enzymes in the whey which may give a false positive result. A time delay or failure of the milk to change color (in comparison to a control) was regarded as phage-inhibition. Infected strains were removed when phage titers rose and persisted above 10^5 pfu/ml using the BCP test. Dilution tubes and controls were run for each of the six strains in the multiple blend.

RESULTS

Table 1.1 shows the initial starter strains used in each cheese plant and the bacteriophage-insensitive mutants used to replace an individual strain as it became phage infected. Cheese manufactured with a six-strain blend containing S. lactis BA-1 and BA-2 was free of bitter flavors and graded as A quality even after nine months of aging. However, strains BA-1 and BA-2 were replaced with S. cremoris strains because as phage appeared, mutants could not be isolated that would remain phage insensitive long enough to be useful in cheesemaking. Strain 108-12 and R6-12 were second-line mutants, i.e. a mutant of the original mutant. The first-line mutant of R6 remained phage insensitive for only 3 days. The second-line R6 mutant (R6-12) has remained stable for over one year. Although still phage-insensitive, it was subsequently withdrawn and replaced with strain 134 to gather further data on this strain. Strain 108-12 has remained stable in plant A for over one year. First-line mutants of strains 290-P, 224, and SK11G also have proven to be quite stable in commercial cheesemaking. Mutant 224-3 has been in continual use in plants A and B for well over one year, and has proved useful in three of the four cheese plants. It has not yet been introduced into the fourth plant. Long-term phage-insensitivity of some mutants has also been reported by New Zealand researchers (9,10).

Figures 1.2 and 1.3 show the phage profiles of plants B and C prior to and after introduction of the multiple-strain blend. One day after introduction, total phage levels in plant B (Figure 1.2) had decreased dramatically. After one week, no phage for any indicator strain was detected. A similar response was noted in plant C (Figure 1.3). Immediately after introduction of the multiple blend, phage titers decreased and within three days titers were almost negligible. A plant oversight required that a commercial starter be reintroduced after three days, while

Table 1.1. Initial starter strains and bacteriophage-insensitive mutant replacements used in four cheese plants.

Initial Strains	Replacement Mutants
PLANT A	
290-P	
108	108-12
R6	R6-12
224	224-3
SK11G	SK11G-C
C13	C13-1
PLANT B	
290-P	290-PC
108	108-C
SK11G-W (BIM of SK11G)	
224-3 (BIM of 224)	
BA-1[a]	134
BA-2[b]	C13-C
PLANT C	
290-P	
108	
SK11G	
C13	
224	
BA-1[a]	134
PLANT D	
290-P	
108-12	
SK11G-D	SK11G-C
224-3	
C13-1	C13-C
BA-1[a]	134

[a] *S. lactis* strain eventually replaced with *S. cremoris* strain 134.

[b] *S. lactis* strain eventually replaced with C13-C (BIM of *S. cremoris* C13).

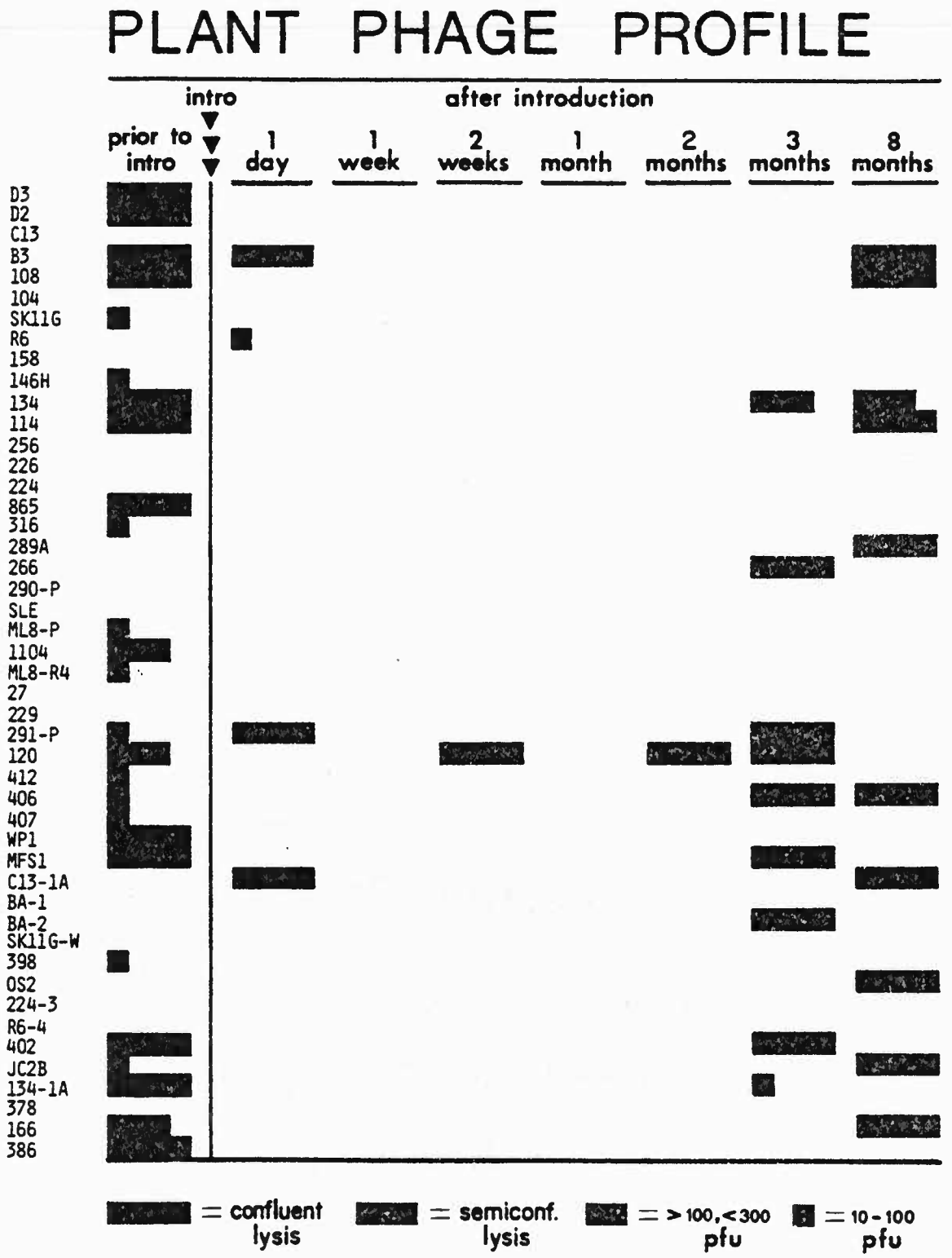

Figure 1.2. Phage incidence profile of whey samples obtained from Cheddar cheese plant B.

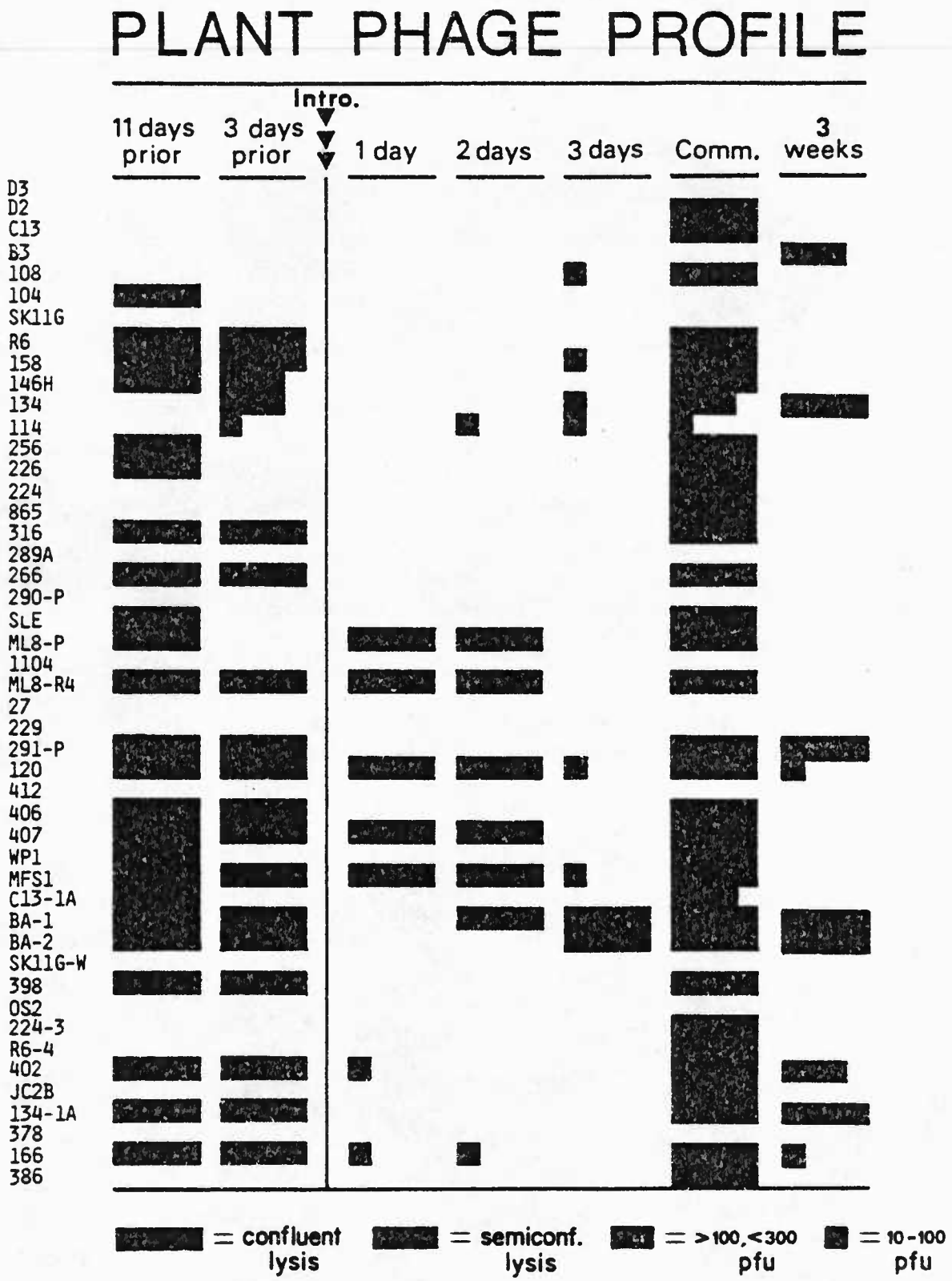

Figure 1.3. Phage incidence profile of whey samples obtained from Cheddar cheese plant C.

additional multiple blend was prepared. Immediately after reintroducing the commercial starter, a substantial increase in phage levels for most all of the strains occurred. When the plant again returned to exclusive use of the multiple-blend, phage titers dropped and became undetectable for most of the strains. By eliminating commercial mixed cultures in each plant, indicator strains were also eliminated.

In actual cheesemaking, phage developing for any one of the six strains did not infect any of the other five strains in the blend. As phage titers for a single strain increased to high levels, cheesemaking with the multiple blend was not affected. Make times, amount of starter and titratable acidities remained the same. As the infected strain was removed and cheesemaking continued with five strains, there was no significant change in starter activity or performance. Cheesemaking continued with five strains until a BIM of the infected strain was reintroduced.

Cheese milk inoculated with the six-strain culture required an average inoculum of 0.7% with resulting make times of 3.5 to 4.0 h, a milling acidity of 0.45% and an average cheese pH of 5.1 after pressing.

Tables 1.2, 1.3, and 1.4 show actual production data obtained using the multiple-strain blend. Plant A has made cheese with this six-strain culture, exclusively, for over 23 months. Culture blends used in all four plants still contain some of the initial starter strains. Total cheese production of the four plants using the multiple blend to date is over 69.3 million lbs (31.5 million kg).

Prior to introduction of the multiple-strain blend, plant C manufactured only four vats of cheese per day. Each vat required a different commercial starter and up to twelve commercial starters were used in a three-day rotation. Cheese make times, amounts of required starter and cheese quality varied substantially from vat to vat. Complete starter failures ("dead vats") occurred

Table 1.2. Cheddar cheese production data from four cheese plants using lactic starters made up of phage-insensitive strains and mutants.[a]

Plant	Consecutive Days	Consecutive Vats	Cheese Amount lbs ($x10^6$)	Cheese Amount kg ($x10^6$)
A	701	9,467	18.9	8.6
B	503	10,060	31.2	14.2
C	380	1,959	7.5	3.4
D	280	3,078	11.7	5.3
Totals		24,564	69.3	31.5

[a]As of March 1, 1981

Table 1.2. Updated from table above.[a]

Plant	Consecutive Days	Consecutive Vats	Cheese Amount lbs ($x10^6$)	Cheese Amount kg ($x10^6$)
A	824	11,370	22.7	10.3
B	626	13,486	41.8	19.0
C	503	2,971	11.3	5.1
D	403	4,768	18.1	8.2
Totals		32,595	93.9	42.6

[a]As of August 1, 1981.

Table 1.3. Grades of Cheddar cheese produced in one factory using a lactic starter made up of six phage-insensitive strains.

Grade	Pounds	Kilograms	Percentage
A	3,241,400	1,473,363	98.3
2nd	57,000	25,909	1.7
B[a]	0	0	0
	3,298,400	1,499,272	100

[a]Due to starter failure.

Table 1.4. Cheese grade and product sales data for two 3-month periods showing comparison of performance for commercial and phage-insensitive multiple-strain cultures.

Dec. 1, 1978 - Feb. 28, 1979 Commercial Cultures				Dec. 1, 1979 - Feb. 28, 1980 OSU Cultures			
Grade	Lbs	Kg	%	Grade	Lbs	Kg	%
A	705,248	320,567	89.2	A	825,918	375,417	90.8
2nd[a]	28,851	13,114	3.6	2nd[a]	83,776	38,080	9.2
B[b]	56,734	25,788	7.2	B[b]	0	0	0
	790,833	359,470	100.0		909,694	413,497	100.0

[a]Usually attributed to high acid.

[b]Due to starter failure.

frequently. Table 1.3 shows the percent of down-graded cheese (1.7%) during 124 consecutive days of manfacture using the multiple blend starter. Since adopting the BIM starter, plant C has experience no "dead vats" and has increased production to 6 vats per day.

DISCUSSION

Although it has been recommended (11) that strain ratios (temperature sensitive versus temperature insensitive) in a blend be adjusted, no attempt was made to do so in this study since it was desirable to make the culture program as simple as possible for use in cheese plants. Work of Daniell (personal communication) indicated that all of the S. cremoris strains used in this study were temperature sensitive. Although it is generally recognized that strains of S. lactis will frequently produce bitter flavor in aged cheese, cheese made with the multiple blend containing two S. lactis strains showed no bitterness after 9 months of aging. New Zealand has successfully incorporated S. lactis strains into a multiple-strain blend (9). They insist, however, that strains of S. lactis should constitute less than 5% of the bulk starter population. The percentage of S. lactis strains used in our study constituted over 33% of the bulk starter population without producing flavor defects in the ripened cheese. This could be due to synergistic effects between the strains of the culture blend, or due to particular characteristics of the selected strains.

In general, BIM of S. cremoris have proven to be more stable against phage than those of S. lactis. Some mutants of S. cremoris, however, show wide variability in acid production as well as in the phage sensitivity (12,13). This is probably due to heterogeneity of the parent strain itself, with the mutant reflecting the stability of the strain from which it was derived (12). Phage-insensitive mutants isolated from strains 108 and C13 have shown the greatest variability in phage sensitivity. Strain 108 has been slow to develop at least four variants which differ in phage sensitivity (12). Although fast-acid producing, BIM can be readily isolated from both strains, mutants isolated thus far show only short-term phage-insensitivity--usually less than one month.

Heap and Lawrence (4) have suggested that the major source of phages is the starter culture itself and that the introduction of starters which can act as indicator strains leads to the establishment of high phage levels in a plant. The New Zealand researchers further noted that once a phage becomes established in a plant, other subsequently detected phages are either the original phage or a modification of it. Lawrence et al. (9) have stated that while commercial mixed starters are the most convenient source of phage-resistant strains, they often contain phage. Therefore, the practice of expanding the number of commercial cultures used in rotation may not offer greater protection, but may instead introduce more indicator strains and phages (as shown in Figure 1.3). The greater the number of phage types present in a cheese plant, the sooner new phages may appear due to cross-phage relationships (3,9). Use of the six-strain blend minimizes cross-phage relationships by selecting strains with non-overlapping phage sensitivities. If strain changes must be made in a cheese culture, new strains that will be compatible with existing strains of a starter blend can be easily selected by the method of Heap and Lawrence (3).

If starter failure can be eliminated in a cheese operation, substantial economic gain can be realized. Table 1.4 summarizes sales data for plant B for two different three-month periods in which cheese was manufactured with commercial cultures, and with the multiple-blend starter one year later. By comparison, the proportion of cheese determined A grade remained the same for both time periods, but manufacture of B grade cheese was completely eliminated when the six-strain starter was used. Simultaneously, the proportion of second-grade cheese (usually high acid) remained the same when the multiple-blend was used. The typical price differential between B-grade and second-grade cheese is $0.15/lb. Extrapolation of such data for a full year would result in an economic gain of over $300,000 which can be directly attributed to

performance of the six-strain blend alone. This figure was based entirely upon the elimination of B-grade cheese resulting from starter failure.

Consistent starter performance allows cheesemakers and management the freedom to "fine tune" the cheesemaking process in order to: (1) increase cheese yields, (2) vary the manufacturing process as required to control cheese composition, and (3) improve product quality. The use of a multiple-strain, phage-insensitive starter results in simplified handling of starter cultures, more consistent starter activity, more uniform cheese composition from day to day and significant economic advantages as a result of improved cheese quality and virtual elimination of starter failures.

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CHAPTER 2

DEFINED STRAINS AND PHAGE-INSENSITIVE MUTANTS FOR COMMERCIAL MANUFACTURE OF COTTAGE CHEESE AND CULTURED BUTTERMILK[a]

ABSTRACT

The use of defined single-strains and their phage-insensitive mutants as starters for cottage cheese and cultured buttermilk is discussed. A culture blend of six strains was used to manufacture consecutive vats of cottage cheese thus eliminating culture rotations. The advantages of this culture system included the elimination of starter failure, better product uniformity, more consistent starter activity, and standardized make procedures. Individual strains used in starter cultures were also used as antigens for a rapid detection test for lactic-streptococcal agglutinins in cheese milk. When sedimentation was encountered, individual agglutinin-sensitive strains were identified and replaced without having to replace an entire culture blend. Strains were also pretested for sensitivity against cheese milk prior to their use in starters. Individual starter strains varied in their sensitivities to agglutinins in cheese milk and colostrum. Phage-infected strains were replaced with phage-insensitive mutants. Defined single-strains and their phage-insensitive mutants have been successfully used for cottage cheese and cultured buttermilk production for more than two years.

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INTRODUCTION

An important challenge facing the cottage cheese industry today is the production of a consistently high quality product (6,17). Cottage cheese uniformity requires careful control of manufacturing variables (4,6,12,14,17). According to Marshall (17), the most frequently encountered defects in cottage cheese--high acid flavor, mealy texture, and shattered curd--are all interrelated, and stem from inadequate control of acid development. Most quality, shelf-life, and production variables can be controlled by good-manufacturing practices. However, phage infection and agglutination, factors that directly influence acid development, have had limited control by cottage cheese manufacturers.

Various starter systems are currently used in cottage cheese production. Traditional propagation of mother cultures has generally been replaced by lyophilized or concentrated frozen bulk cultures. While most commercial cottage cheese cultures are mixtures of undefined lactic strains, some attempt has been made to utilize defined strains for cottage cheese production. One culture service (28) provides lyophilized, mixed-strain cultures that contain three different lactic strains and a Leuconoctoc sp. A different pair of mixed-strain cultures is used each day in a four-day rotation. Phage infected cultures are replaced by an entirely different three-strain culture.

Phage control programs for cottage cheese making presently include starter rotations, good sanitation, and the use of phage-inhibitory media (14). Two-stage homogenization and prescribed heat-treatment of skimmilk is partially effective in reducing agglutination, but the most effective measure is the selection of agglutinin-insensitive starter cultures (8,10,12). However, this task may be complicated by the number of strains present in a culture, and by the number of cultures used in a rotation. Tests

have been developed to detect the presence of lactic-streptococcal agglutinins in milk (7,9), but they have proven inadequate in terms of simplicity, reliability, and antigen shelf-life (21).

The formation of curd by direct addition of acidulants to skim milk (5,18) has been successful for cottage cheese manufacture since phage, agglutinins, and antibiotics have no effect on cheese-making with this method of production (23). However, the results of comparative studies of yield and quality of cottage cheese made by cultured and direct-set methods are divided over a preferred method of manufacture (22,23,27). Cheese manufactured by the addition of starter culture to pre-acidified milk has also been reported (3).

This study reports on the use of defined single-strains, and their phage-insensitive mutants, as cottage cheese starters. Individual strains used in starter culture combinations, were also used as antigens for a rapid detection test for lactic-streptococcal agglutinins in cheese milk (21), thus allowing selection of agglutinin-insensitive strains under most conditions.

METHODS AND PROCEDURES

Cultures

Starter strains of Streptococcus cremoris and their respective phage-insensitive mutants were selected as reported previously (25). Cultures were prepared and supplied to cottage cheese factories as frozen bulk cultures (26). The lactic cultures used for cultured buttermilk manufacture were combined with aroma-producing strains of S. diacetylactis and Leuconostoc citrovorum at inoculation.

Antigen preparation

Antigens were prepared as outlined by Salih (21). Individual strains of S. cremoris were streaked and grown anaerobically on M17 agar (24) for 48 h at 30 C. A single colony was transferred into 100 ml of M17 broth and incubated for 18-24 h at 25 C. This 100 ml quantity was used to inoculate a larger quantity (250 ml) of M17 broth which was incubated as above. Grown cultures were then refrigerated at 4 C until cell harvesting began.

Cells were harvested by centrifugation at 10,000 rpm for 10 min. Cell pellets were resuspended and washed twice with 10 ml aliquots of phosphate buffer (0.1 M, pH 7.0) to remove residual broth. Washed cells were resuspended in buffer then heat inactivated in a 60 C water bath for 1 h before filtering through loosely packed funnels loaded with glass wool. Filtered cell suspensions were again centrifuged at 10,000 rpm for 10 min and the pellets resuspended in 10 ml of phosphate buffer prior to staining. A 5% (w/v) stock solution of rose bengal dye was prepared and filtered through Whatman No. 1 filter paper.

The antigens (bacterial cells) were stained by adding 0.1 ml of fresh dye stock solution to the 10 ml cell suspensions, mixing, and storing at 4 C overnight. Excess dye was removed after overnight staining by centrifuging and resuspending the cell

pellets several times in phosphate buffer, as described in the washing steps above. Packed cell volumes (PCV) were determined by centrifuging the washed cells in 12 ml calibrated conical centrifuge tubes for 10 min at 5,000 rpm. After decanting, the PCV was estimated and the final volumes of the cell suspensions were adjusted to 2% PCV with tris-maleate buffer (0.5 M, pH 7.0). Refrigerated antigens should remain stable for up to three years (21).

Whey preparation

Cheese milk or colostrum, to be tested for the presence of agglutinins, was prepared by adding 0.1 ml of single strength rennet to a 100 ml sample and incubating in a 30 C water bath until coagulation occurred (approx. 5 min). The coagulum was physically broken by vigorous shaking then reincubated for an additional 5 min to allow syneresis to occur.

Milk and colostrum samples

Cheese milk samples were collected from cheese plants experiencing agglutination. Colostrum samples were collected from four individual cows from the Oregon State University dairy herd.

Agglutinin testing

Whey from cheese milk was tested against individual streptococcal antigens using the method of Salih (21). This involved spotting 25 ul of whey onto wells of a Brewer's diagnostic card (Brucellosis card test; Hynson, Westcott and Dunning, Inc., Baltimore, MD, 31301). In addition, 25 ul of undiluted stained-antigen for an individual strain were added to each well. The two drops were thoroughly mixed with a sterile toothpick and the card was hand-rocked for 4 min to allow maximum cellular clumping. Positive agglutination reactions showed severe to slight cell aggregation, while negative samples showed no cell clumping.

Relative agglutinin-titers were estimated by observing the degree of cell-clumping and assigning a value of one to four; four representing the most severe agglutination (no stained-cells left in solution). The absence of agglutination was represented by a zero value. Agglutinin endpoint titrations were performed by spotting 25 ul of serial 1/2-dilutions onto a diagnostic card and reacting with respective antigens as outlined above.

Cheesemaking

Cheese plants used the short-set inoculation method for cottage cheese manufacture. Bulk culture was propagated in externally-neutralized whey-base medium and cheese milk inoculum levels were 3 to 3.5%.

Strain changes

Strain changes necessitated by phage infection were made as previously reported (25). Whenever agglutination was encountered, cheese milk was tested against a bank of potential strains, including phage-insensitive mutants of parent strains, and the agglutinin-sensitive strain was replaced with an insensitive one.

RESULTS

Figure 2.1 shows the manufacturing times (make times) for 100 consecutive vats of cottage cheese, made in plant A over a three-week period. Similar results have been reported by other cottage cheese plants using this culture system. Although total cheese manufacturing times varied between factories depending upon the inoculum levels and manufacturing procedures used, all plants showed a similar uniformity in culture activity from vat to vat.

The use of defined-strain cultures allowed cheesemakers the use of a single culture blend to ferment every vat of cheese milk, thus eliminating culture rotations. Depending upon bulk tank capacities, bulk starter production was reduced from a daily operation to two or three times weekly.

The rapid agglutinin-detection test of Salih has been used successfully in several cottage cheese plants to remedy agglutination problems. The milk supply for each cheese plant exhibited its own characteristic agglutinin profile. Strains showing agglutinin-sensitivity in one cottage cheese plant did not necessarily show sensitivity in other plants. In all cases encountered, agglutination was overcome with the first change of strains. Subsequent strain changes were not needed.

Table 2.1 shows the relative agglutinin-titers of four different colostrum whey samples tested against individual starter strains. Colostrum samples varied in the specificities of their immunoglobulins. Even with elevated concentrations of both specific and non-specific antibodies, individual strains varied signficantly in their sensitivities to these antibodies.

Table 2.2 reports the relative agglutinin-titers of cheese milk tested against single starter strains. Again, strains varied in their sensitivities to immunoglobulins in the different milk samples. There was no correlation between strain-sensitivities to agglutinins derived from cheese milk or colostrum. For example,

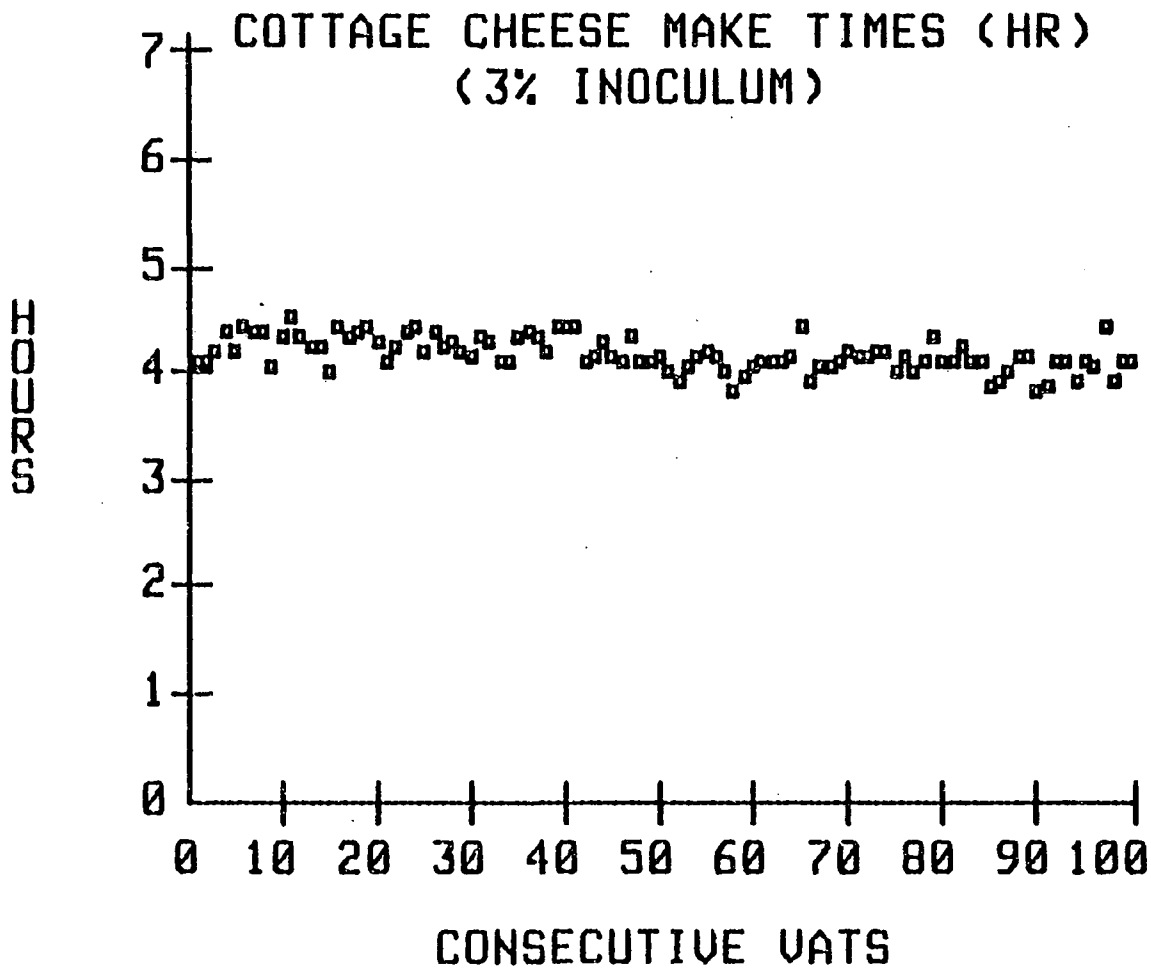

Figure 2.1. Cottage cheese production showing consistency in make times obtained using defined-strain cultures.

Table 2.1. Relative agglutinin-titers of four colostrum wheys tested against selected defined-strains and phage-insensitive mutants.

Strain	Sample 1	2	3	4
290-P	0	2-3	4	0
290-PC	0	2-3	3	0
M290-PC	0	3	3	0
108	0	4	4	0
108-C	4	3	3	3
108-12	0	4	4	0
SK11G	3-4	4	2	1
SK11G-C	0	4	2	0
224	1	4	0	2-3
224-3	1	0-1	0	4
C13	0	0	2	0
C13/25	0	1-2	3	0
U-134	4	3	3	1
31 N	0	4	4	0
229	3	2	3	0
284	0	4	4	0
288	0	2-3	0	1
289-C	4	4	4	2-3
291	2	4	4	1

4 = most severe; 0 = no agglutination.

Table 2.2. Relative agglutinin titers of cheese milk wheys tested against selected defined-strains and phage-insenstive mutants.

	Sample	
Strain	1	2
290-P	2	0
290-PC	2	-
M290-PC	2	-
108	0	1
108-C	2-3	-
108-12	1	0
SK11G	2	1
SK11G-C	0	0
224	2	-
224-3	0	-
C13	1	0
C13/25	0	1
U-134	1	1
31 N	2-3	1
229	3-4	2-3
284	1	2
288	0	-
289-C	2	-
291	2	-

4 = severe; 0 = no agglutination.

strains 290-P and 108 were sensitive to two out of four colostrum samples but only one out of two cheese milk samples (Tables 2.1 and 2.2). Although feasible with this technique, no attempt was made to correlate end-point titers (Table 2.3) with the degree of agglutination observed in cheesemaking. The results of relative agglutinin titers (Table 2.2) were sufficient to eliminate agglutination problems by identifying strains sensitive to the immunoglobulins present in the cheese milk.

Table 2.3. Agglutinin-titers obtained by dilution end-point determination for colostrum whey against selected single strains.

	Agglutinin end point						
Strain	1/2	1/4	1/8	1/16	1/32	1/64	1/128
290-P	-	-	-	-	-	-	-
108-C	+	+	+	+	-	-	-
SK11G-C	-	-	-	-	-	-	-
224-3	+	+	+	+	+	+	-
C13/25	-	-	-	-	-	-	-
U-134	+	-	-	-	-	-	-

+ = agglutination; - = no agglutination.

DISCUSSION

Defined single-strains and their phage-insensitive mutants have been successfully used in cottage cheese and cultured buttermilk production for over two years. Many of the advantages previously reported (25) for their use in Cheddar cheesemaking also apply to cottage cheese and cultured buttermilk manufacture. Some of these advantages include the elimination of starter failure, better product uniformity, elimination of starter rotations, more consistent culture activity and standardized make procedures.

Milk used for cultured buttermilk has traditionally been vat pasteurized at elevated temperatures to improve body and texture of the final product. However, because of increasing energy costs, more and more milk is being HTST pasteurized. This higher-heat, shorter-time treatment (HTST) is not as effective in inactivating phage or agglutinins in milk, and frequently results in a weak-bodied product. With present culture technology, phage can be successfully controlled using defined single-strains and phage-insensitive mutants. Weak body can be overcome by using cultures that impart viscous or ropy characteristics. A method for inducing stable ropy cultures has been reported (13).

Kanno et al. (15) isolated the agglutinating factor for lactic streptococci in milk which was characterized as immunoglobulin M (IgM). More recently, Salih (21), using immunofluorescent techniques showed immuoglobulin G (IgG) to also be a major agglutinating factor in milk. Reiter et al. (20) showed that strains vary widely in their sensitivities to immunoglobulins (antibodies). Milk secreted during normal lactation contains low levels of immunoglobulins (16,19). However, the concentration of antibodies in milk increases significantly with infection, injury, or irritation to the udder (19). Immunoglobulins in milk are derived either from blood serum or they are produced locally in the udder (16). Most of the IgG in milk is derived from blood serum,

whereas IgA and IgM are synthesized locally in response to antigens (16). Immunoglobulin titers in milk and blood, however, are not necessarily related. Emmons et al. (11) reported that while blood titers remained constant over a two-week period, agglutinin titers for milk were three times higher in the first week compared to the second week.

Colostrum and mastitis milk (from both clinical and subclinical mastitis) represent major sources of antibodies which could elicit starter agglutination (11). Results from this study showed that colostrum and cheese milk varied, according to their sources, in both the concentrations and specificities of their immunoglobulins. Emmons et al. (11) showed that agglutinin titers varied within the lactation cycle, and that titers were different between cows during the same stage of lactation. Although starter strains, such as S. lactis C2, which are reportedly "resistant" to agglutinins, did not agglutinate in normal milk, they did show agglutinin-sensitivity in colostrum milk (11).

Cross-sensitivities can be obtained under stimulation by invading bacteria possessing antigens common with those of starter bacteria (11). Briggs and Newland (2) observed cross-reactions between antisera for S. cremoris and strains from nearly all of the Lancefield groups. Auclair and Vassel (1) found that after extended subculturing in autoclaved milk, resistant starter strains might become sensitive to natural inhibitors in milk, and would eventually be predominated by inhibitor sensitive strains. In light of the above, it is questionable whether starter strains can be found that are agglutinin-resistant under all cottage-cheesemaking conditions.

Because wide variations are found in the agglutinin-sensitivities of starter strains and also in the antibodies in cheese milk, the use of a large number of strains or cultures containing several strains may predispose cheese milk to agglutination difficulties. The use of defined single-strains

offers the advantage of identifying agglutinin-sensitive strains as sedimentation is encountered, without having to replace the entire culture, and also allows the testing of strains against cheese milk prior to their use in starters.

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CHAPTER 3

CHARACTERIZATION OF PARENT STRAINS AND PHAGE-INSENSITIVE MUTANTS OF LACTIC STREPTOCOCCI

ABSTRACT

Cultural characteristics of phage-insensitive mutants of lactic streptococci were compared to those of their respective parent strains. Traits examined included acid-producing activity, optimum growth temperature, generation time, proteolysis, phosphate and sodium chloride tolerance, phage adsorption, agglutination, morphology, and phage induction. Mutant strains showed variations in individual characteristics, but no general pattern of variation was observed. Overall, the phage-insensitive mutants were found to possess similar desirable characteristics and stabilities of the parent strains; consequently they have been successfully used in Cheddar and cottage cheese manufacture for more than three years without the need to rotate cultures as a phage control measure.

INTRODUCTION

The flavor of good quality Cheddar cheese is largely dependent on the starter strains selected for cheesemaking (35). Desirable strain characteristics for lactic starters include rapid acid production (14,42), phage insensitivity (15), absence of bacteriocins (4), and sensitivity to Cheddar cheese cook temperatures (30,35).

Phage-resistant mutants have been used in Cheddar cheese manufacture for approximately 10 years with varying success (8,20,26,33). Most mutant-associated difficulties have centered around reversions in phage sensitivity (20,24), increased host-ranges, sensitivity to new phages, and insufficient activity (26).

The present study compared a variety of characteristics of mutant strains with those of their parent strains. This was done in an effort to better understand the incidence and magnitude of altered strain properties as a consequence of selection for phage insensitivity and fast acid-production together in the same strains.

METHODS AND PROCEDURES

Mutant strain selection

Phage-insensitive mutants of Streptococcus cremoris and Streptococcus lactis parent strains were isolated as previously described (52). All strains were maintained in sterile reconstituted skim milk (RSM) and subcultured weekly.

Acid production

All parent strains and mutants examined were capable of coagulating RSM in 15 to 18 h at 21 C when inoculated at a 1% level. Acid production under simulated cheesemaking conditions was determined using a test similar to that described by Pearce (42) except that pH was measured directly after 5 h, rennet was omitted, and cook and cheddaring temperatures were increased 1 C to 39 and 38 C respectively.

Flavor

Whole milk cultures of parents and mutants were incubated overnight at 22 C and organoleptically evaluated to assess development of any off-flavor.

Antibiotic production

Compatibility of strains and possible bacteriocin production were checked by cross-streaking strains against each other on M17 agar (49), followed by incubation at 25, 30 and 37 C. In addition, whey from milk tubes coagulated with individual strains was spotted onto plates of M17 agar seeded with the individual strains used in this study. The absence of inhibitory zones indicated the strains likely were compatable. Whey from coagulated milk tubes was also used in the bromcresol purple milk test (BCP test) (44). Color development comparable to that of the control indicated compatability and absence of bacteriocins.

Optimum temperature

Strains were tested in one degree increments over a temperature range of 26 to 40 C. Bottles containing 100 ml of pasteurized skim milk were placed in a water bath held at the desired temperature (+/- 0.25 C) by a constant temperature immersion circulator. After tempering, individual bottles were inoculated (5%) with a single strain and mixed.

Cell growth was measured using a method similar to that of Kanasaki et al. (21) as modified by Heap and Lawrence (14). In this procedure, 0.5 ml of milk was aseptically drawn from an incubating sample and added to 4.5 ml of EDTA (0.2%, pH 12.5) to solubilize the casein micelles. The resulting solution was vigorously mixed on a vortex mixer and allowed to stand 3 to 5 min before reading to allow dissipation of incorporated air bubbles. A blank was prepared as above using pasteurized milk to which starter was added. Turbidity was read at 410 nm with a Perkin-Elmer 35 spectrophotometer.

Samples were taken hourly for the first two hours, then in half-hour intervals until coagulation occurred. Linear regression curves of the last five to seven readings before coagulation were determined and the reciprocal of slope was plotted against temperature to generate optimum temperature curves for each strain.

Generation time

Standard plate counts were made at 0, 2, 4 and 6 h on cultures grown in milk at 30 C (approximate optimum temperature for all strains examined). Growth rates (k) were determined by the first order rate equation $k = (\ln X2 - \ln X1)/(t2-t1)$, in which X2 and X1 are cell counts at times (t) 4 and 0 h respectively. Generation times (g) were calculated from k values with the equation: $g = .693/k$.

Proteolysis

The proteolytic activities of parent strains and mutants were determined colorimetrically using the Hull method (18) as modified by Citti et al. (6). Proteolysis was measured both after 16 h of incubation at 22 C, and after simulated cheesemaking conditions.

Pasteurized milk was divided into 50 ml aliquots which were individually inoculated with a single strain. Samples were incubated for 16 h at 22 C. The degree of casein hydrolysis was immediately determined after incubation.

Proteolysis under simulated cheesemaking conditions, was determined by inoculating duplicate 10 ml aliquots of pasteurized milk with a 2% inoculum of a single strain after which tubes were immediately subjected to the Pearce activity test as described above. Casein hydrolysis was measured upon completion of the activity test (after 5 h).

Phosphate tolerance

Phosphate milk. Milk solutions were prepared by reconstituting 62 g non-fat dry milk in 400 ml H_2O. Varied amounts (X) of phosphate salts (from Table 3.1) were added to 100-X ml of distilled water and mixed. Phosphate solutions and reconstituted milk (RSM) were autoclaved separately, and cooled in an ice bath. Phosphate-milk solutions were prepared by combining sterile RSM (462 g) and individual 100 g phosphate solutions (total wt 562 g). Phosphate concentrations (as PO_4 ion, rather than total phosphate salt) for phosphate-milk solutions were 0.5, 1.0, 1.5, and 2.0%.

Table 3.1. Amounts of added phosphates and final solution concentrations used in making milk and broth media for phosphate-tolerance determinations.[a]

Final % PO_4 ion	Combined wt (X)		% total salt final conc.
	g K_2HPO_4	g NaH_2PO_4	
0.5	3.39	2.05	0.97
1.0	6.78	4.10	1.94
1.5	10.16	6.15	2.90
2.0	13.55	8.20	3.87

[a]Approximately 50% of the PO_4 ion at each concentration was derived from each phosphate salt used.

Phosphate-milk aliquots of 10 ml were pipetted into tubes and then inoculated (2% level) with individual parent and mutant strains. Samples were incubated for 12 h at 30 C after which pH of sample tubes was recorded. Tubes of RSM were inoculated (2% level) from the incubated phosphate milk samples and incubated 10 h at 30 C. Coagulation of RSM tubes was recorded after incubation.

M17 phosphate broth. M17-phosphate broth was prepared as follows: A stock solution of M17 broth was prepared by adding to 1.6 liters H_2O the ingredients, except glycerophosphate, needed for 2 liters of broth. Phosphate solutions were prepared by adding varied amounts (X) of phosphate salts from Table 3.1 to 100-X ml H_2O. Broth stock solution and phosphate solutions were individually autoclaved and cooled. Respective phosphate solutions were added to 460 ml aliquots of broth stock solution and mixed to give final phosphate ion concentrations of 0.5, 1.0, 1.5, and 2.0%. Aliquots of 10 ml were pipetted into tubes and inoculated with 2% of an overnight M17 broth culture of the respective parent or mutant strain. The pH values were recorded after incubation for 12 h at 30 C. Tubes of RSM were inoculated (2% level) from

incubated M17-phosphate samples and incubated 10 h at 30 C. Coagulation of RSM tubes was recorded after incubation.

Titration curves were prepared for milk and broth solutions at both 0 and 2% phosphate ion (3.87% total phosphate) concentrations. Respective solutions in duplicate 10 ml quantities, were titrated with 1.0 M lactic acid. The pH was recorded after addition of each ml of acid. Titration curves for 0 and 2% phosphate ion levels were generated by plotting pH versus millimoles of acid added.

Salt tolerance

Reagent grade sodium chloride was added to sterile RSM and M17 broth in half-percent increments over the range of 0 to 5% (w/v) and thoroughly mixed. Milk and broth were pipetted in 10 ml quantities into sterile tubes and inoculated with 2% overnight culture of respective parent and mutant strains. Samples were incubated 15 to 17 h at 25 C after which milk coagulation and pH were recorded.

Phage adsorption

Adsorption of phage to bacteria was measured by the method of Terzaghi and Terzaghi (50). Bacteriophage and culture volumes were increased from 0.05 ml to 0.1 ml to reduce volume errors.

Phage induction

Parent strains and mutants were examined for lysogeny by treatment with Mitomycin C (MC). A range of MC-doses including 1, 2, and 4 μg/ml was used to induce temperate phages as recommended by Meister and Ledford (39). Tubes of M17 broth were inoculated with 3% overnight culture of a respective strain then incubated for 3 h at 30 C to allow the strain to reach mid-exponential phase of growth. Doses of MC were added to the broth cultures which were further incubated overnight at 30 C.

Screening for indicators and lysogens was accomplished by the method of Reyrolle et al. (46). Filtrates were prepared and pooled in groups of five from individual cultures of MC-induced strains. Twelve groups total represented three strain-groupings with a control and three MC-dose levels for each group of strains. Filtrates were spotted onto M17 plates seeded with the individual strains used in the study. Plates were incubated at 25, 30 and 37 C. When plaques were observed, individual filtrates were tested.

Agglutination

Sensitivities of parent strains and their phage-insensitive mutants to agglutinins in milk were determined as previously described by Thunell et al. (53).

RESULTS

Mutant stability

Table 3.2 lists parent strains and their phage-insensitive mutants used in this study. All parent and mutant strains have been used in commercial cheesemaking. Mutant stability (phage insensitivity), as represented by the months used in actual cheesemaking, is listed in Table 3.3. While mutants M290-PC, 108-1, SK11G-W, SK11G-D and BA-1 RM displayed shorter stabilities than other phage-insensitive mutants, used in this study, the duration of their phage-insensitivity extended long enough to justify their use as starter strains. Strain 134 is a phage-resistant mutant of AM2 isolated in New Zealand (26).

Acid production

Acid production by parent strains and mutants under simulated cheesemaking conditions was compared by the Pearce Activity Test (Table 3.4). Acid production by mutant strains varied with each respective mutant. With exception of strains 108-C and C13/25, acid production by mutant strains was approximately equal to or greater than that of the parent strain under simulated cheesemaking conditions. It should also be noted that acid production varied widely between individual parent strains.

Growth characteristics

Figures 3.1 through 3.7 show growth characteristics of parent and mutant strains grown in pasteurized skim milk over the temperature range of 26 to 40 C. All of the strains examined, both parents and mutants, displayed temperature optima in the range of 31 to 34 C. None of the *S. cremoris* parent or mutant strains examined were capable of growth above 38 C. However, *S. lactis* strain BA-1 and its mutant BA-1 RM showed only slightly slower growth at 40 C (Figure 3.7). Strain 224 possessed a narower growth

Table 3.2. Parent strains and phage-insensitive mutants used in comparative evaluations of mutant strain characteristics.

Parent	Mutant
290-P	290-PC M290-PC
108	108-C 108-12
SK11G	SK11G-C
224	224-3
C13	C13/25
U-134[a]	---[b]
BA-1	BA-1 RM

[a]Phage resistant mutant of AM_2 isolated in New Zealand (26).

[b]The need to select a mutant for this strain has not arisen.

Table 3.3. Mutant stability as indicated by number of continuous months used in cheesemaking.

Mutant	Plant A	Plant B	Plant C
290-PC	17+	27+	27+
M290-PC	6	--	--
108-12	--	29+	--
108-1	--	3	--
108-C	14+	--	--
SK11G-W	9	--	--
SK11G-C	26+	26+	27+
SK11G-D	--	10	--
224-3	35+	38+	28+
C13/25	14+	23+	24+

+ = still in use as of October, 1982.

Table 3.4. Comparison of acid production by single strains and their phage-insensitive mutants under simulated cheese-making conditions (Pearce activity test).

Strain	Δ pH	
	$\bar{X}$	s
290-P	.72	.06
290-PC	.90	.08
M290-PC	.65	.03
108	1.33	.08
108-C	.49	.04
108-12	1.34	.05
SK11G	.67	.04
SK11G-C	1.20	.10
SK11G-W	.68	.05
224	.82	.04
224-3	.79	.07
C13	1.05	.06
C13/25	.61	.03
U-134[b]	.91	.07
BA-1	1.40	.05
BA-1 RM	1.53	.03

[a] $\bar{X}$ = Mean of repeated in-duplicate (4 tubes in all) trials.

[b] The need to select a mutant for this strain has not arisen since no phage for it has appeared.

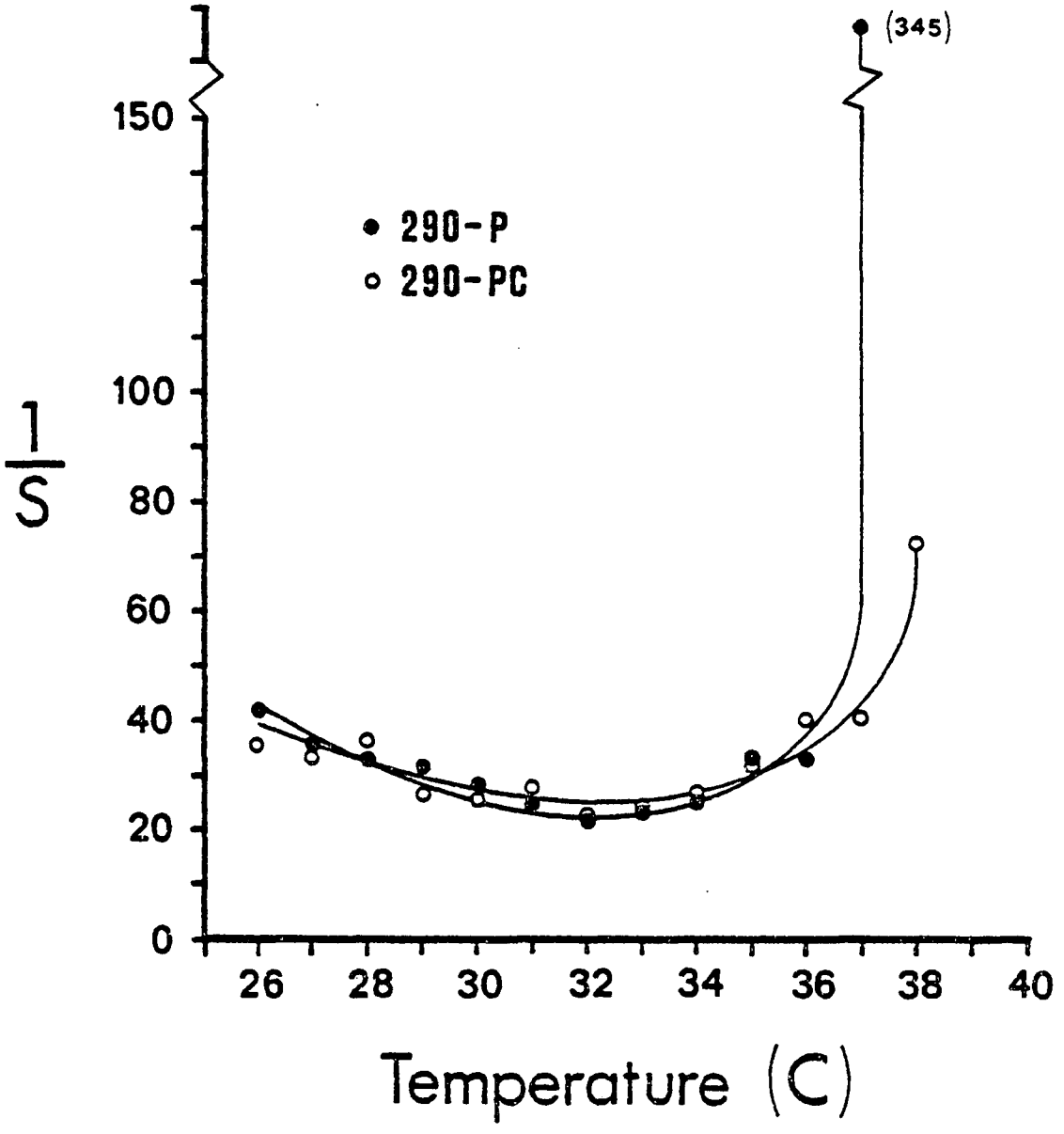

Figure 3.1. Growth of strain 290-P and its phage-insensitive mutant 290-PC in pasteurized skim milk at 26 to 40 C.

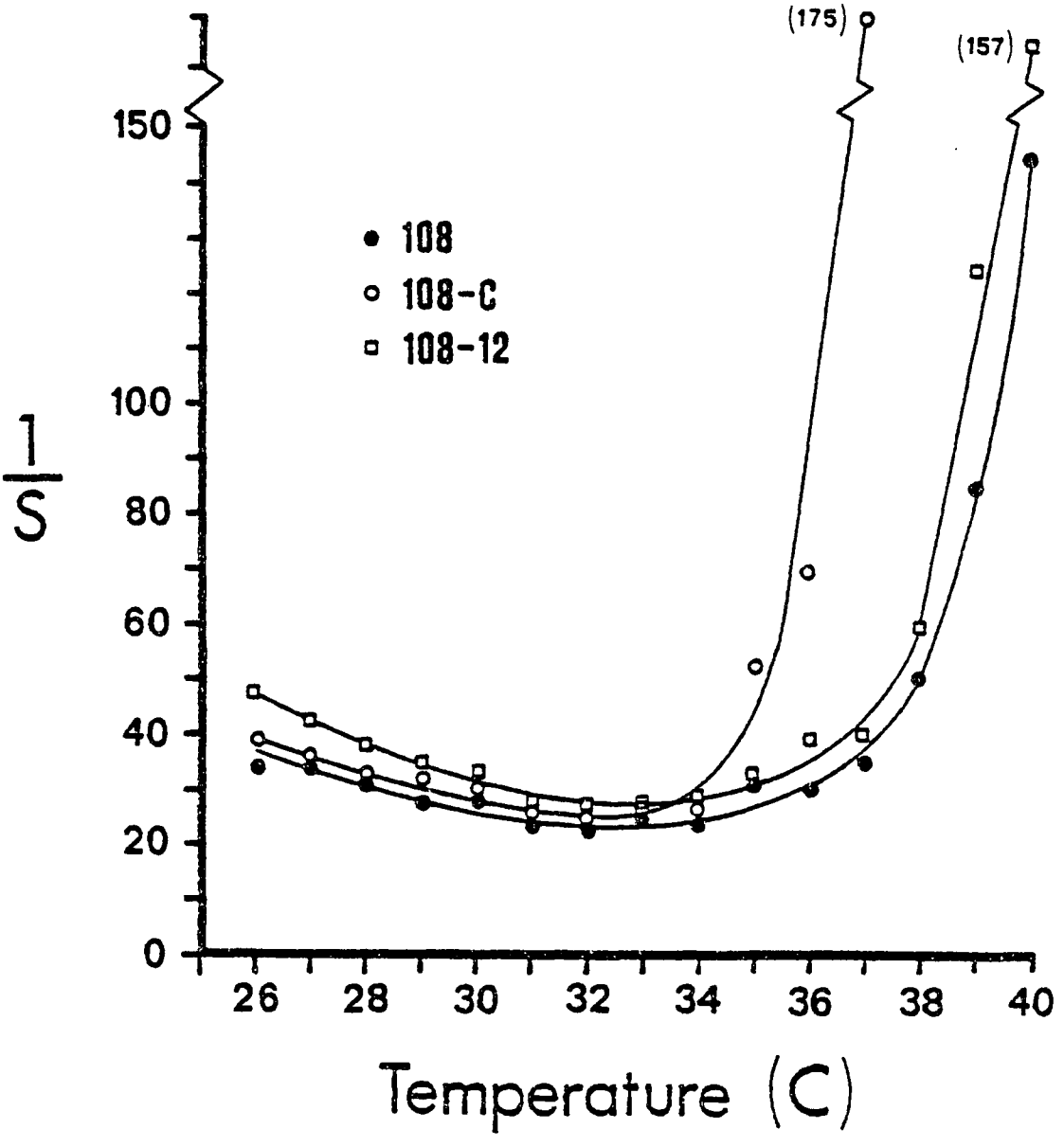

Figure 3.2. Growth of strain 108 and its phage-insensitive mutants 108-C and 108-12 in pasteurized skim milk at 26 to 40 C.

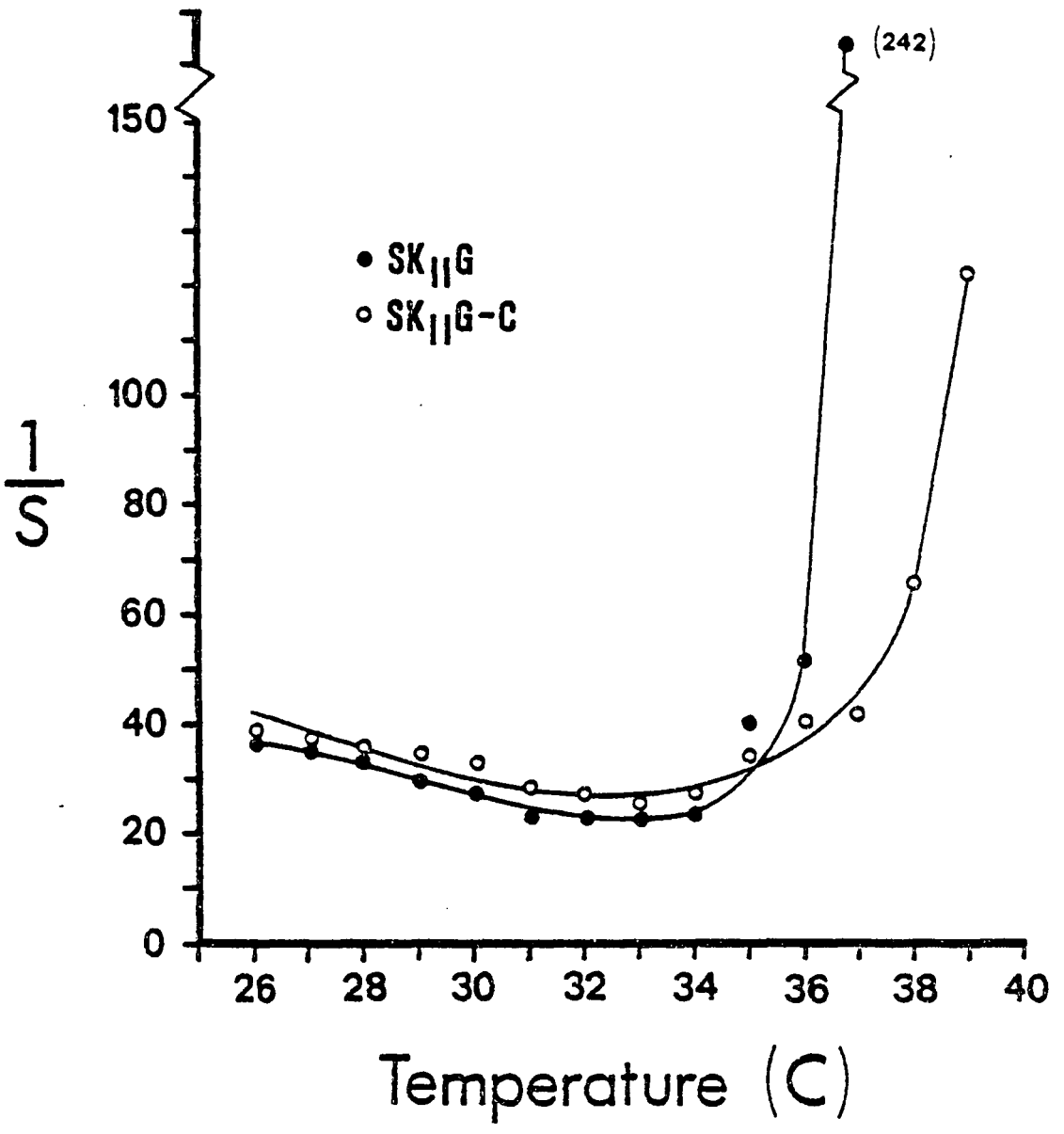

Figure 3.3. Growth of strain SK11G and its phage-insensitive mutant SK11G-C in pasteurized skim milk at 26 to 40 C.

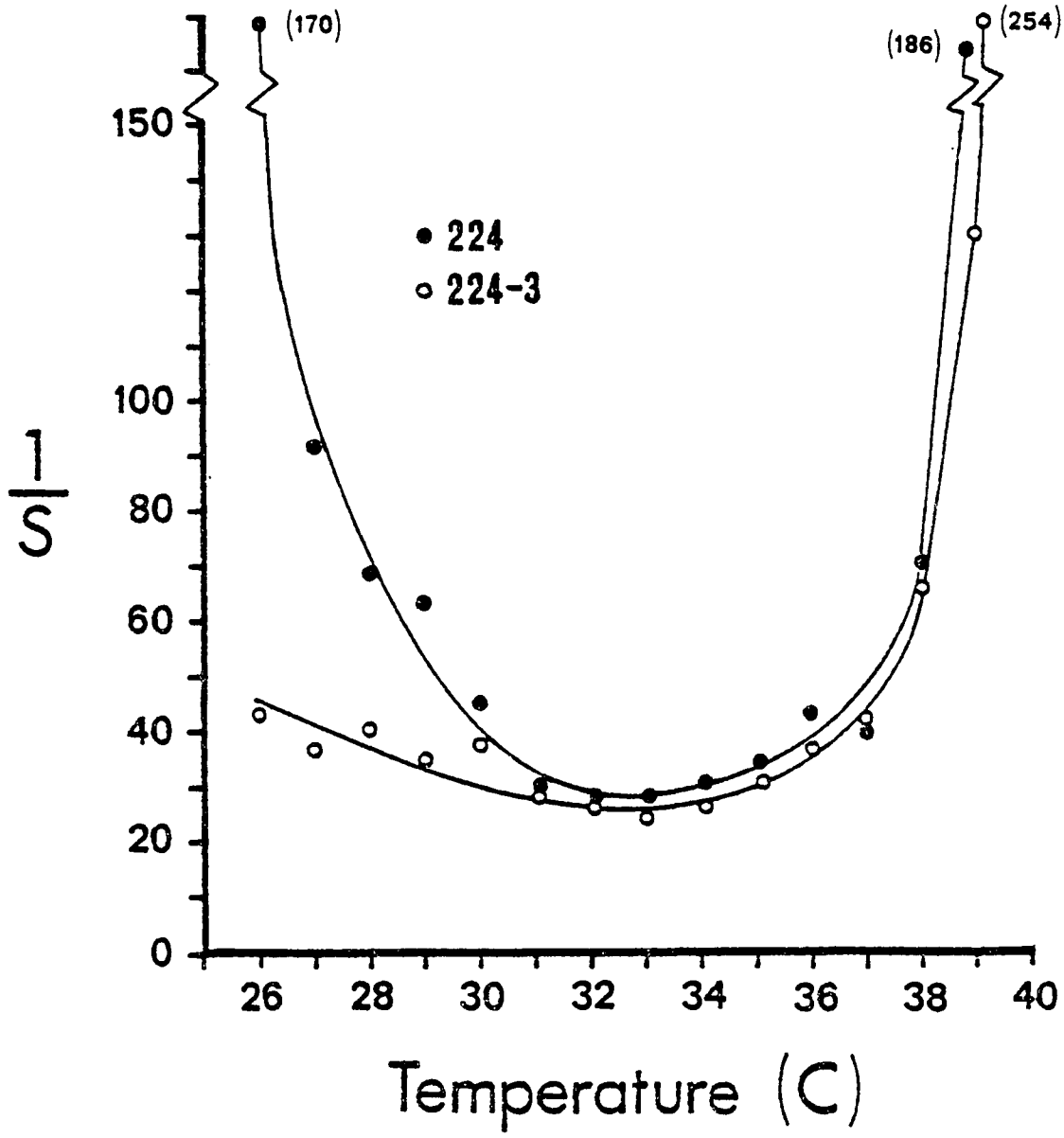

Figure 3.4. Growth of strain 224 and its phage-insensitive mutant 224-3 in pasteurized skim milk at 26 to 40 C.

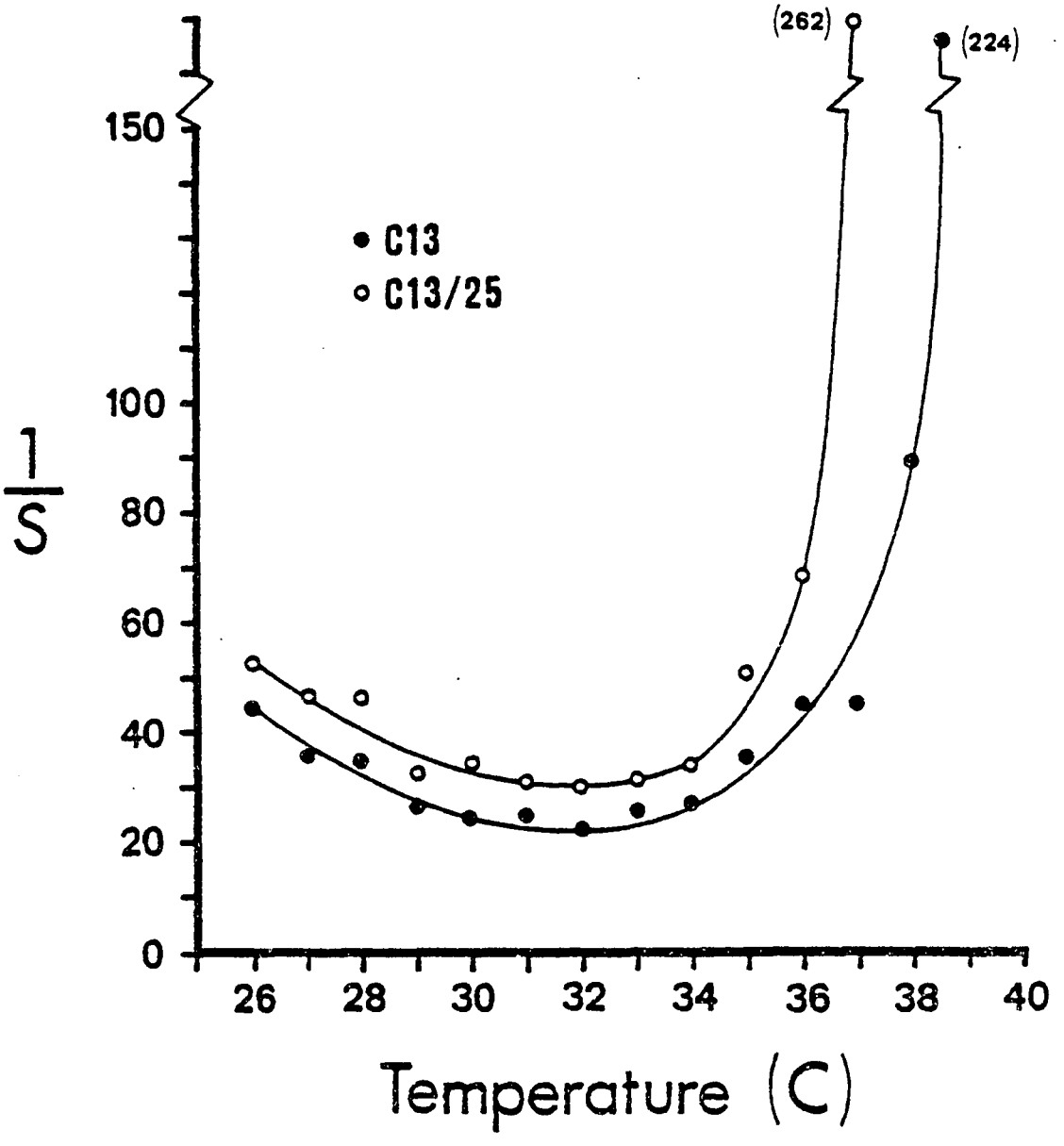

Figure 3.5. Growth of strain C13 and its phage-insensitive mutant C13/25 in pasteurized skim milk at 26 to 40 C.

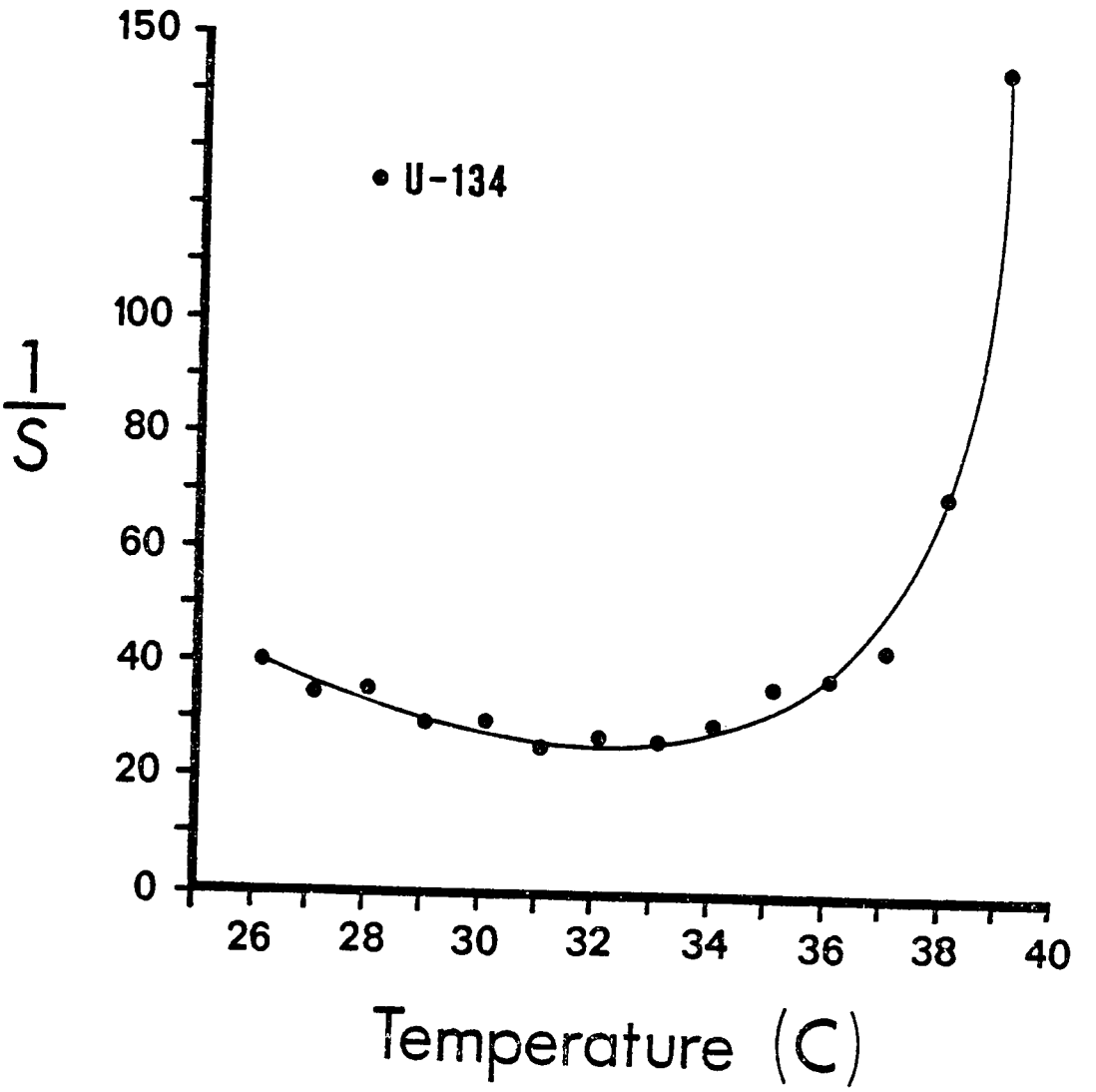

Figure 3.6. Growth of strain U-134 in pasteurized skim milk at 26 to 40 C.

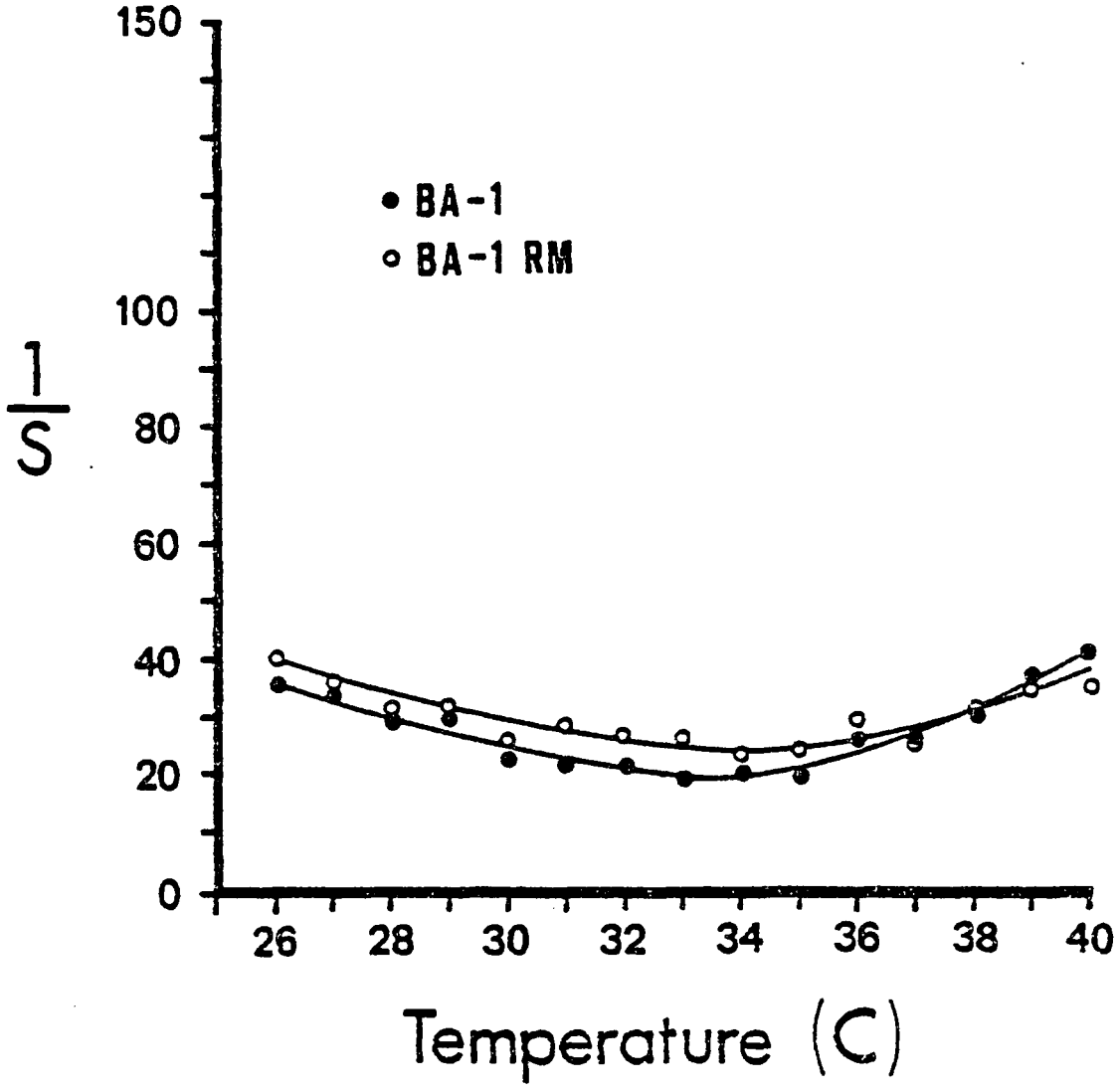

Figure 3.7. Growth of strain BA-1 and its phage-insensitive mutant BA-1 RM in pasteurized skim milk at 26 to 40 C.

Table 3.5. Generation times for selected parent and mutant strains grown at 33 C (approximate optimum temperature) in pasteurized skim milk.

Strain	Gen. Time (min)
290-P	50.0
290-PC	56.7
108	56.1
108-C	58.6
108-12	31.25
SK11G-C	54.0
224	64.2
224-3	50.8
C13	46.2
C13/25	54.8
U-134	73.2
BA-1	46.0
BA-1 RM	50.6

range than its phage-insensitive mutant 224-3 (Figure 3.4). Growth characteristics within the temperature range of 26 to 31 C were approximately the same for all parents and mutants, with exception of parent strain 224 which did not grow well in this lower temperature range.

Generation times

Generation times for parent and mutant strains grown in pasteurized milk at 33 C, are listed in Table 3.5. Strain 108-12, a second-line mutant (mutant of a mutant) possessed the shortest generation time of the examined strains; 31.25 min, which was almost half that of its parent 108 and of a first-line mutant 108-C. Generation times for other examined strains ranged between 46 and 73 min.

Proteolysis

Table 3.6 summarizes the extent of proteolysis of pasteurized skim milk by single strains and mutants after 16 h of incubation at 21 C, and after growth under simulated Cheddar cheesemaking conditions (Pearce activity test). The extent of casein proteolysis, after 16 h incubation, was approximately equal for both parent and mutant strains, with exceptions of strains 290-P and C13. Parent strain 290-P exhibited approximately one-third of the proteolytic activity exhibited by other parental strains, while its phage-insensitive mutant (290-PC) showed proteolytic activity similar to that of other parent-strains. Mutant strain 290-PC was more proteolytic than its parent, whereas mutant strain C13/25 showed a ten-fold decrease in proteolytic activity compared to the parent strain.

Proteolysis under simulated cheesemaking conditions was understandably less than that found during extended incubation due to shorter incubation and higher temperatures involved. Proteolysis under these conditions was one-fourth to one-half of that after

Table 3.6. Proteolysis of pasteurized skim milk by single strains as measured by the Hull method after growth for 16 h at 21 C and after simulated Cheddar cheesemaking conditions (Pearce activity test).[a]

Strain	16 h, 21 C	Simulated cheesemaking
Control	0	0
290-P	13	8
290-PC	52	16
108	48	23
108-C	45	13
108-12	45	22
SK11G	52	18
SK11G-C	50	13
224	43	10
224-3	47	14
C13	61	26
C13-25	6	6
U-134	50	16
BA-1	48	27
BA-1 RM	48	24

[a]2% inoculum

incubation at 21 C for 16 h. Mutant strain C13/25 was a notable exception since its proteolytic activity (least displayed by any examined strains) was similar under both incubation conditions.

Phosphate tolerance

Tables 3.7 and 3.8 show the growth responses (indicated by pH achieved) of various single strains grown in milk and M17 broth containing different levels of phosphates. Acid production, indicated by millimoles of lactic acid produced, were calculated from titration curves (Figures 3.8 and 3.9) and are summarized in Table 3.9. Of seven phage-insensitive mutants examined, four showed greater tolerance than parents to high phosphate levels in milk, one was equally tolerant, and two were less phosphate tolerant. When grown in phosphated-M17 broth, three mutants exhibited equivalent phosphate tolerance, and four mutants were less tolerant of phosphate, compared to their respective parent strains. Inocula from incubated milk and broth tubes containing 2% PO_4 ion were sufficiently active to coagulate RSM in 10 h at 30 C. Although some strains were slightly inhibited by high phosphate levels, and any cellular damage was apparently reversible, and did not result in prolonged lag phases when inoculated into RSM.

Sodium chloride tolerance

Figures 3.10 through 3.16 show salt tolerance patterns for various lactic strains and mutants, as the result of growth (measured by pH attained) in broth and milk, which contained increment percentages of sodium chloride. Of seven mutants examined, two showed higher salt tolerance in milk than their parents, two were equivalent, and three were less tolerant. When grown in broth, three were more tolerant than their parents, three equivalent to, and one less tolerant than their parents. Acid production in milk by the various S. cremoris parent strains and mutants, was sharply decreased in the NaCl concentration range of

Table 3.7. Growth responses of various single strains grown in skim milk containing different levels of added phosphates.[a]

	pH				
		Percent phosphate as PO_4			
Strain	RSM Control	0.5	1.0	1.5	2.0
290-P	4.58	4.76	4.99	5.55	6.38
290-PC	4.58	4.73	4.93	5.87	6.37
108	4.46	4.66	4.84	5.28	5.70
108-C	4.53	4.66	4.88	6.95	6.53
108-12	4.45	4.63	4.82	5.21	5.46
SK11G	4.45	4.64	4.94	5.60	6.16
SK11G-C	4.47	4.69	4.88	5.20	5.80
224	4.57	4.85	5.34	6.18	6.43
224-3	4.57	4.82	5.06	5.95	6.37
C13	4.49	4.66	4.90	5.52	6.17
C13/25	4.52	4.72	5.09	5.76	6.44
U-134	4.51	4.68	4.90	5.65	6.24
BA-1	4.49	4.66	4.85	4.91	5.62
BA-1 RM	4.50	4.66	4.82	4.98	5.27
Uninoculated milk	6.40	6.70	6.70	6.70	6.70

[a]2% inoculum, incubated 12 h at 30 C

Table 3.8. Growth response of various single strains grown in M17 broth containing different levels of added phosphates.[a]

	pH				
		Percent phosphate as PO_4			
Strain	M17 Control	0.5	1.0	1.5	2.0
Control	5.43	6.79	6.82	6.83	6.85
290-P	4.36	4.58	5.58	6.12	6.34
290-PC	4.47	4.98	6.08	6.45	6.54
108	4.26	4.47	5.53	6.10	6.32
108-C	4.56	5.22	6.17	6.48	6.59
108-12	4.32	5.44	6.28	6.53	6.63
SK11G	4.41	4.64	5.56	6.16	6.38
SK11G-C	4.25	4.49	5.52	6.10	6.32
224	4.34	4.53	5.52	6.09	6.32
224-3	4.40	4.68	5.62	6.15	6.44
C13	4.31	4.70	5.58	6.23	6.46
C13/25	4.55	5.61	6.35	6.60	6.72
U-134	4.41	4.76	5.68	6.17	6.35
BA-1	4.26	4.39	5.28	5.97	6.24
BA-1 RM	4.27	4.41	5.28	5.97	6.24

[a]2% inoculum, incubated 12 h at 30 C.

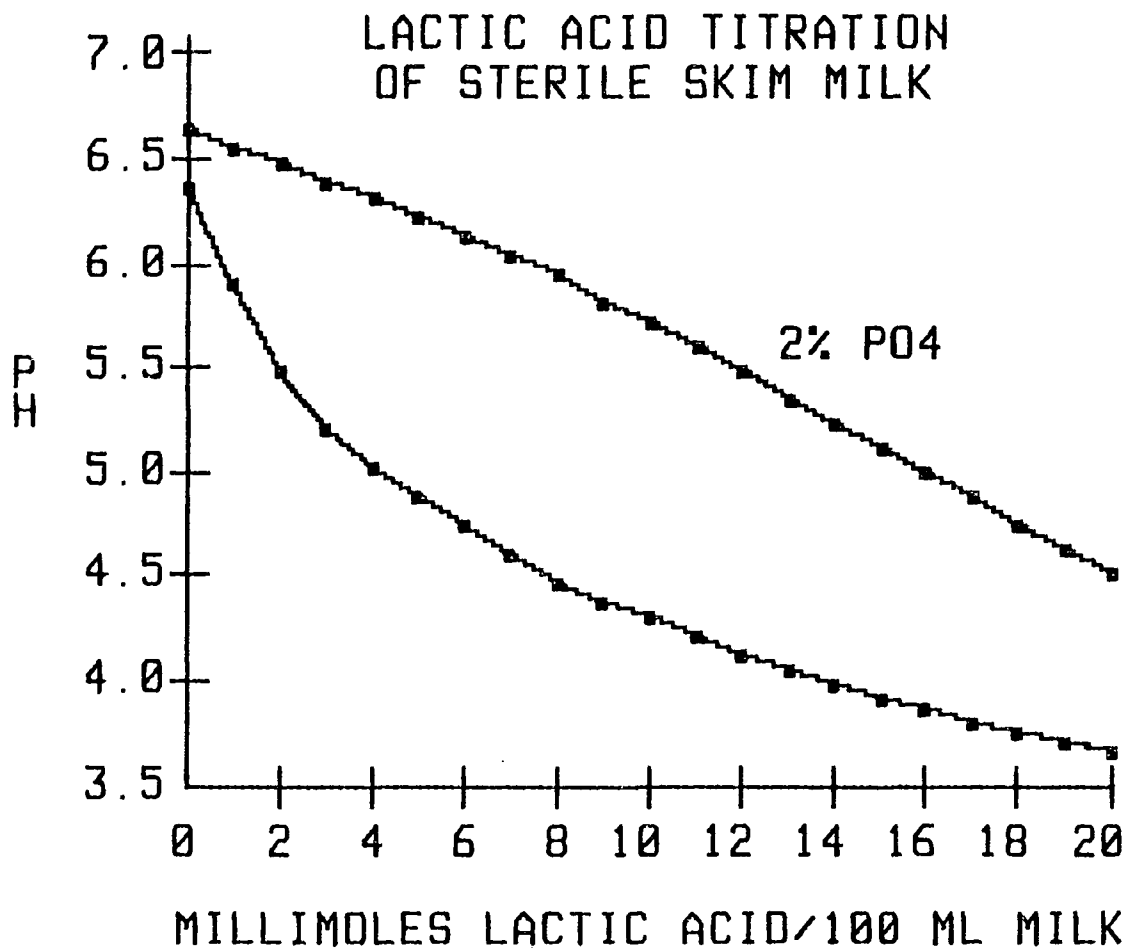

Figure 3.8. Titration curves for skim milk, with and without 2% added phosphate ion (3.87% total phosphate), titrated with 1.0 N lactic acid.

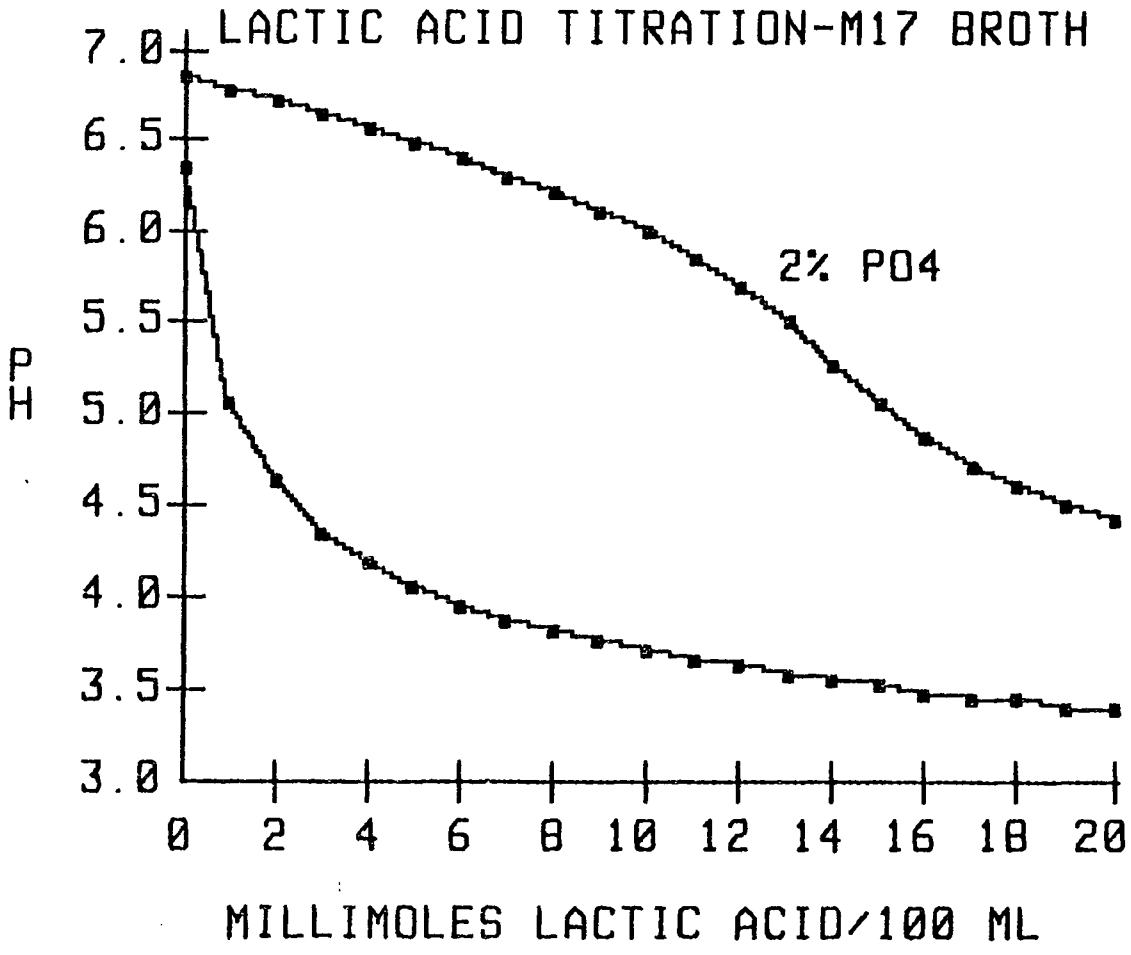

Figure 3.9. Titration curves for M17 broth, with and without 2% added phosphate ion (3.8% total phosphate), titrated with 1.0 N lactic acid.

Table 3.9. Comparison of acid production by single strains grown in milk and broth controls and in 2% PO_4-ion buffered media.[a]

	Millimoles lactic acid produced			
	Milk		M17 Broth	
Strain	Control	2% PO_4	Control[b]	2% PO_4
290-P	.70	.40	.10	.65
290-PC	.70	.40	.30	.40
108	.80	1.00	.37	.68
108-C	.55	.20	.25	.36
108-12	.80	1.20	.35	.35
SK11G	.80	.60	.28	.60
SK11G-C	.80	.92	.32	.68
224	.70	.25	.30	.68
224-3	.70	.40	.28	.58
C13	.80	.55	.30	.52
C13/25	.76	.30	.24	.20
U-134	.75	.50	.28	.65
BA-1	.80	1.10	.37	.76
BA-1 RM	.78	1.36	.36	.76

[a]Values determined from titration curves

[b]Unbuffered

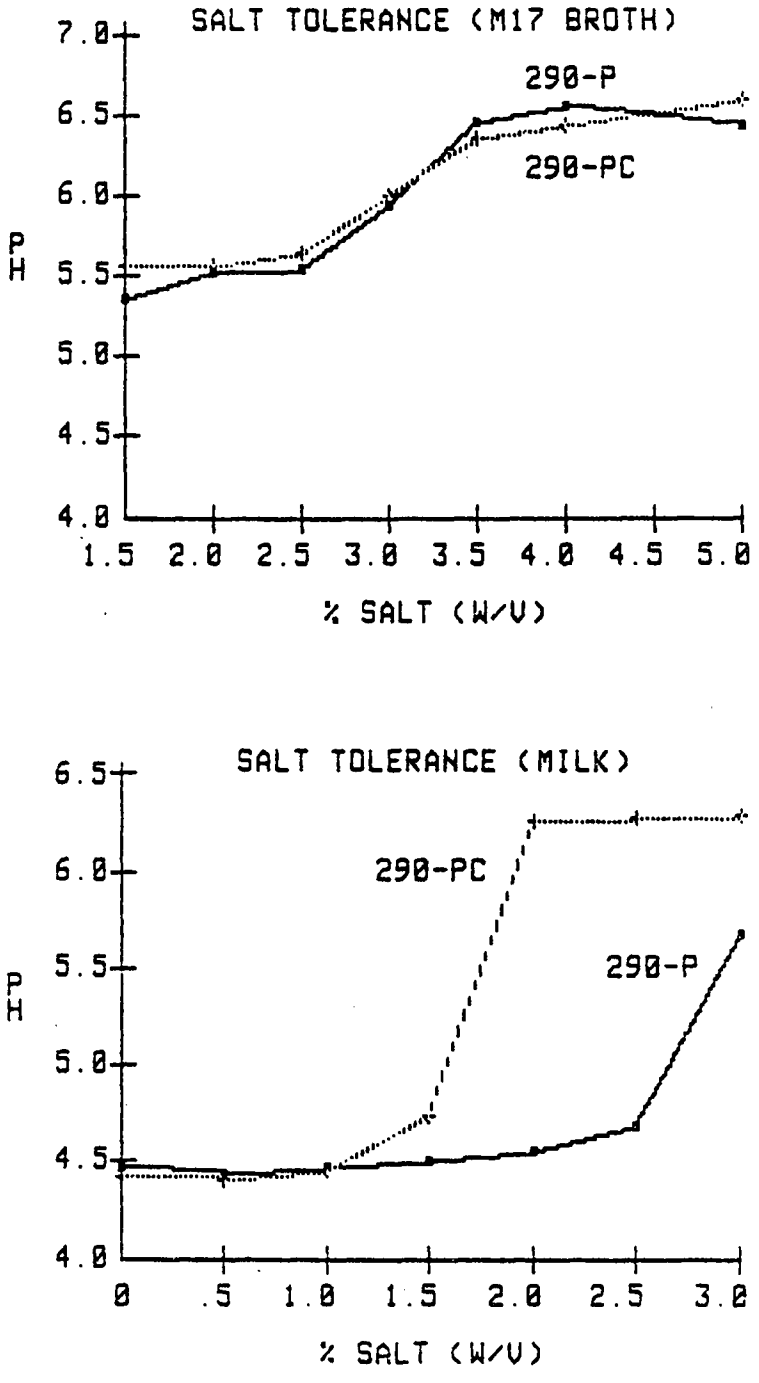

Figure 3.10. Salt tolerance of S. cremoris 290-P and its phage-insensitive mutant 290-PC as measured by pH attained during growth at 25 C for 15 h in broth and milk containing the indicated levels of sodium chloride.

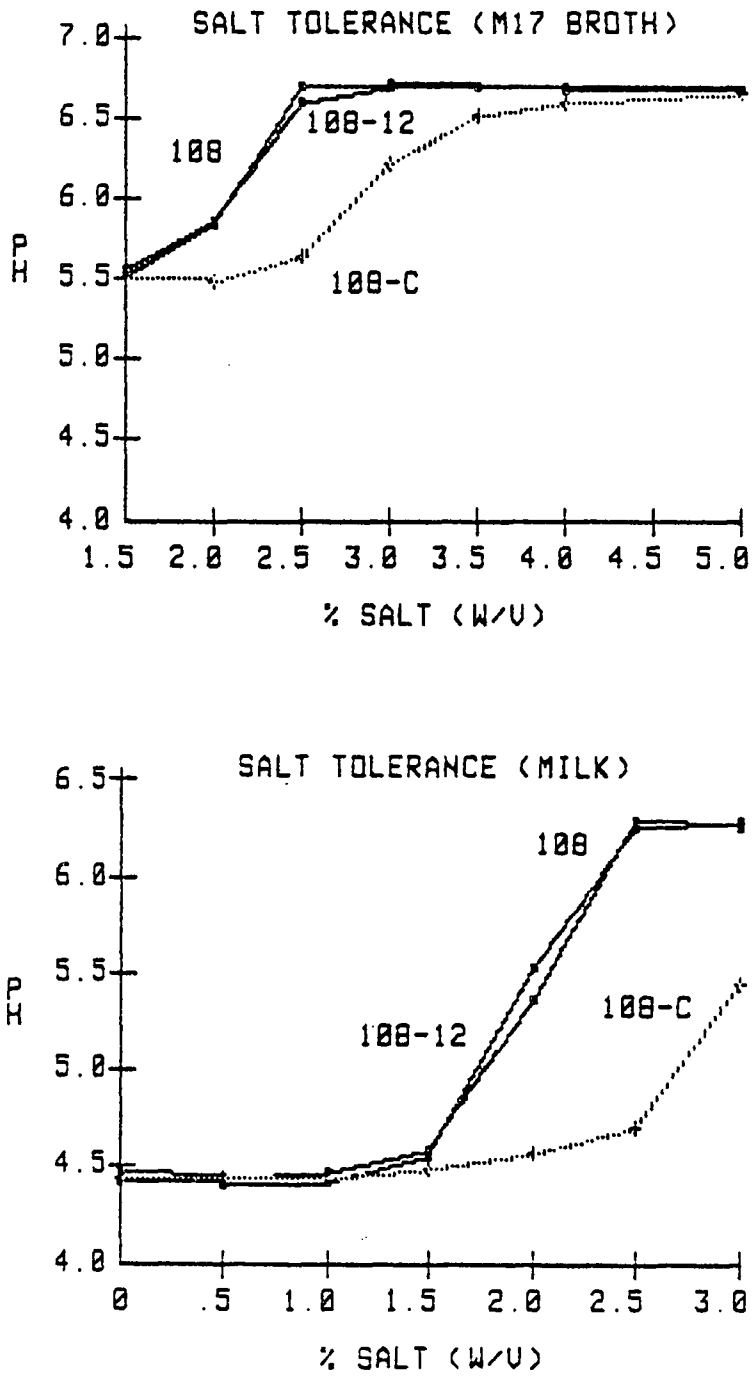

Figure 3.11. Salt tolerance of S. cremoris 108 and its phage-insensitive mutants 108-C and 108-12 as measured by pH attained during growth at 25 C for 15 h in broth and milk containing the indicated levels of sodium chloride.

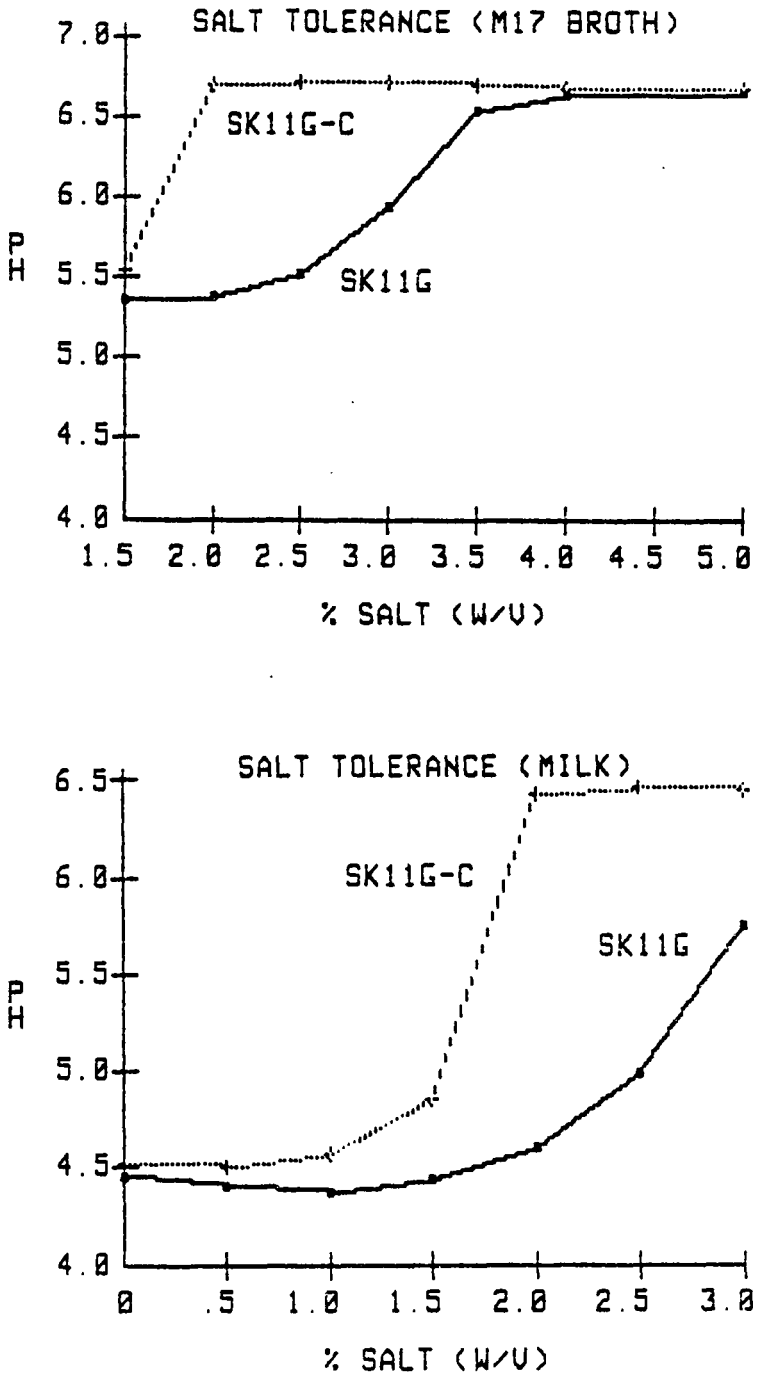

Figure 3.12. Salt tolerance of S. cremoris SK11G and its phage-insensitive mutant SK11G-C as measured by pH attained during growth at 25 C for 15 h in broth and milk containing the indicated levels of sodium chloride.

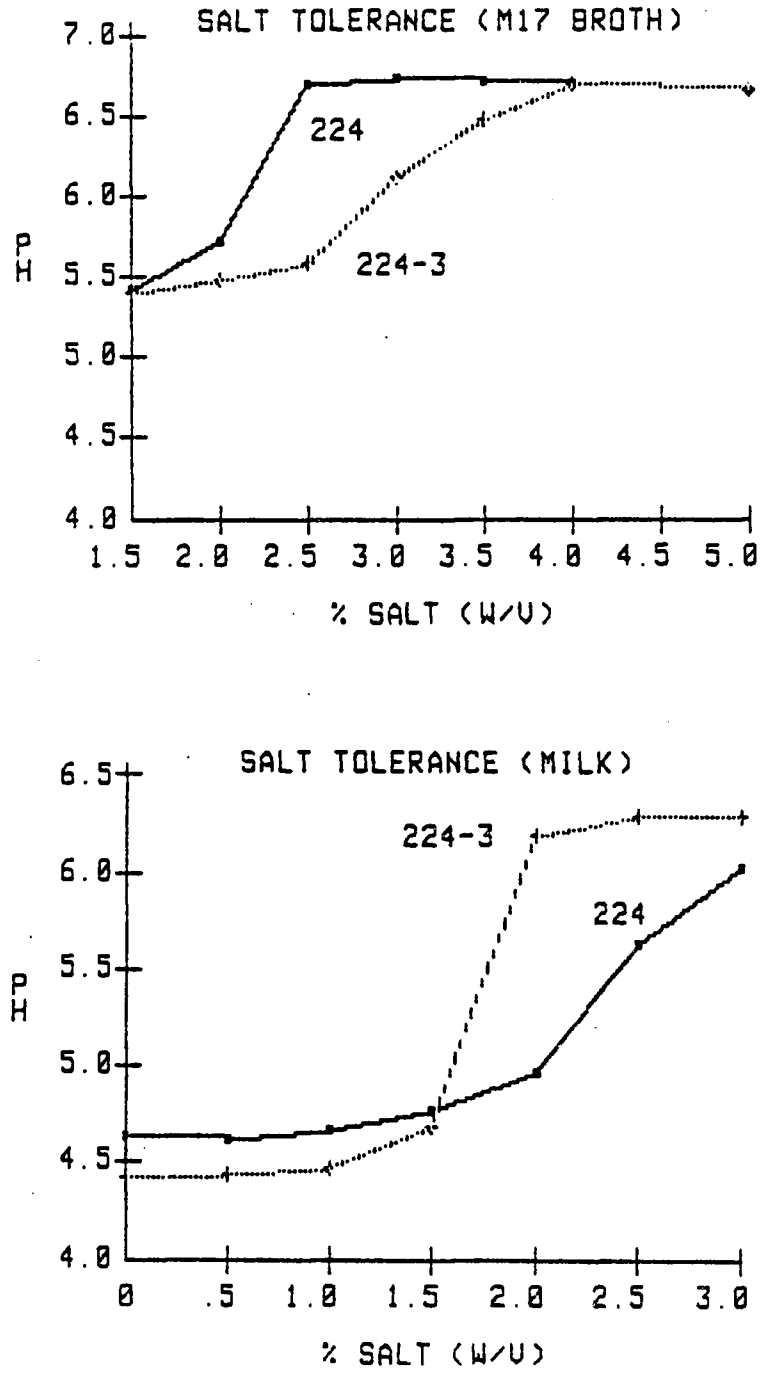

Figure 3.13. Salt tolerance of S. cremoris 224 and its phage-insensitive mutant 224-3 as measured by pH attained during growth at 25 C for 15 h in broth and milk containing the indicated levels of sodium chloride.

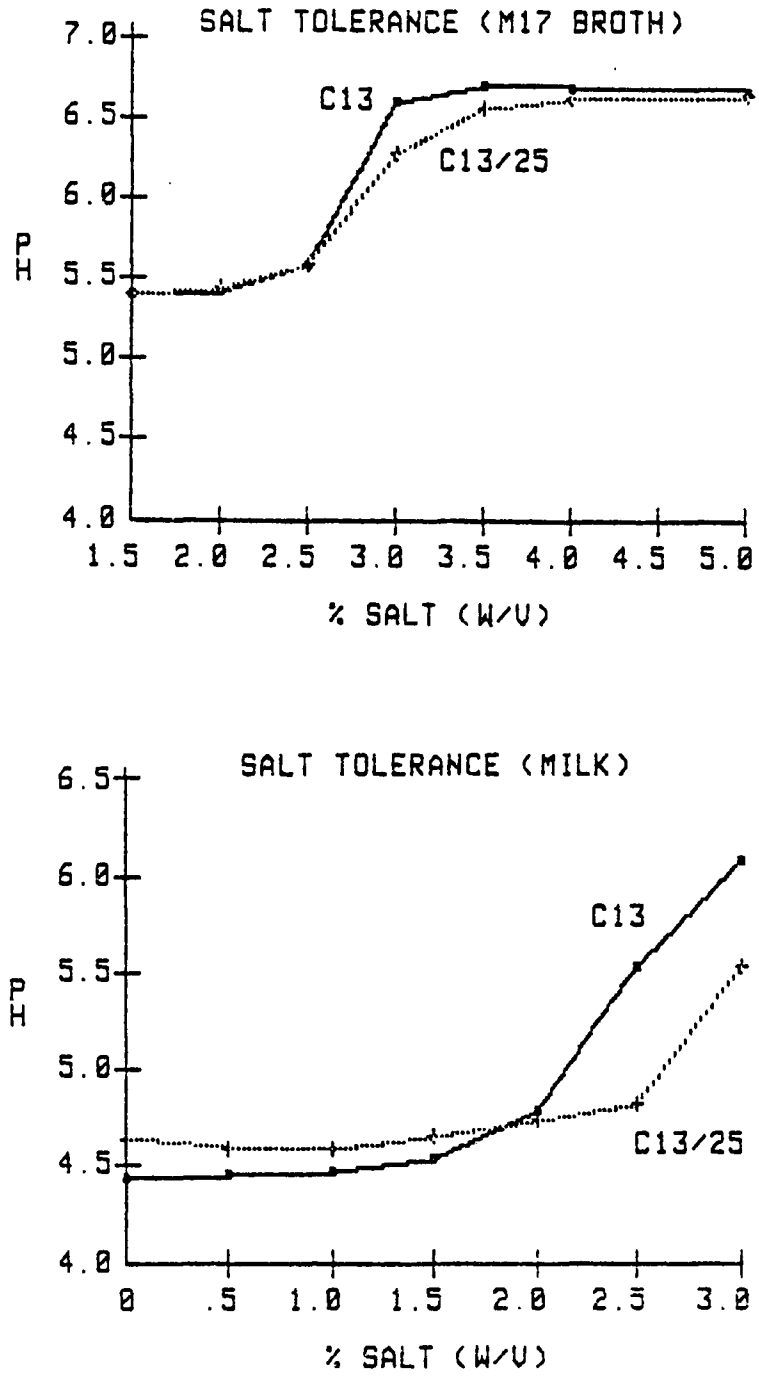

Figure 3.14. Salt tolerance of S. cremoris C13 and its phage-insensitive mutant C13/25 as measured by pH attained during growth at 25 C for 15 h in broth and milk containing the indicated levels of sodium chloride.

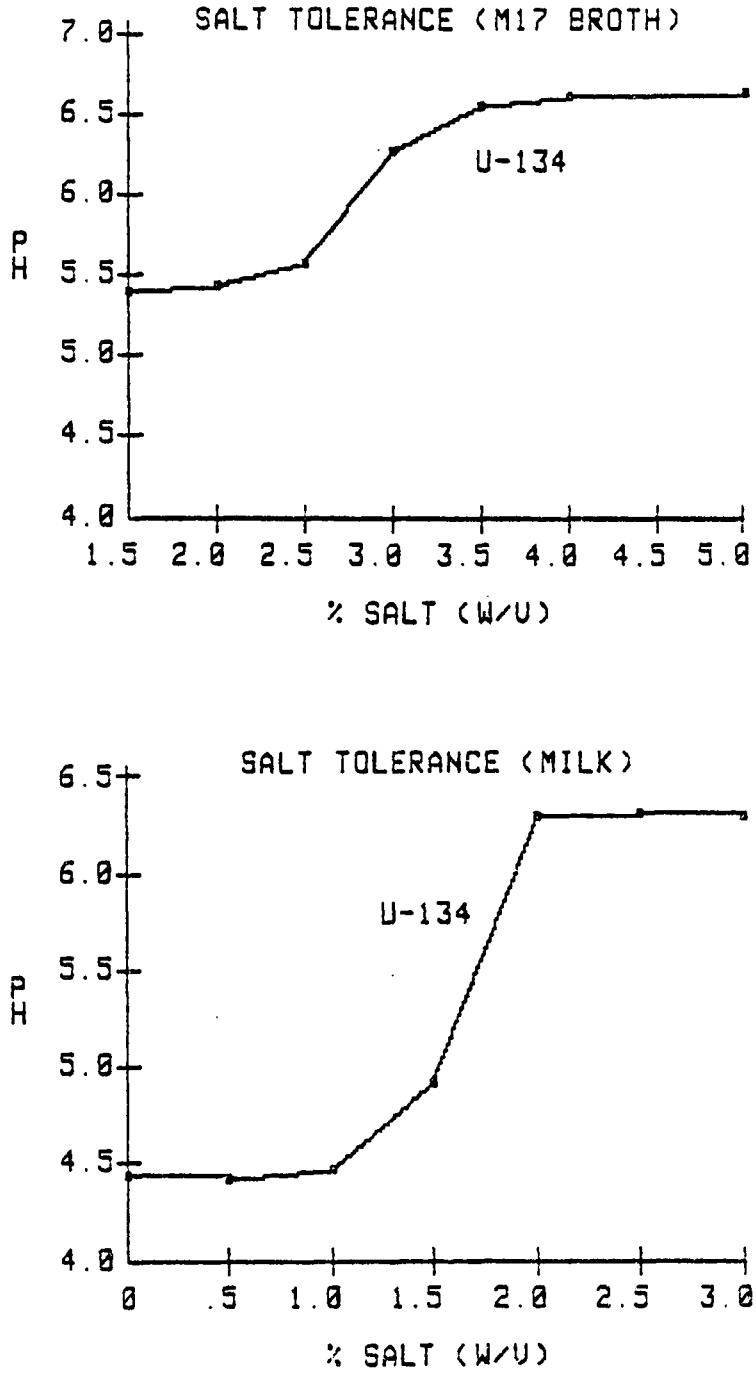

Figure 3.15. Salt tolerance of S. cremoris U-134 as measured by pH attained during growth at 25°C for 15 h in broth and milk containing the indicated levels of sodium chloride.

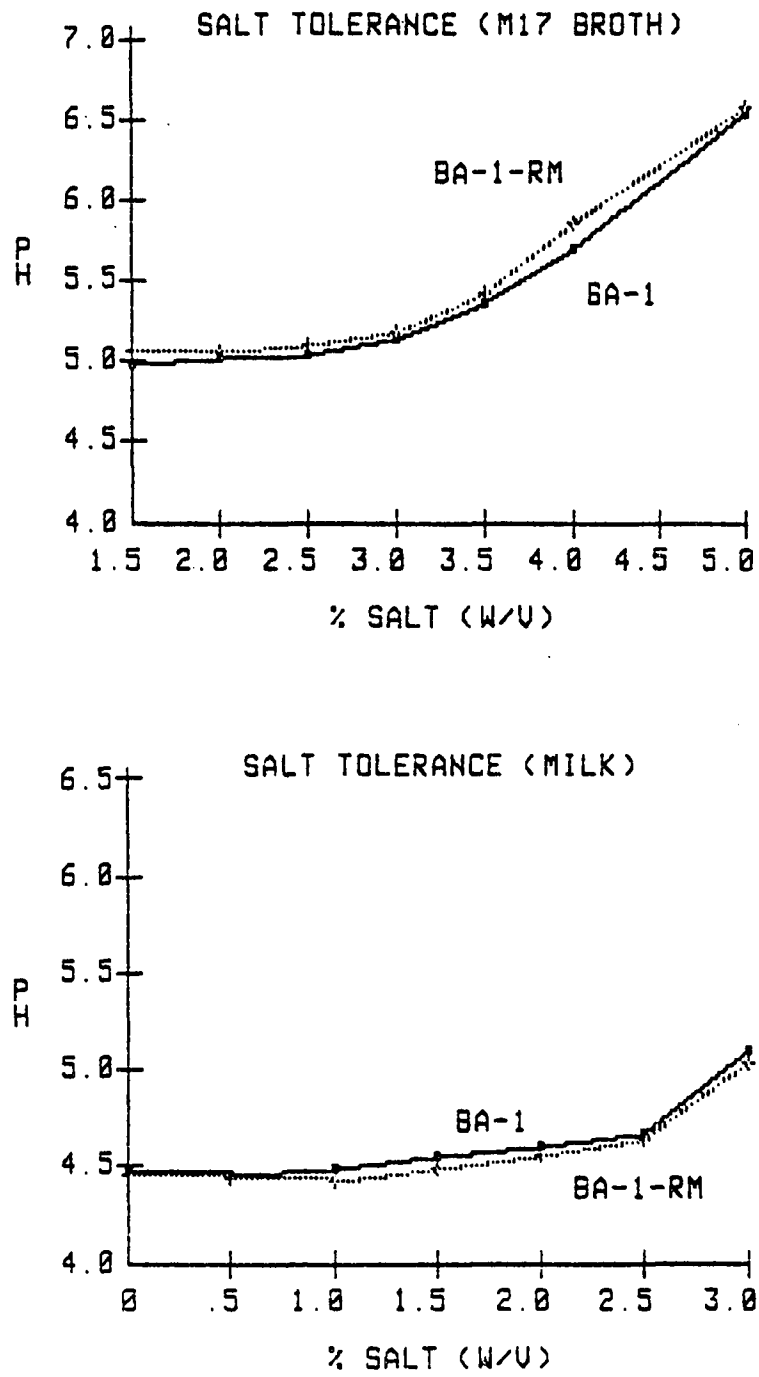

Figure 3.16. Salt tolerance of S. lactis BA-1 and its phage-insensitive mutant BA-1 RM as measured by pH attained during growth at 25 C for 15 h in broth and milk containing the indicated levels of sodium chloride.

1.5 to 2.5%, while S. lactis BA-1 and its mutant were affected by NaCl concentrations between 2.5 and 3.0%. Acid production and growth in M17 broth, which contained various levels of salt, was somewhat better than in milk. All strains, except SK11G-C and 224, could tolerate 0.5 to 1.0% higher salt in broth than in milk. Salt tolerance for strains SK11G-C and 224 remained the same in both growth media (Figures 3.12 and 3.13).

Phage adsorption

Table 3.10 compares the phage adsorption patterns for selected strains and their phage-insensitive mutants. With the exception of strain 224-3, which adsorbed only 38% of phage 205, all of the other mutant strains were resistant to adsorption by respective phages. From these results, it would appear that mutant phage-insensitivity was due primarily to alteration of the receptor sites on bacterial cell surfaces rather than to a host restriction-modification system.

Phage induction

Phage-induction with mitomycin C revealed no apparent inducible phages in either the parent or the mutant strains, using all parent and mutant strains examined in this study as indicator strains. Supernatants from induced strains tested with the BCP activity test showed no inhibition of acid production which was interpreted as an absence of lytic phages.

Morphology

Morphologies of parent strains and phage-insensitive mutants used in this study are shown in Figures 3.17 to 3.23. Mutant strains 290-PC, 108-C, and 108-12 displayed increased chaining. Other mutant morphologies were similar to their parents.

Table 3.10. Comparison of phage adsorption patterns for selected single strains and their phage-insensitive mutants.

Strain	Phage	% Adsorption
290-P	210	89
290-PC	210	0
108	219	40
108-C	219	0
SK11G-C	170	80
SK11G-C	170	0
224	205	31
224-3	205	12
C13	178	42
C13/25	178	0
BA-1	101	16
BA-1 RM	101	0

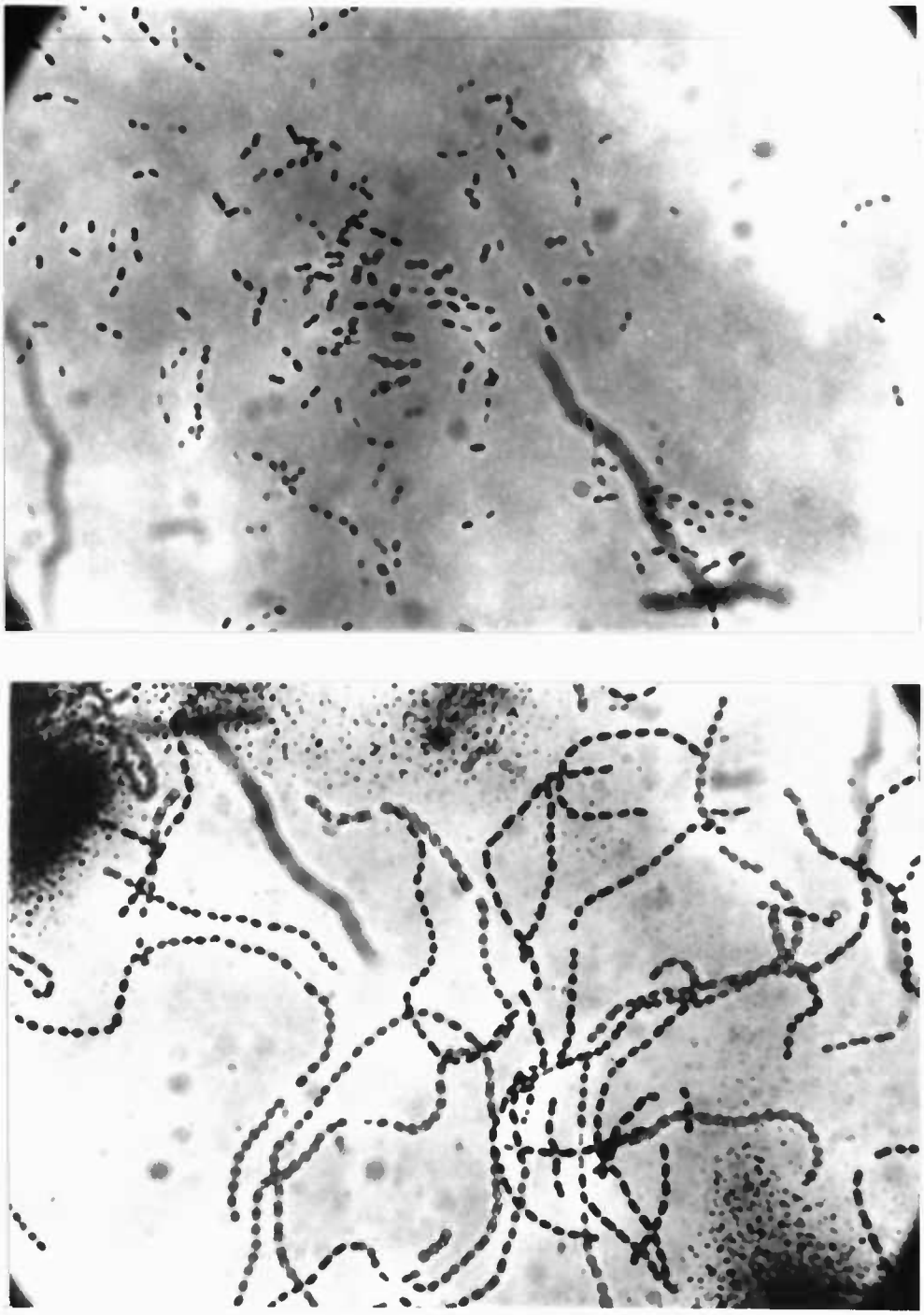

Figure 3.17. Morphological comparison of S. cremoris parent strain 290-P (above) and its phage-insensitive mutant 290-PC (below).

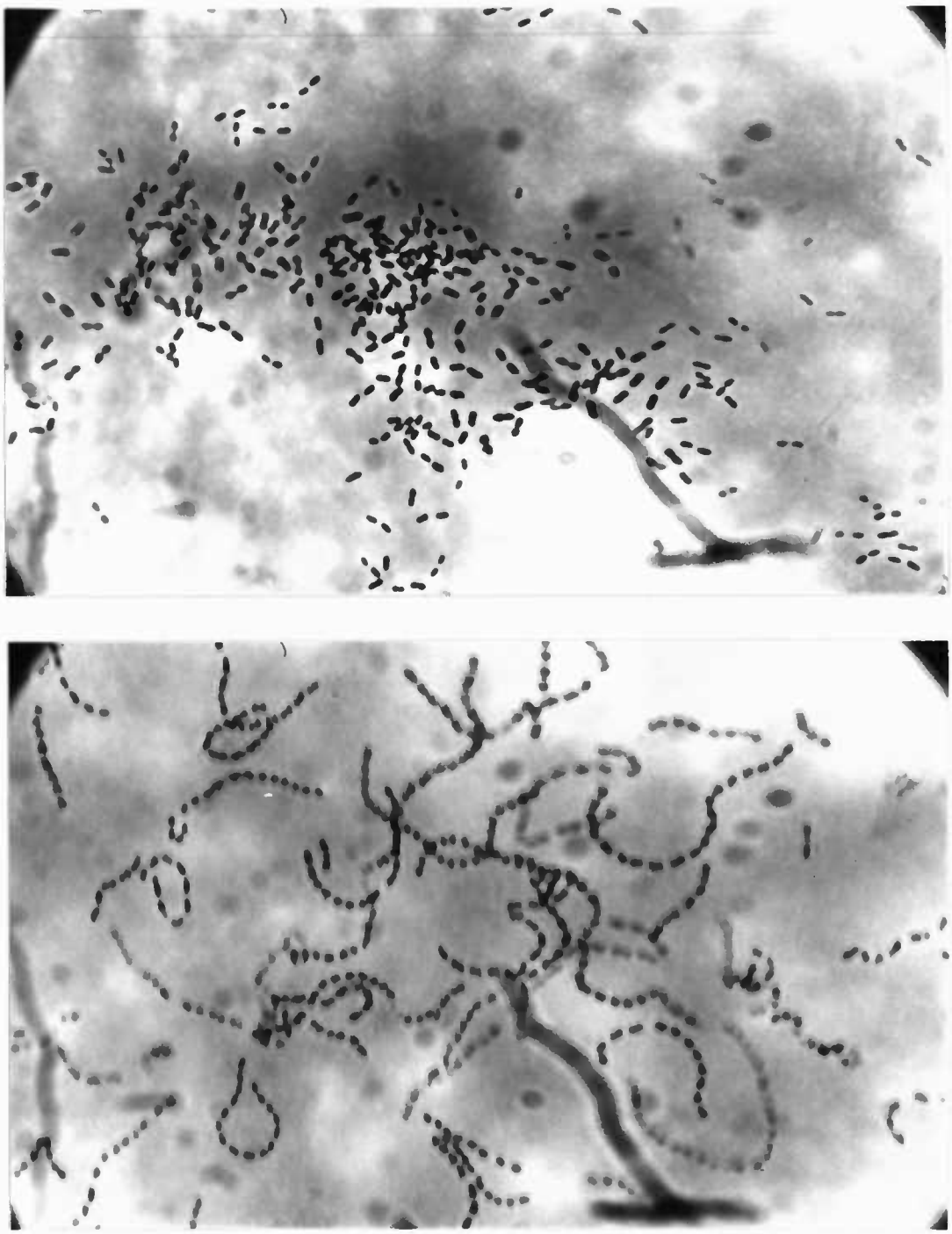

Figure 3.18. Morphological comparison of S. cremoris parent strain 108 (above) and its phage-insensitive mutants 108-C, (below).

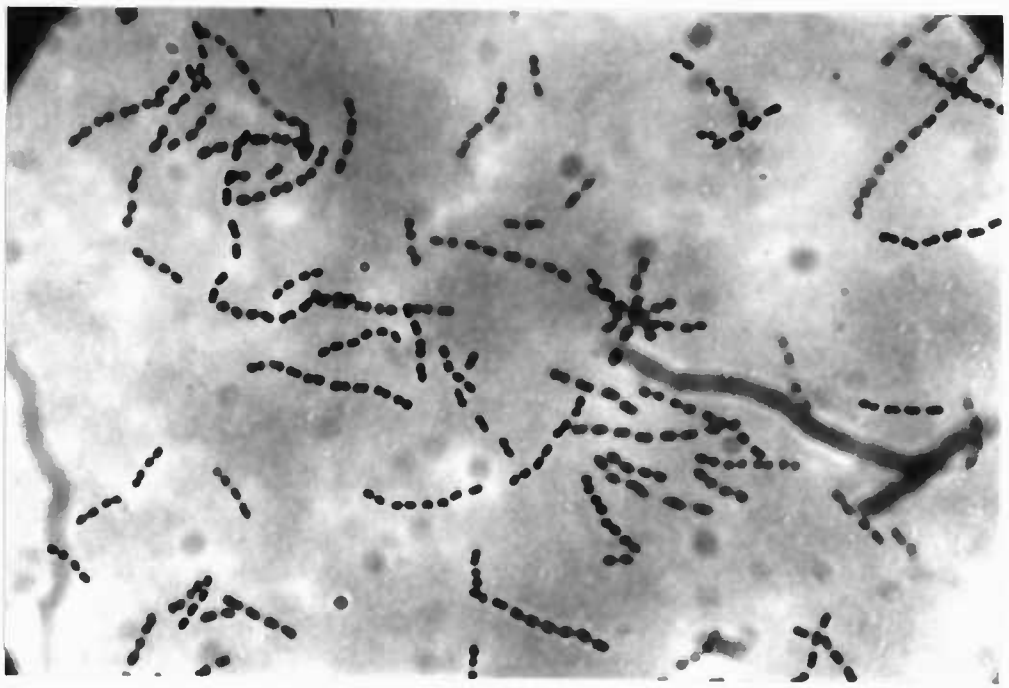

Figure 3.18a. Morphological characteristics of S. cremoris phage-insensitive mutant 108-12.

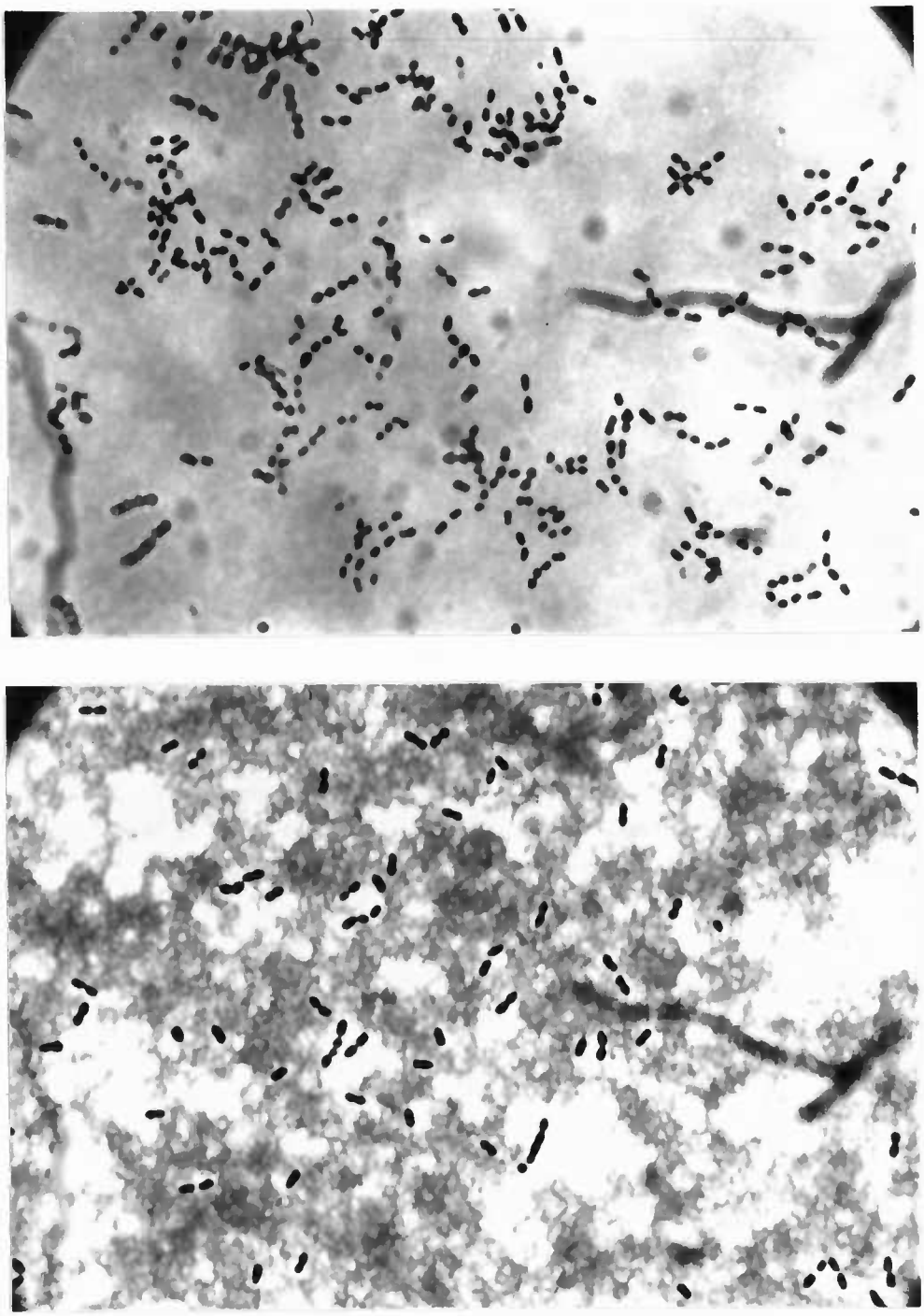

Figure 3.19. Morphological comparison of S. cremoris parent strain SK11G (above) and its phage-insensitive mutant SK11G-C (below).

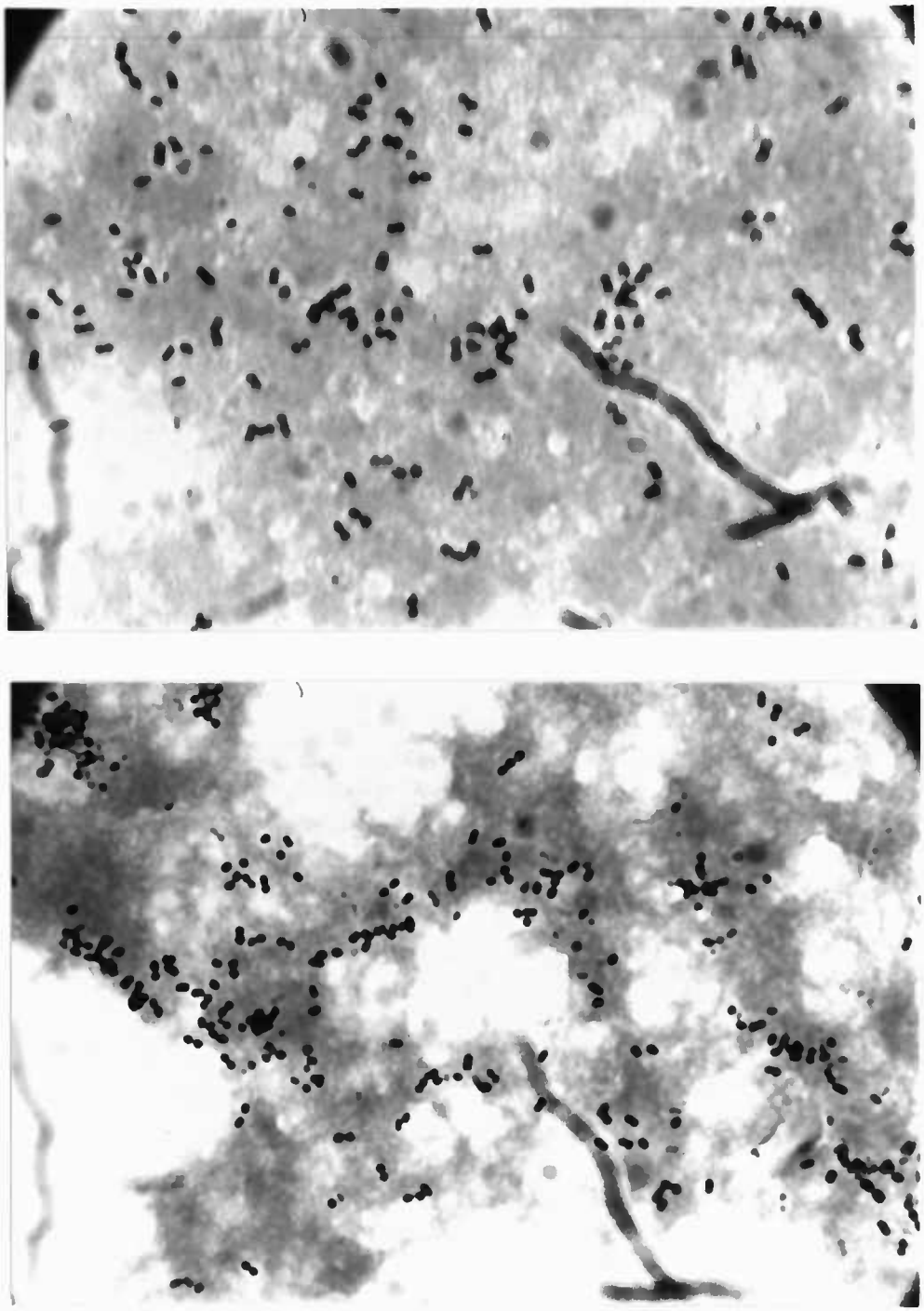

Figure 3.20. Morphological comparison of S. cremoris parent strain 224 (above) and its phage-insensitive mutant 224-3 (below).

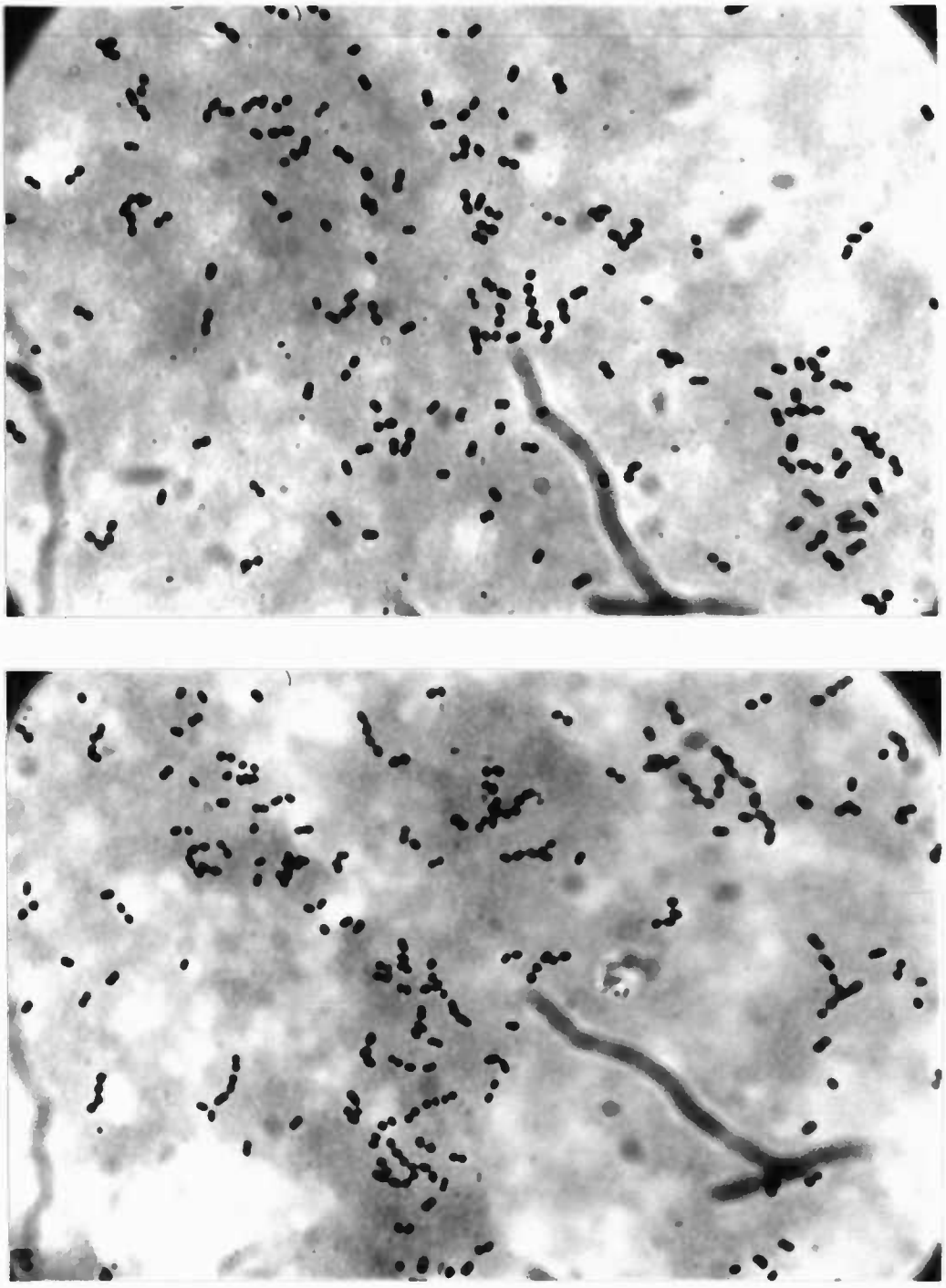

Figure 3.21. Morphological comparison of S. cremoris parent strain C13 (above) and its phage-insensitive mutant C13/25 (below).

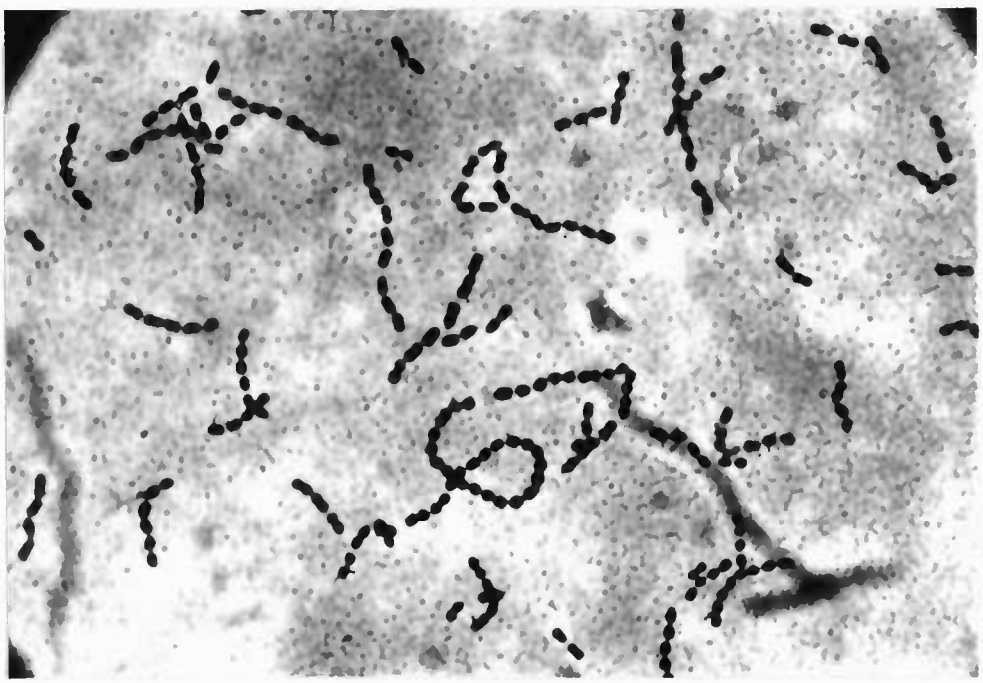

Figure 3.22. Morphological characteristics of S. cremoris strain U-134.

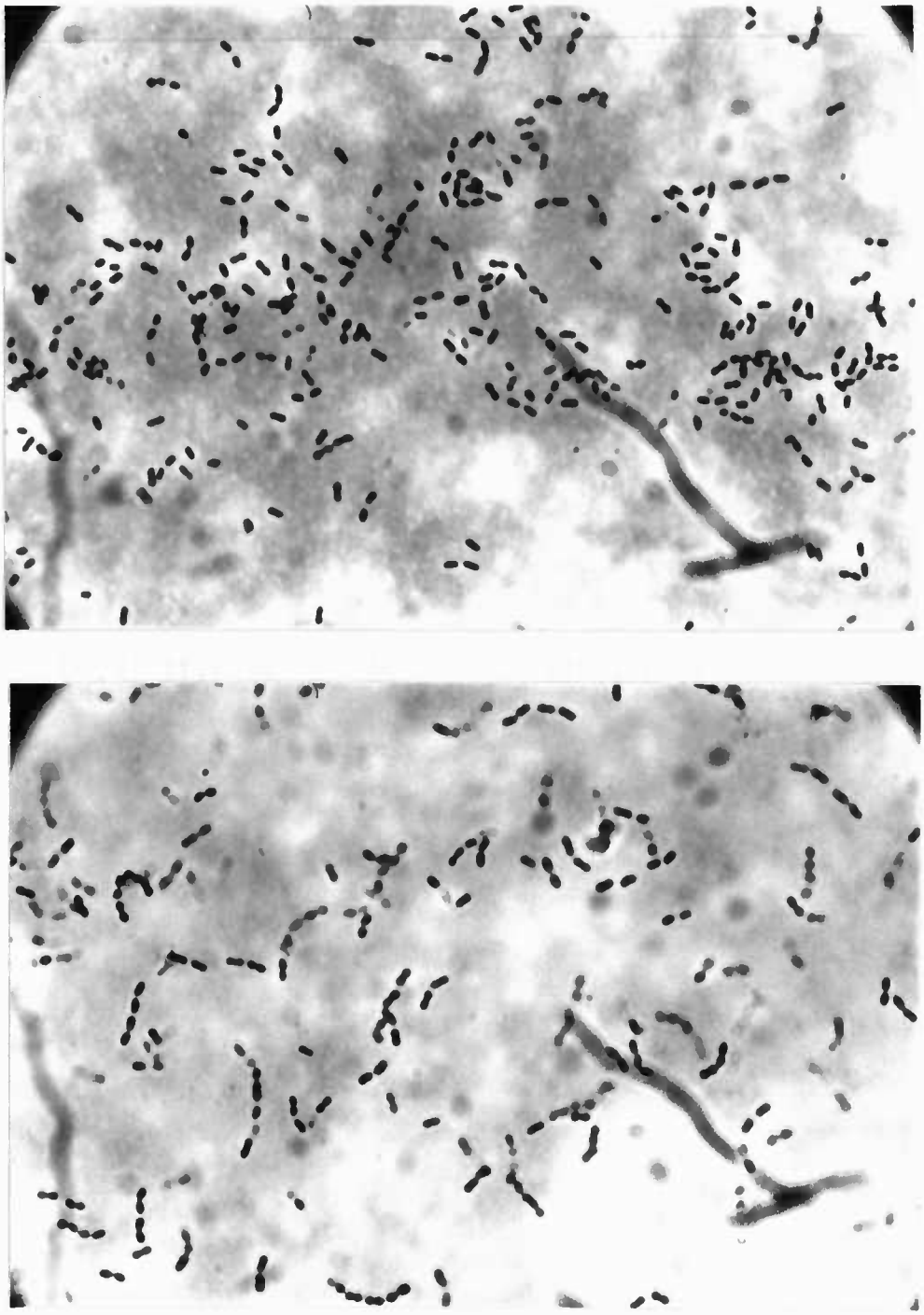

Figure 3.23. Morphological comparison of S. lactis parent strain BA-1 (above) and its phage-insensitive mutant BA-1 RM (below).

DISCUSSION

Mutant stability

The results of this research, as well as other studies (8,20,26) have shown the potential for selection and use of phage-insensitive mutants in commercial cheesemaking.

Phage-insensitive mutants have been successfully used to replace phage-infected parent strains in multiple-strain blends. A multiple-strain starter composed of six, phage-insensitive mutants has been used in Cheddar cheesemaking for nearly two years without need for strain changes. Some of the mutant strains developed within this study remain in cheesemaking use afer 38 mo. While not all mutants selected have displayed this long-range stability, the majority of those introduced for commercial cheese manufacture have remained sufficiently stable to justify their use as starter strains. Once stabilized in a cheese plant, most mutants have characteristically shown long-range stability against phage infection (Table 3.3). In most cases, phages which appeared for mutant strains differed in host sensitivity from the original phage. This observation has also been reported by Jarvis (20) and Limsowtin and Terzaghi (26).

Heterogeneity

Heterogeneity among strains of lactic streptococci has been previously reported (7,10,27,28) and it is not surprising to find heterogeneity also among phage-insensitive mutants of a particular strain. While all isolates of a mutant strain may show phage-insensitivity, individual isolates can and will differ from each other in traits such as acid production, phosphate and salt tolerance, and temperature maxima. They also may differ in these characteristics when compared to the parent strain. Mutant characteristics may be enhanced above those of the parent, remain equal to, or be reduced somewhat below parental traits. Table 3.11

Table 3.11. Summary of data illustrating trait differences and similarities between parent and mutant lactic streptococcal strains.[a]

Mutant Strain	Acid production (Simulated cheesemaking)	Optimum temperature	Maximum temperature	Generation time	Proteolysis		Phosphate tolerance		NaCl tolerance		Phage adsorption	Agglutination	Induction
					16 h, 21 C	Simulated cheesemaking	Milk	Broth	Milk	Broth			
290-PC	+	=	+	=	+	+	=	-	-	=	-	+/-	=
108-C	-	-	-	=	=	-	-	-	+	+	-	+/-	=
108-12	=	=	=	-	=	=	+	-	+	+		+/-	=
SK11G-C	+	-	+	=	=	-	+	=	-	-	-	+/-	=
224-3	=	+	=	-	=	+	+	=	-	+	-	+/-	=
C13/25	-	=	-	+	-	-	-	-	+	+	-	+/-	=
BA-1 RM	=	=	=	=	=	=	+	=	=	=	-	+/-	=

[a] "+" = greater than parent
"-" = approximately equal to parent
"-" = less than parent

[b] Agglutination reaction of mutants is not strain dependent but depends on milk supply

summarizes some of the characteristics of mutant strains, compared to those of their parent strains.

The presence of slow and fast acid-producing variants in a single-strain culture has been reported by Garvie (10) and by Citti et al. (7). During selection of phage-insensitive mutants, the presence of slow and fast acid-producing variants is often encountered. Fast acid-producing clones selected from a culture were, for the most part, equal to or greater in acid-producing ability than the parent strain, under simulated cheesemaking conditions. Only two mutant strains, 108-C and C13/25 produced acid at lower rates than their respective parents. In the presence of stimulatory substances such as peptone or autolyzed yeast extract, both mutant strains produced acid at rates similar to their parent strains.

The positive influence of added growth supplements has been reported by other researchers (11,16). Comparative studies by Citti et al. (7) on fast and slow acid-producing variants linked slow acid-production with decreased proteolysis. They noted that while fast and slow acid-producing cells exhibited similar generation times and acid-production per cell, fast variants were four times more numerous because of extended log phases due to higher proteolytic activity. The amount of total acid produced in milk culture was apparently dependent upon available nitrogen; thus, these organisms became dependent upon their proteolytic enzymes to provide nitrogen. As seen in Table 3.11, mutants 108-C and C13/25, which were slower in rate of acid production, were also lower in proteolytic activity under cheesemaking conditions (Pearce test), than were their parent strains. Low proteinase activity in slow acid-producing, proteinase-negative variants has been attributed to loss of surface-bound proteinase activity (41) and to loss of protease controlling plasmids (9). Both of these mutant strains, although slow-acid producers, are proteinase positive. Mills and Thomas (40) reported that cell wall-associated

proteinases play a role in the formation of bitter peptides, while intracellular peptidases have a role in removing bitter peptides. The plasmid profile for 108-C appears to differ slightly from the 108 parent, whereas plasmids for C13/25 and its parent appear the same (Paulo Orberg, personal communication). The exact nature of slow acid-production by these mutant strains, whether linked to plasmid or cell wall proteinases, should be investigated by future research.

Growth temperatures

The multiplication of non-bitter starter strains is inhibited by cooking temperatures used in Cheddar cheesemaking, thus resulting in lower bacterial populations than those of bitter starters (30). If temperature sensitive starter strains (non-bitter) are allowed to increase to high numbers, bitter cheese results (30,31). The standard cooking temperature of 37.8 C for Cheddar cheese, is above the optimum growth temperature for non-bitter strains, and near that of identified bitter-strains (30). A simple test has been devised by Sullivan et al. (48) which classifies starter strains as potentially bitter or non-bitter according to their growth in milk at cooking temperatures.

Lee and Collins (25) reported an optimal growth temperature range of 28 to 31 C for nine S. cremoris and one S. lactis strain grown in yeast extract broth. Maximal growth temperatures were 34.4 to 38.9 C in litmus milk and broth. In contrast, all 12 of the S. cremoris parent and mutant strains examined in the present study, exhibited temperature optima in the range of 31 to 34 C, and temperature maxima between 36 and 38 C (Figures 3.1 through 3.6). S. lactis strain BA-1, and its mutant BA-1 RM, showed temperature optima between 30 and 36 C, with a maximum temperature greater than 40 C (Figure 3.7). Because the cooking temperature of 37.8 C is above the optimum temperature, all of the S. cremoris strains and mutants used in this study were regarded as "temperature sensitive"

and "non-bitter". Although BA-1 and BA-1 RM could be classified as potentially-bitter strains, because of their insensitivity to cook temperatures, their use in a six-strain multiple-blend, where they contributed only a part of the total bacterial population, did not result in bitter-flavored cheese even after extended aging (52). It should be noted that under stress from pH, temperature, or salt, strains BA-1 and BA-1 RM could potentially grow and predominate other strains in a blend, whose growth, but not acid production, could be inhibited (13,55).

Generation times

Generation times (at optimum temperature) reported in this study (Table 3.4) were similar to those reported by Lee and Collins (25) for nine S. cremoris strains grown at their optimum temperatures. Optimum temperatures for strains and mutants in this study averaged three degrees higher than those reported for strains used by Lee and Collins. It is unknown whether the growth medium (milk versus broth) had an influence on temperature optima.

Phosphate tolerance

Acid production, in the presence of 2% phosphate ion (3.87% total phosphate salt), varied widely according to the strain, and did not follow a perceivable pattern (Table 3.9). Some strains showed greater acid production in the presence of phosphate, than in the non-phosphate control. Although several strains showed reduced acid production in the presence of 2% phosphate ion, cellular damage, if any, was minor and reversible. All strains grown in phosphated milk and broth retained sufficient activity to clot milk normally. Hargrove et. al., (13) reported reduced activity in several mixed-strain cultures after they were subcultured twice in milk containing only sodium phosphate salts. Potassium phosphate salts, in the same concentrations (2 or 3%) and pH, had no inhibitory effects, nor did equal ratios of sodium and

potassium salts. Zottola and Marth (57) reported on a phage inhibitory medium containing 1% each of Na_2HPO_4 and KH_2PO_4 that was not inhibitory to starter cultures. Ausavanodom et al. (3) showed that significantly lower phosphate levels could be used in phage inhibitory media if the pH was continuously held between 6.0 and 6.2 during culture growth. Ledford and Speck (24) found injury occurring to cultures grown in commercial phage-inhibitory media containing high levels of phosphates. Injury resulted in diminished proteinase activity, but not in enzyme inactivation or decreased cell counts. Skim milk supplemented with 1 and 2% phosphates, also resulted in attenuated proteinase activity.

No attempt was made in the present study to establish whether or not reduced acid production was related to decreased proteinase activity or inactivation, however; greatest acid development was observed for five strains in 3.87% phosphated milk, suggesting that proteinase activity was not impaired in these strains. Acid production and growth would be expected to be higher from strains where greater amounts of nitrogen are available (7). Thus, higher acid production would be expected in M17 broth, as compared to milk, due to peptone supplementation. Results showed, however, that just the opposite was true. Acid production, by nine of fourteen strains grown in phosphated milk, was equal to or greater than that observed in phosphated broth (Table 3.9). Lower acid production in unbuffered broth controls was likely due to growth in a low pH environment, which resulted in reduced enzyme activities (13). Higher acid production in the milk controls, compared to broth controls, was likely due to greater buffering capacity exhibited by milk. Although nine of fourteen strains showed significantly lower acid production in buffered as compared to control milk, all strains grown in presence of high phosphates were capable of coagulating RSM normally without subculturing. Strains propagated in high-phosphate broth showed similar activity. Table 3.9 summarizes phosphate-tolerance characteristics of parent and mutant strains.

Salt tolerance

While small proportions of NaCl have stimulatory effects on lactic cultures, larger proportions exhibit toxic properties (37). Walter et al. (56) reported that strains of S. lactis were not significantly inhibited in Cheddar cheese curd by 1.6 to 2.0% NaCl, whereas most S. cremoris strains are inhibited slightly at 1.4%, definitely at 1.6% and almost completely at 2.0%. Variations in salt-in-moisture (S/M) levels in Cheddar cheese between 4% and 6% significantly affected lactose metabolism by starter bacteria (22,51,54). Lactose was completely fermented in about 8 days at 1.42% (4% S/M) salt levels, whereas starter metabolism was virtually stopped at 2.13% (6% S/M) salt (54). Commercial mixed strain cultures displayed reduced acid development in skim milk in the presence of 1.5 to 2.0% salt (19,34).

All fourteen parent and mutant strains examined showed greater salt tolerance when grown in M17 broth, than when grown in milk. Salt tolerance was improved with increased nutritive value of broth versus milk. Under these increased nutritive conditions, half of the strains examined were tolerant of salt levels up to one percent greater than those for milk.

Phage adsorption and induction

Bacteria may become insensitive to phage attack either by mutation or by lysogenization (1). Adsorption data collected in this study indicated that all selected mutants were of the receptor-site type, rather than lysogenic mutants, because of their inability to adsorb phage which infected the parent strain. Although optimum induction conditions (39) were employed, no lytic phages were induced which impaired acid development by any of fourteen mutant and parent strains examined. This does not exclude the possibility of induced or defective phages (29); had other indicator strains been used, inducible phages might have been detected. Of interest, however, is the fact that developed mutants

did not show increased host ranges for existent or newly appearing phages in cheese plants. Most of the mutant strains selected have remained remarkably stable in commercial cheesemaking (Table 3.3). Newly emerging phages have not displayed multiple-strain infectivity. Research has shown that lysogenic strains may serve as reservoirs for phages that will attack sensitive strains in mixed or multiple-strain starter cultures (5,17,45). For this reason, the use of cultures of known composition and reduced strain numbers would be most advantageous.

Agglutination

As previously reported by Thunell et al. (53), individual starter strains varied in their sensitivities to agglutinins in cheese milk and colostrum. Parent and mutant strains did not follow a predictable agglutinin-sensitivity pattern. Agglutinin-sensitivity shown by a parent strain did not necessarily reflect sensitivity by its mutant strain, and visa versa. Sensitivity appears to be dependent upon the source of the specific antibodies contained in the milk or colostrum. Agglutinin-sensitivity may be displayed by culture strains in one cheese factory, but not in another factory, again dependent upon the sources of antibodies in the milk supply.

Morphology

Increased chain lengths for mutant strains 290-PC, 108-C, and 108-12 (Figures 3.17 and 3.18) most likely resulted from lowered autolysin (peptidoglycan hydrolase) activity (36,43,47). Filamentous forms of *S. cremoris* and *S. lactis* were reported by McDonald (36) and apparently resulted from the failure of autolysin to separate daughter cells after division. Chained-cells were less susceptible to autolysis and to lysis by lysozyme than were normal cells. The role of cell wall autolysin in chain formation has also been reported for mutant strains of *S. faecalis* (43,47). Soper and

Winter (47) found that chained forms contained lower levels of autolysin with no apparent differences in form of the cell wall substrate. Pooley et al. (43) reported on a long-chain S. faecalis mutant that was affected either at the activation step (latent to active autolysin) or in the transport of autolysin to the cell wall. This mutant contained four times more latent autolysin than active autolysin compared to the wild type.

With selection of phage-insensitive mutants for use as cheese starters, the possibility arises that such mutants could possess undesirable characteristics as a result of phage-conversion or transduction (2,38). Characteristics which could conceivably be transferred by these means might include malty flavor, citrate fermentation, agglutination, increased temperature tolerance, diacetyl production, and increased protease or lipase activity. While the mutants selected in this study did not exhibit any such characteristics which would preclude their use as cheese starters, it nevertheless is recommended that mutants be carefully screened for desirable and undesirable traits before their introduction into cheesemaking.

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CHAPTER 4

FROZEN BULK STARTERS FROM INTERNAL-PH-CONTROL-GROWN CULTURES[a]

ABSTRACT

Frozen bulk starters were prepared by growing and freezing unconcentrated individual S. cremoris strains in an internal-pH-control medium (PHASE 4). Frozen cultures were stored for 3 mo, with and without a cryoprotectant, and assayed monthly for cell viability and activity. Individual strains showed variations in their ability to survive storage at -20 C. Addition of glycerol helped preserve cell viability and activity at -20 C. Storage at -40 C and -80 C in PHASE 4 preserved activity and viability without need of cryoprotectants. Unfrozen PHASE 4 cultures retained original activity and viabilility after 1 mo refrigerated storage. Frozen PHASE 4 bulk starters have been successfully used in Cheddar and cottage cheese making for over 1 yr.

[a]Technical Paper No. 6427, Oregon Agricultural Experiment Station.

INTRODUCTION

Traditionally, lactic bulk starter preparation requires several subculturing steps to prepare large quantities of starter. Such steps often are performed on a daily, time-consuming basis and require skilled personnel to transfer and propagate stock cultures. Current trends in cheesemaking favor large-scale, multiple-fill operations in which large amounts of bulk starter are needed, thus making traditional methods of starter preparation impractical. For several years, frozen concentrated starters have been commercially available for both bulk and direct-to-vat inoculation.

Bacteria differ in their susceptibility to freezing and thawing, as influenced by species, strain, growth conditions, age, the nature of the growth medium, and the conditions of freezing, storage, and thawing (20,21). Much research has been undertaken to determine the optimum conditions for the maintenance of maximum activity in frozen cultures. Several general principles regarding freeze-injury to bacterial cells have emerged from this research: a) cell death increases with extended storage time (8,20,21), b) lower storage temperatures reduce the cell death rate (2,8,21), c) the higher the cell concentrations the more resistance to freeze-injury (21), d) cells in the stationary growth phase survive freezing better than log-phase cells (14,20,21), e) cryoprotective agents provide protection against freeze-injury (2,7,9,12,14,17,21,24).

Studies on starter culture storage without cryoprotective agents have established that optimal temperatures are -40 C and below (23,24). While freezing and storing in liquid nitrogen (-110 to -196 C) provides best cell viability (2,5,4,9,17,24), storage temperatures of -20 C and -40 C are more commonly encountered in cheese factories. Culture storage at temperatures at or above -20 C results in marked decreases in cell viability and activity

and often requires the addition of cyroprotective agents (2,3,9,16,24). However, even in the presence of such agents, cultures stored at temperatures above -40 C exhibit survival differences between strains (8,9,15,17,24).

The rate of freezing and thawing is important in determining maximum cell viability and culture activity. Rapid freezing accompanied by fast thawing is favored for maximum bacterial cell survival (2,3,9,16,17). Current theories on cryoinjury to bacteria indicate that cellular damage is most likely due to elevated solute concentrations (which results from cell dehydration), and to membrane destruction by intracellular ice crystals (16,21). On rapid cooling, water is released at a rate faster than cell membrane permeability allows, which leads to the formation of small holes in the cell membrane permitting leakage of cellular components, or total membrane destruction (21). If the system temperature is lowered too quickly during freezing, water is unable to leave the cell rapidly enough by dehydration mechanisms to maintain equilibrium between intra- and extracellular solutions. Hence, upon sufficient supercooling, intracellular ice formation begins (6). Rapid freezing rates produce small ice crystals which result in less cellular damage (2,3,6,16,21). However, if sufficient cellular water is retained during rapid freezing, small intracellular ice crystals tend to be unstable and will grow in size by recrystallization, even at low storage temperatures and during thawing. Ice crystals formed during both freezing and recrystallization can produce cell membrane damage (3,21).

The addition of a cryoprotectant results in greater survival rates during frozen storage by binding water and inhibiting either intracellular or extracellular ice crystal formation (2,16). The presence of most cryoprotectants results in the formation of extracellular amorphous ice (glass), instead of crystalline ice, upon freezing (16).

This paper reports on the growth of lactic cultures to high numbers ($>10^9$ CFU/ml) in an internal-pH-control medium (PHASE 4) and freezing these cultures as bulk starters without further concentration. This study also tested the feasibilty of shipping these bulk starters over long distances, either in frozen or unfrozen forms.

METHODS AND PROCEDURES

Cultures

Streptococcus cremoris strains 290-P, 108, SK11G, 224, C13, and U134 were obtained from the Oregon State University culture collection. Strains were carried and transferred on a regular basis in sterile reconstituted skim milk (RSM). All strains had been previously characterized (27).

Growing and Freezing

PHASE 4^{TM} (22), a commercially available internal-pH-control medium (Galloway West, Fond du Lac, WI) was reconstituted from a single lot according to manufacturers directions (75 g/liter) in seven 3-liter portions of tap water and pasteurized at 92.2 C for 45 min. The pasteurized media were quickly cooled to ambient temperature in ice water and each portion was inoculated (1% level) with one of six different single strains. The last portion was inoculated with an equal mixture (5 ml each) of all six individual strains, equivalent to a 1% inoculum. The inoculated media were incubated 14-16 h at 25 C with continual agitation (100 rpm) on a multistation magnetic stirrer (Lab-line Instruments, Inc., Melrose Park, Ill.). Following incubation, all cultures had pH values of 5.35 ± .03. Each grown culture was divided into two portions. The first portion was canned in 100-ml aliquots in sterile aluminum pull-top cans. Glycerol (10% level) was added to the second portion, which was canned as above after thorough mixing. For control purposes, each culture was sampled before freezing to determine viable cell counts and activity. Canned cultures were then frozen at -20 C, -40 C or -80 C, and stored at these temperatures for up to three months.

Cell Counts

Cultures were sampled monthly for viable cell counts and activity. Cans of each culture and treatment were thawed for 20 min in cool (20 C) tap water containing 200 ppm chlorine to simulate cheese-factory thawing conditions. Can contents were mixed by shaking before opening. Samples (0.1 ml) of thawed cultures were aseptically transferred into 99.9 ml of cold (2-5 C), sterile peptone water (0.1%) contained in a 250 ml blender jar. The diluted culture was blended at high speed for 65 sec to break up bacterial chains (18) and serial dilutions of the blended cultures were made in sterile 0.1% peptone water. Dilutions of each culture were spotted in 25 µl amounts onto predried M17 agar plates (25) with an Oxford Micro-doser pipette (Lancer Division of Sherwood Medical, St. Louis, MO 63103). Each dilution plate contained 5 to 7 replicate spots. Inoculatd plates were incubated upright at 30 C for 48 h, after whch colony forming units per ml (CFU/ml) were determined and mean values of observations reported.

Activity Tests

Coagulation test. Tubes of RSM (10 ml) were inoculated at 0.1, 0.5, and 1.0% levels with each of the thawed cultures. Inoculated tubes were incubated at 21 C for 18 h and examined for coagulation. Immediately after sampling, thawed cans were covered and refrigerated at 4 C for 1 wk, then the activity was reexamined as above using the 1% inoculum level only. Tubes which failed to coagulate in 18 h were incubated a further 2 h then observed for coagulation.

Modified Horral-Elliker Activity Test (13). Thawed culture cans were thoroughly shaken to mix the contents, and the individual cultures were diluted 1:1 with 0.1 M phosphate buffer. Tubes containing 10 ml RSM were tempered at 37 C, inoculated with 0.3 ml of each culture dilution, mixed and incubated in a 37 C water bath for 3.5 h. At the end of incubation, tubes were chilled and

titrated against 0.1 N NaOH. Results were expressed as ml of 0.1 N NaOH required to neutralize acid to the phenolphthalein end point. Starters capable of producing a change of 0.35 ml or greater were considered active. A change of 0.30 to 0.35 ml was considered slow acid production, and cultures producing changes less than 0.30 ml were considered too slow for use in cheese making.

Cottage cheese activity test. Direct bulk starter inoculation was simulated by diluting individual cultures 1:9 in 0.1 M phosphate buffer and inoculating bottles containing 100 ml of RSM with 2 ml of the diluted culture (inoculum rate was equivalent to 757 ml of canned culture/100 gal milk). After incubation at 24 C for 18 h, grown cultures were used to inoculate a second set of bottles of tempered RSM at a 5% level. After incubation for 4 h at 32 C, the pH value of each culture was measured.

Treatment for Transported Cultures

Strains were grown as above, canned without cryoprotective agent, and separated into three treatment groups. Treatments were as follows:

Treatment 1. Cultures not frozen, packed without ice and not refrigerated during shipment, nor upon arrival until tested.

Treatment 2. Cultures frozen overnight at -40 C, shipped without refrigeration or dry ice and refrigerated upon arrival until tested.

Treatment 3. Cultures frozen overnight at -40 C, shipped with excess dry ice and placed in freezer upon arrival.

Triplicate samples of each treatment were packed and shipped in similar-sized Freeze-safe insulated containers (Polyfoam Packers Corp., Chicago, Il.). Cultures were shipped by common carrier in July and were in transit three days between Oregon and Florida. Upon arrival, Treatment 2 cultures had completely thawed, but were

still cold. Cultures from Treatments 1 and 2 were tested directly, whereas Treatment 3 cultures were thawed in chlorinated water, then immediately tested.

After initial testing, a set of cultures from Treatments 1 and 2 was held for 1 mo at 5 C, after which the activity of the refrigerated cultures was determined as above.

RESULTS

Figures 4.1 through 4.7 show viable cell counts for individual strains held under frozen storage at three temperatures for three months. Individual strains showed differences in their abilities to survive prolonged frozen storage, even with added glycerol.

Strain 290-P (Figure 4.1) showed a one-half log decrease in viable cells when stored at -20 C for three months. The same decrease was seen in the culture stored with glycerol. A corresponding loss of activity, with increased storage time (Table 4.1), was noted for cells stored without glycerol. Cells stored in the presence of glycerol, in spite of viable cell losses, retained good activity. No decrease in viable cell counts were seen for strain 290-P stored at -40 C and -80 C. At these temperatures, added glycerol did not enhance cell survival, and activities were similar for cultures both with and without the cryoprotectant (Tables 4.2 and 4.3).

Strains 108 and C13 (Figures 4.2 and 4.5) showed no decreases in viable cell counts under any of the storage conditions. Activities remained constant over the storage period for all temperatures (Tables 4.1, 4.2 and 4.3).

Strain SK11G (Figure 4.3) showed an apparent, but inexplainable, increase in viable cell counts during storage at -80 C. Similar increases were seen at both -40 C and -80 C in glycerol-protected cultures. Cell counts of unprotected SK11G cultures at -40 C and -20 C showed insignificant decreases, as did the -20 C glycerol-protected culture. Activities (Tables 4.1, 4.2 and 4.3) remained constant for SK11G cultures at all temperatures and treatments. However, a decrease in activity of -20 C cultures, both glycerol-protected and unprotected, appeared after cultures were thawed and refrigerated for one week (Table 4.1). No corresponding decrease in activity was seen in SK11G cultures stored at the two lower temperatures after subsequent thawing and week-long refrigeration.

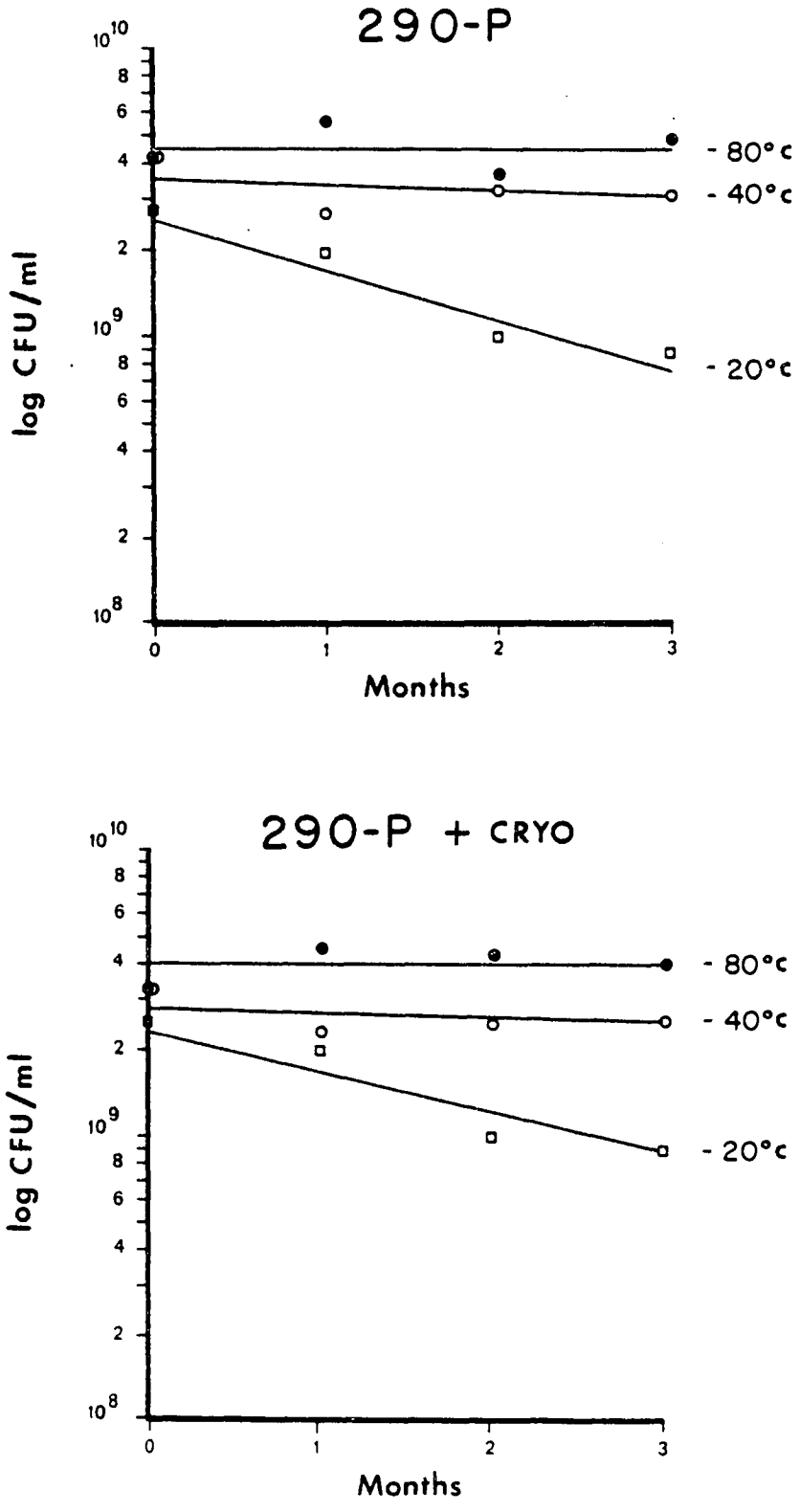

Figure 4.1. Effect of storage temperature on the viability of S. cremoris 290-P grown and frozen in PHASE 4, both with and without cryoprotectant (glycerol).

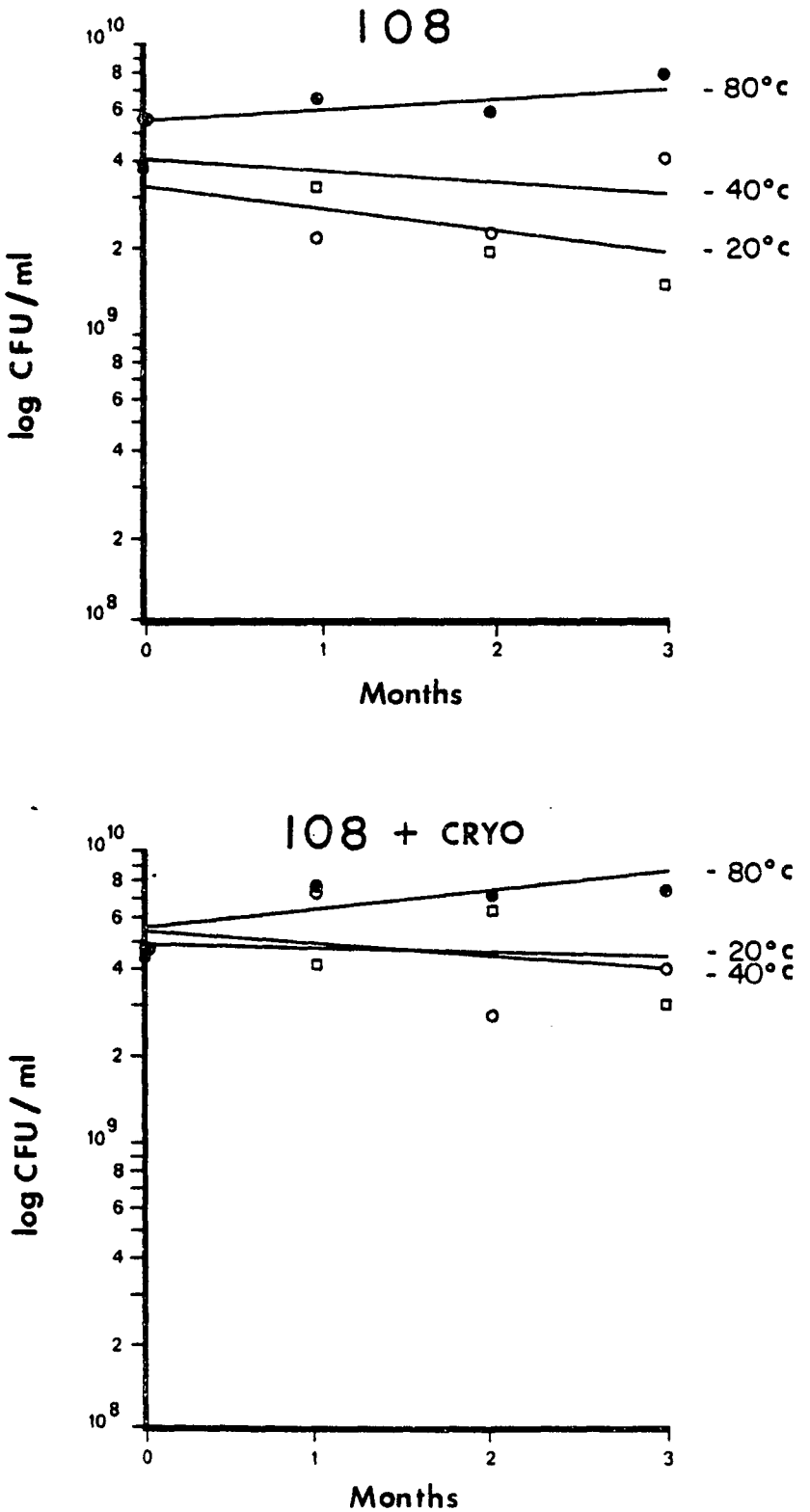

Figure 4.2. Effect of storage temperature on the viability of S. cremoris 108 grown and frozen in PHASE 4, both with and without cryoprotectant (glycerol).

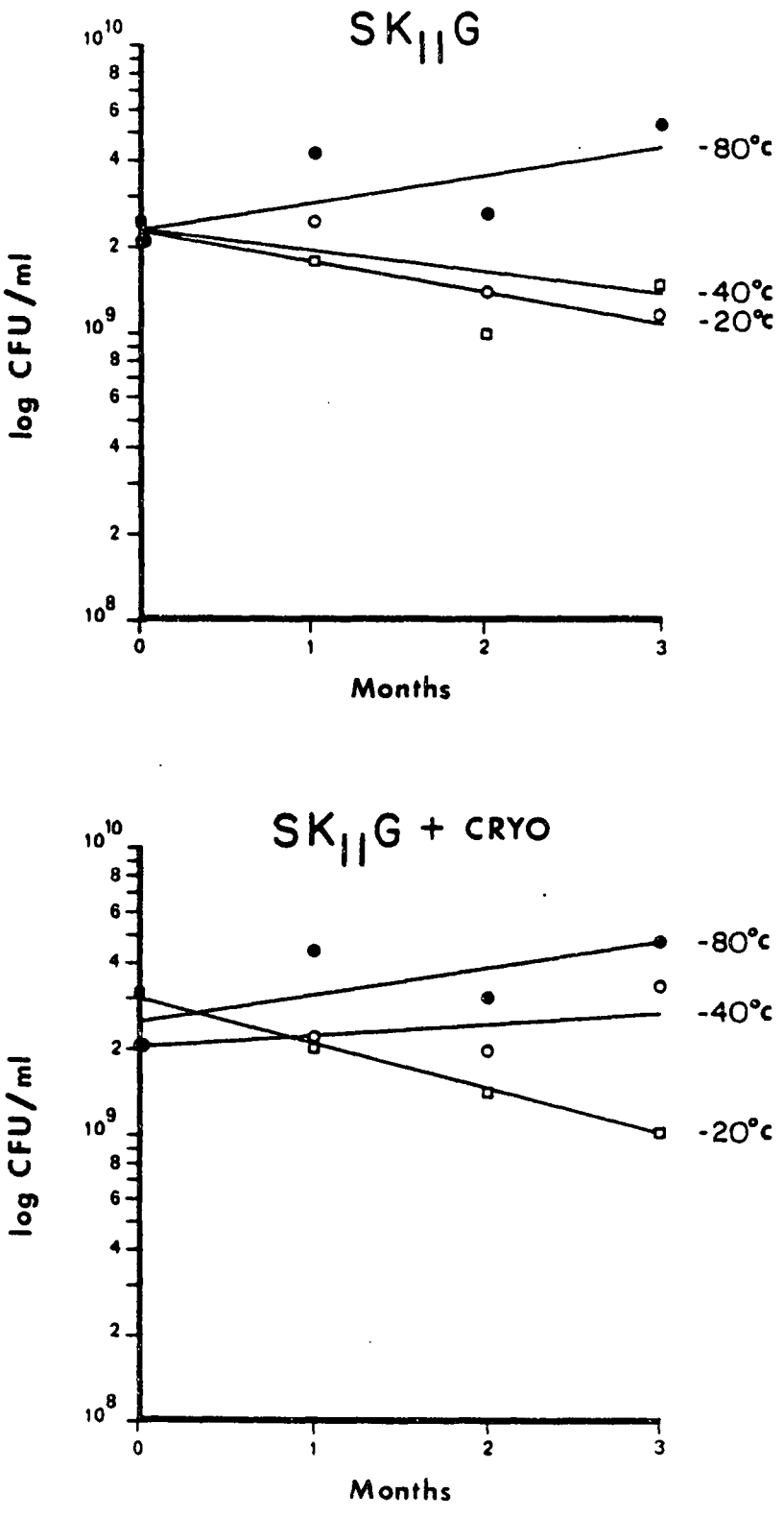

Figure 4.3. Effect of storage temperature on the viability of S. cremoris $SK_{11}G$ grown and frozen in PHASE 4, both with and without cryoprotectant (glycerol).

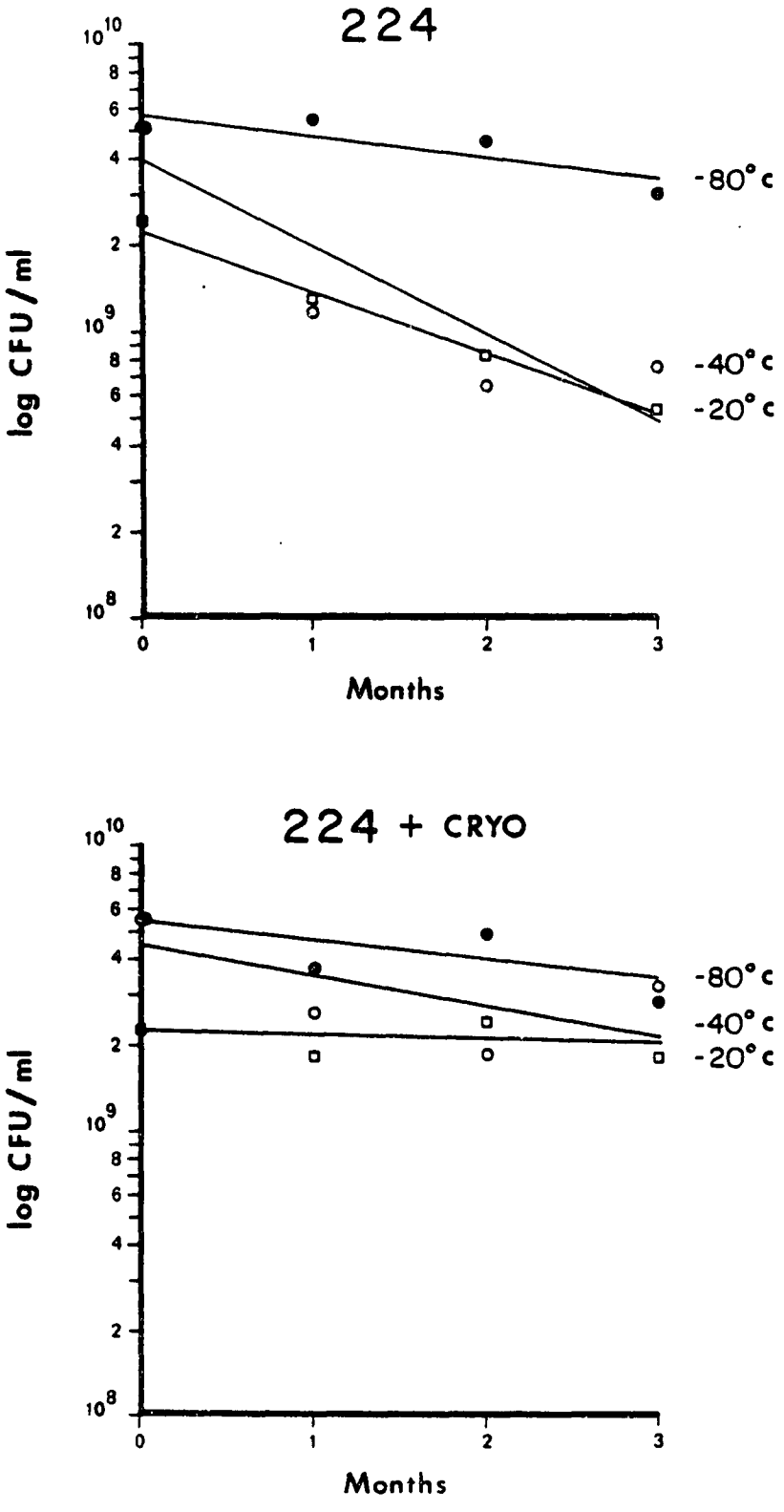

Figure 4.4. Effect of storage temperature on the viability of S. cremoris 224 grown and frozen in PHASE 4, both with and without cryoprotectant (glycerol).

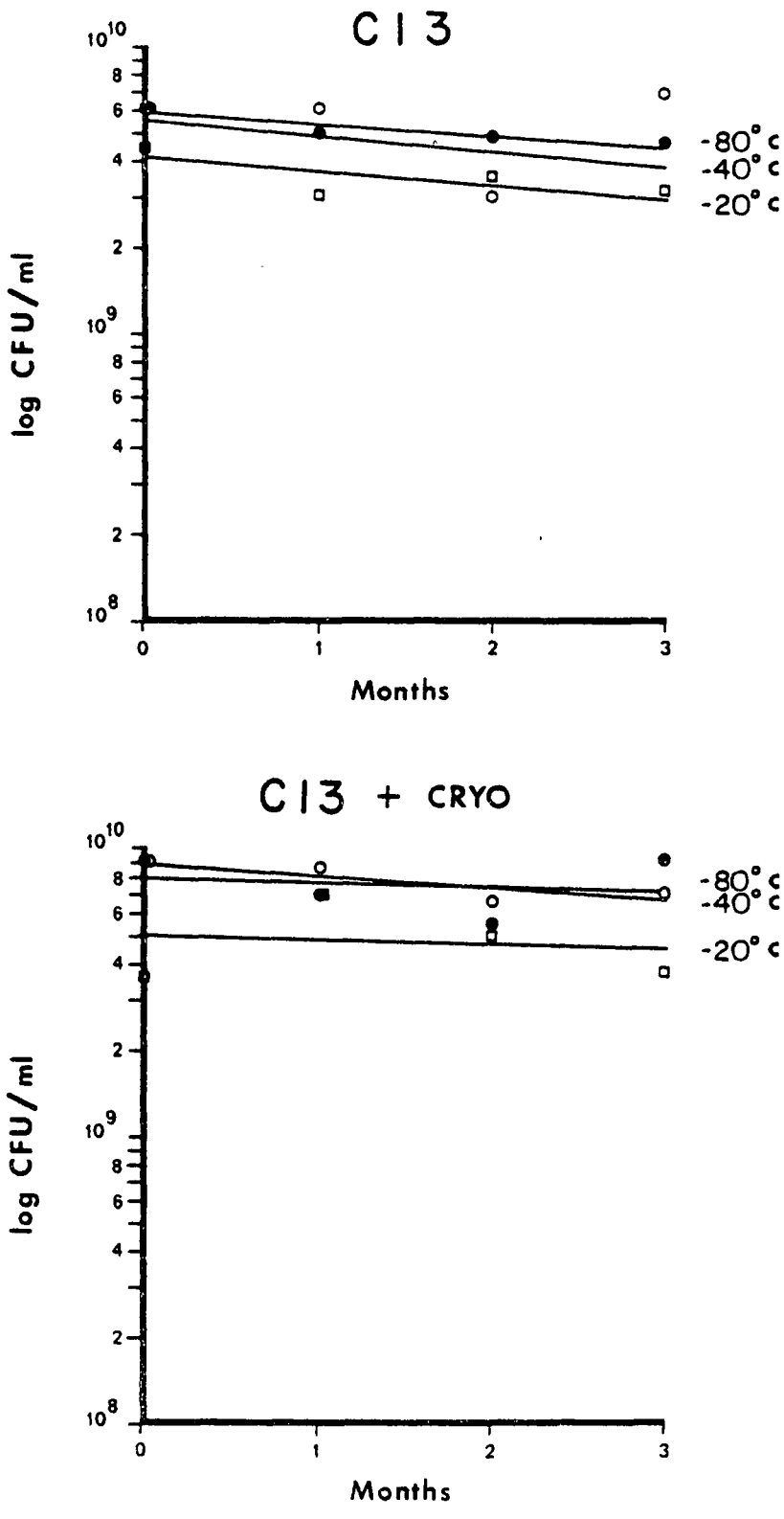

Figure 4.5. Effect of storage temperature on the viability of S. cremoris C13 grown and frozen in PHASE 4, both with and without cryoprotectant (glycerol).

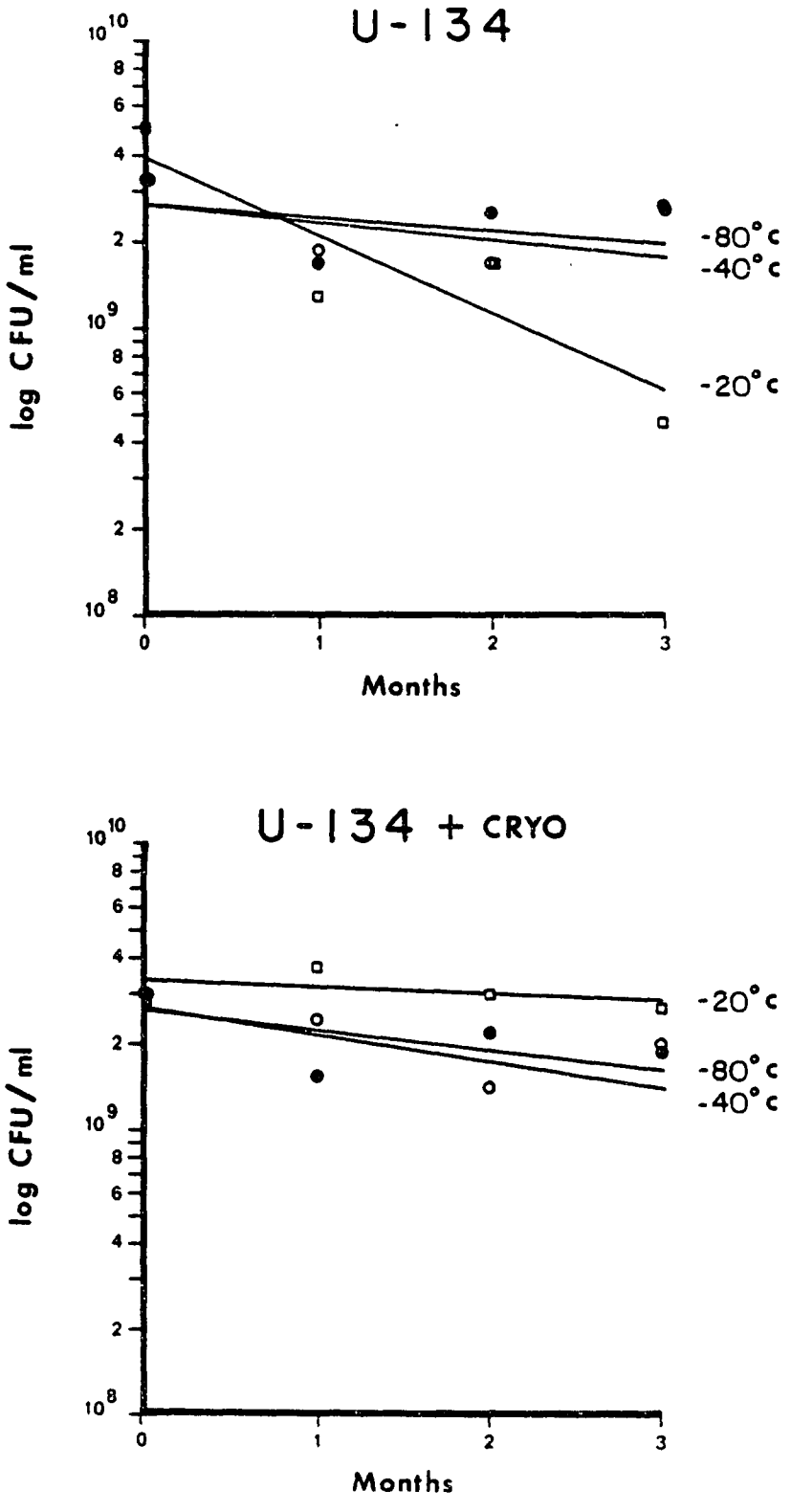

Figure 4.6. Effect of storage temperature on the viability of S. cremoris U-134 grown and frozen in PHASE 4, both with and without cryoprotectant (glycerol).

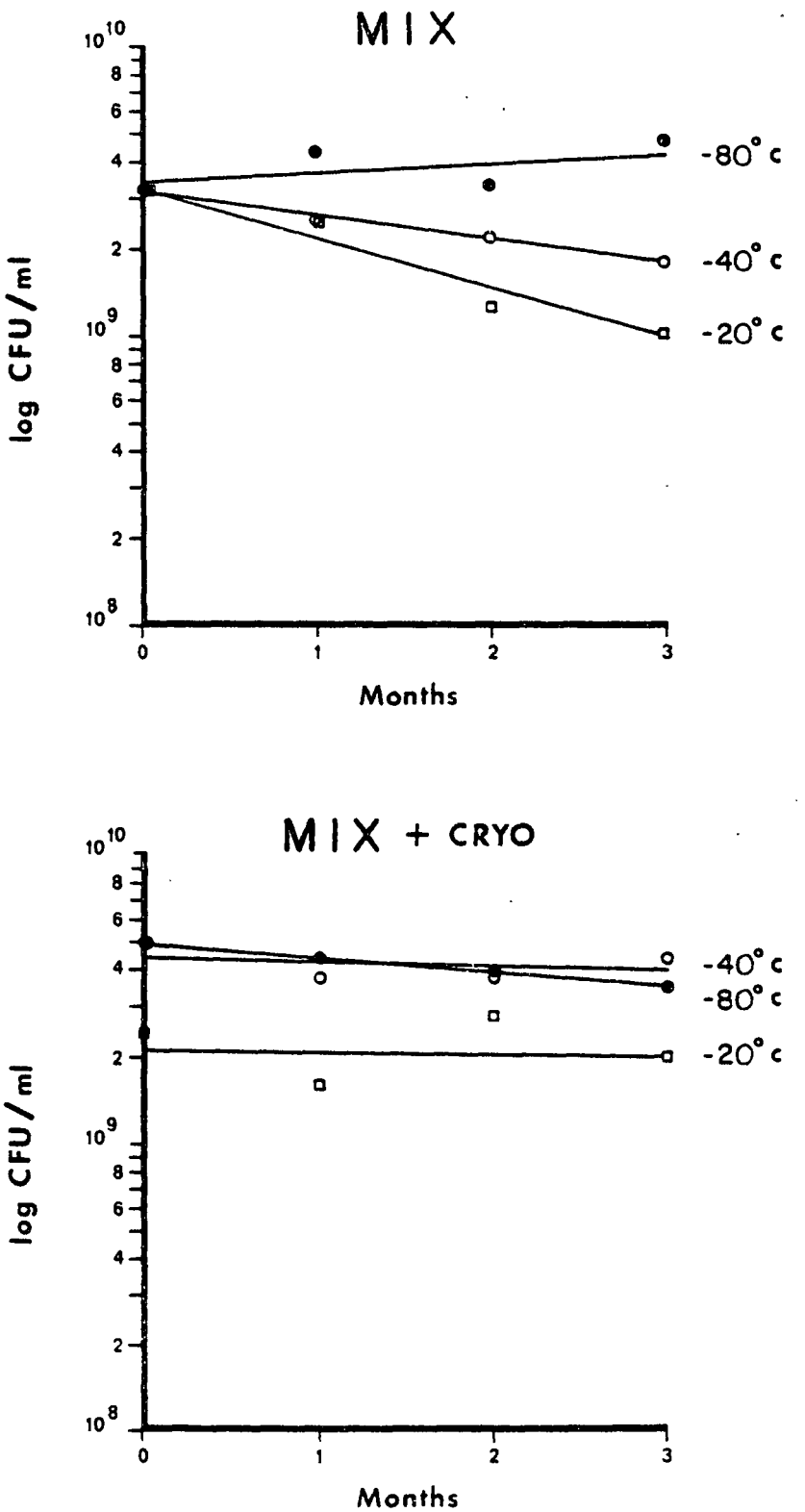

Figure 4.7. Effect of storage temperature on the viability of S. cremoris six-strain mix grown and frozen in PHASE 4, both with and without cryoprotectant (glycerol).

Table 4.1. Acid-producing activity of PHASE 4-grown cultures after different storage periods at -20°C[a].

	1 Month				2 Months				3 Months			
	Thawed (% inoculum)			Thawed and held 1 wk @ 4 C (% inoculum)	Thawed (% inoculum)			Thawed and held 1 wk @ 4 C (% inoculum)	Thawed (% inoculum)			Thawed and held 1 wk @ 4 C (% inoculum)
Strain	1.0	0.5	0.1	1.0	1.0	0.5	0.1	1.0	1.0	0.5	0.1	1.0
290-P	+	+	+	b	+	+	-	-	-	-	-	-
108	+	+	+	+	+	+	+	+	+	+	+	
SK11G	+	+	+	-	+	+	+	-	+	+	+	-
224	+	b	b	+	+	-	-	-	-	-	-	
C13	+	b	+	+	+	+	-	+	+	-	+	
U-134	+	+	b	-	+	+	-	-	-	-	-	-
MIX	+	b	+	+	+	+	+	+	+	-	-	
Cryo-protected												
290-P	+	+	+	+	+	+	+	+	+	+	+	-
108	+	+	+	+	+	+	+	+	+	+	+	
SK11G	+	+	+	-	+	+	+	-	+	+	+	-
224	+	b	+	+	+	+	+	+	+	+	+	
C13	+	+	+	+	+	+	+	+	+	+	+	
U-134	+	+	+	+	+	+	+	+	+	+	+	+
Mix	+	+	+	+	+	+	+	+	+	+	+	

[a]Coagulation test. Coagulated RSM in 18 h at 21°C (+ = coagulation; - = no coagulation)
[b]Coagulated RSM in 20 h at 21°C

Table 4.2. Acid-producing activity of PHASE 4-grown cultures after different storage periods at -40°C[a].

	1 Month				2 Months				3 Months			
	Thawed (% inoculum)			Thawed and held 1 wk @ 4 C (% inoculum)	Thawed (% inoculum)			Thawed and held 1 wk @ 4 C (% inoculum)	Thawed (% inoculum)			Thawed and held 1 wk @ 4 C (% inoculum)
Strain	1.0	0.5	0.1	1.0	1.0	0.5	0.1	1.0	1.0	0.5	0.1	1.0
290-P	+	+	+	+	+	+	+	+	+	+	+	+
108	+	+	+	+	+	+	+	+	+	+	+	
SK11G	+	+	+	+	+	+	+	-	+	+	+	+
224	+	b	+	+	+	+	-	+	+	b	b	
C13	+	+	+	+	+	+	b	+	+	+	+	
U-134	+	+	+	b	+	+	+	+	+	+	+	+
MIX	+	+	+	+	+	+	+	+	+	+	+	
Cryo-protected												
290-P	+	+	+	+	+	+	+	+	+	+	+	+
108	+	+	+	+	+	+	+	+	+	+	+	
SK11G	+	+	+	+	+	+	+	+	+	+	+	+
224	+	+	+	+	+	+	+	+	+	b	+	
C13	+	+	+	+	+	+	+	+	+	+	+	
U-134	+	+	+	+	+	+	+	+	+	+	+	+
MIX	+	+	+	+	+	+	+	+	+	+	+	

[a]Coagulation test. Coagulated RSM in 18 h at 20°C (+ = coagulation; - = no coagulation)
[b]Coagulated RSM in 20 h at 21°C

Table 4.3. Acid-producing activity of PHASE 4-grown cultures after different storage periods at -80°C[a].

	1 Month				2 Months				3 Months			
	Thawed (% inoculum)			Thawed and held 1 wk @ 4 C (% inoculum)	Thawed (% inoculum)			Thawed and held 1 wk @ 4 C (% inoculum)	Thawed (% inoculum)			Thawed and held 1 wk @ 4 C (% inoculum)
Strain	1.0	0.5	0.1	1.0	1.0	0.5	0.1	1.0	1.0	0.5	0.1	1.0
290-1P	+	+	+	+	+	+	+	+	+	+	+	+
108	+	+	+	+	+	+	+	+	+	+	+	
SK11G	+	+	+	+	+	+	+	+	+	+	+	+
224	+	+	+	+	+	+	+	+	+	b	+	
C13	+	+	+	+	+	+	+	+	+	+	+	
U-134	+	+	+	b	+	+	+	+	+	+	+	+
MIX	+	b	+	+	+	+	+	+	+	+	+	
Cryo-protected												
290-P	+	+	+	+	+	+	+	+	+	+	+	+
108	+	+	+	+	+	+	+	+	+	+	+	
SK11G	+	+	+	+	+	+	+	+	+	+	+	+
224	b	b	+	+	+	+	b	+	+	b	+	
C13	+	b	+	+	+	+	+	+	+	+	+	
U-134	+	+	+	+	+	+	+	+	+	+	+	+
MIX	+	+	+	+	+	+	+	+	+	+	+	

[a]Coagulation test. Coagulated RSM in 18 h at 21°C. (+ = coagulation; - = no coagulation)
[b]Coagulated RSM in 20 h at 21°C

Table 4.4. Results of modified Horrall-Elliker activity test on 1:9 dilution of transported cultures upon arrival.

	ml of 0.1 N NaOH for neutralization					
Strain	Treatment 1[a]	Δ	Treatment 2[b]	Δ	Treatment 3[c]	Δ
290-P	2.10	.30	2.10	.30	2.35	.55
108	2.10	.30	2.30	.50	2.50	.70
$SK_{11}G$	2.00	.20	2.00	.20	2.05	.25
224	2.05	.25	2.05	.25	2.30	.50
C13	2.10	.30	2.25	.45	2.30	.50
U-134	2.00	.20	2.10	.30	2.25	.45
CONTROL	1.80	---	1.80	---	1.80	---

[a]Shipped unfrozen, unrefrigerated.

[b]Frozen -40 C, shipped without refrigeration or dry ice.

[c]Frozen -40 C, shipped with excess dry ice.

Table 4.5. Results of modified Horrall-Elliker activity test using 1:1 dilutions of transported cultures upon arrival and after one month storage at 5 C.

	ml 0.1 N NaOH for neutralization									
	Upon Arrival						Stored 1 month			
Strain	Treatment 1[a]	Δ	Treatment 2[b]	Δ	Treatment 3[c]	Δ	Treatment 1[a]	Δ	Treatment 2[b]	Δ
290-P	2.50	.70	2.40	.60	3.05	1.25	2.40	.40	2.40	.40
108	2.90	1.10	3.60	1.80	4.40	2.60	2.55	.55	2.35	.35
$SK_{11}G$	2.40	.60	2.20	.40	2.30	.50	2.20	.20	2.30	.30
224	2.80	1.00	2.40	.60	3.00	1.20	2.30	.30	2.50	.50
C13	2.70	.90	3.20	1.40	3.90	2.10	2.60	.60	2.50	.50
U-134	2.50	.70	2.60	.80	2.80	1.00	2.40	.40	2.30	.30
CONTROL	1.80	---	1.80	---	1.80	---	2.00	---	2.00	---

[a]Shipped unfrozen, unrefrigerated.

[b]Frozen -40 C, shipped without refrigeration or dry ice.

[c]Frozen -40 C, shipped with excess dry ice.

Table 4.6. Titratable acidities of milk inoculated with transported cultures upon arrival and after one month storage at 5 C.

Strain	Percent Titratable Acidity[a]				
	Upon Arrival			Stored 1 Month	
	Treatment 1[b]	Treatment 2[c]	Treatment 3[d]	Treatment 1[b]	Treatment 2[c]
290-P	0.68	0.75	0.75	0.62	0.29
108	0.82	0.80	0.78	0.70	0.61
$SK_{11}G$	0.75	0.69	0.70	0.32	0.36
224	0.75	0.73	0.76	0.18	0.17
C13	0.76	0.77	0.74	0.68	0.60
U-134	0.76	0.79	0.77	0.18	0.16

[a]2% inoculum of 1:9 culture dilution, incubated 18 h at 24 C.

[b]Shipped unfrozen, unrefrigerated.

[c]Frozen -40 C, shipped without refrigeration or dry ice.

[d]Frozen -40 C, shipped with excess dry ice.

Table 4.7. Results of cottage cheese activity test using transported cultures upon arrival and after one month storage at 5°C.

	pH after 4 hr at 32 C (5% inoculum of 18 h culture)				
	Upon Arrival			Stored 1 month	
Strain	Treatment 1[a]	Treatment 2[b]	Treatment 3[c]	Treatment 1[a]	Treatment 2[b]
290-P	4.65	4.65	4.70	4.50	5.10
108	4.40	4.45	4.45	4.50	4.40
$SK_{11}G$	4.55	4.50	4.55	4.80	4.80
224	4.70	4.70	4.70	5.70	5.80
C13	4.60	4.65	4.55	4.65	4.45
U-134	4.45	4.80	4.60	6.10	6.30
CONTROL	6.40	6.40	6.40	6.40	6.40

[a]Shipped unfrozen, unrefrigerated.

[b]Frozen -40 C, shipped without refrigeration or dry ice.

[c]Frozen -40 C, shipped with excess dry ice.

Of the six individual strains, strains 224 and U-134 (Figures 4.4 and 4.6, Table 4.1) showed the greatest decreases in viable cell counts and activity during -20 C storage. Both of these strains showed cell count decreases of nearly one log. Strain 224 exhibited a similar log-decrease in cell counts at -40 C, but without corresponding loss in culture activity (Table 4.2). The addition of glycerol to both strains was necessary to preserve culture viability and activity during storage at -20 C. The addition of glycerol also enhanced the viability and activity of strain 224 for the -40 C storage (Figure 4.4 and Table 4.2).

Viable cell counts for the six-strain mixture (Figure 4.7) showed a slight decrease under -20 C storage without glycerol. Over a three month period, a corresponding slight decrease in activity (Table 4.1) was seen at the 0.1% level upon thawing and at the 1% level after 1 week of refrigeration. No major changes in cell counts or activity was noted in the six-strain mixture stored without glycerol at -40 C and -80 C (Tables 4.2 and 4.3). The mixed culture with added glycerol consistently showed good activity and viability at all storage temperatures as well as after thawing and refrigerating 1 week.

Tables 4.4, 4.5, 4.6, and 4.7 report the results of various activity tests conducted on single strain cultures prepared under the three treatment conditions and shipped transcontinentally. Table 4.4 shows the results of the Modified Horrall-Elliker Activity Test conducted on individual cultures upon arrival. Cultures prefrozen and shipped with excess dry ice (Treatment-3) showed significantly higher activities on arrival than cultures prepared and shipped under treatment conditions 1 and 2. Results for Treatment-2 cultures did not differ greatly from those for Treatment-1. Variations between strains were noted under all three conditions. Table 4.5 summarizes additional Horrall-Elliker Test results for transported cultures upon arrival and after 1 month storage at 5 C. These results reflect higher inoculum levels (1:1

dilution) than Table 4.4 results (1:9 dilution), but show similar trends and strain variations for individual cultures. None of the refrigerated cultures retained full activity. Activity after 1 month was strain and treatment dependent.

Table 4.6 shows titratable acidities (T.A.) of milk tubes inoculated with transported cultures upon arrival and after 1 month storage at 5 C. Upon arrival, T.A. of milk inoculated with an individual strain showed no major difference due to treatment. However, strain 290-P showed T.A. differences due to treatment after 1 month refrigerated storage. Treatment-1 culture (unfrozen, unrefrigerated in shipment) showed activity comparable to that upon arrival, while Treatment-2 culture showed an activity decrease of over one-half after 1 mo of storage. Strains 108 and C13 showed only slight activity losses with either treatment after 1 mo refrigeration, whereas strains SK11G, 224 and U-134 showed substantial activity losses. For a given strain these losses did not vary significantly with treatment.

Table 4.7 contains the results of a simulated cottage cheesemaking trial which used bulk starter prepared from transported cultures, upon arrival (unstored) and after one month refrigeration (stored). Activity of individual unstored cultures did not vary significantly between treatments, with the exception of strain U-134, which showed highest activity in the unfrozen, unrefrigerated culture. Strain 290-P showed the most variation between treatments after refrigerated storage. Strain 290-P (Treatment 1; unfrozen) exhibited higher activity than the same culture which had been frozen and thawed (Treatment 2). For an individual strain, activity varied only slightly between treatments. Strain U-134 appeared to be most affected by prolonged refrigerated storage followed by strain 224. All unstored strains, for all treatment conditions, were capable of producing sufficiently active bulk starter to make cottage cheese in 4 h or less with a 5% inoculum. After 1 month refrigerated storage, three

of the six strains were capable of producing active bulk starter (Table 4.7).

DISCUSSION

Leach and Sandine (15) reported that undesirable strain dominance occurred in mixed-strain commercial starters held at -20 C. This dominance was due to differences in freezing survival and cell recovery after thawing. In the present study, individual PHASE-4-grown cultures demonstrated some variation in their ability to survive frozen storage at -20 C. Even with the addition of 10% glycerol, two of the six strains examined showed decreases in activity and viable cell counts when stored at -20 C. However, storage at -40 C proved better for preserving culture activity and viability. This has been substantiated by other researchers (17,23,24,26). At this temperature, all strains except 224 were successfully stored for 3 months without need of added cryoprotectants. When stored at -40 C or below, no strain dominance due to differences in freezing survival would be expected, except for strain 224, whose differences might be overcome by addition of a cryoprotective agent.

After freezing and thawing, bacterial suspensions contain cells that are lethally and nonlethally injured, as well as uninjured cells. The extended lag phase displayed by some cultures upon thawing may be due to time required for injury-repair in non-lethally-injured cells prior to cell multiplication (21). Lloyd (17) categorized damage effects from frozen storage on lactic acid bacteria into three types: 1) a decrease in viability with no effect on acid production 2) no change in cell numbers, with a decrease in acid production, and 3) a decrease in both viability and acid production. Table 4.8 categorizes the strains used in this study according to these effects suggested by Lloyd. Strains 108 and C13 showed no storage effects at -20 C. Strain SK11G displayed decreased viability, but no change in acid production. Strains 290-P, 224, and U-134 showed drops in both viability and acid production. The six-strain mixture showed a similar, but only

Table 4.8. Effects of storage at -20 C on viability[a] and activity[b] of individual strains stored without cryoprotectant.

Strain	No change	Viability decrease Activity constant	Viability constant Activity decrease	Viability decrease Activity decrease
290-P				+
108	+			
$SK_{11}G$		+		
224				+
C13	+			
U-134				+
MIX				+/-

[a]Viable cell counts.

[b]Ability to coagulate RSM in 20 h at 21 C.

slight, decrease at the end of the third month of storage. Of the strains stored at -40 C, only strain 224 showed injury effects; a decrease in viable cell counts with no corresponding drop in acid production.

It is presently unknown why some bacteria lose their ability to divide after freezing and thawing. It is yet to be determined whether this is due to alteration of a single vital component, or whether it is due to cumulative effects of structural and functional changes. At least four types of changes were noted by Ray and Speck (21): These include changes in gross morphology, changes in finer structures (eg. cell membrane), changes in cell function, and changes in genetic stability. With regard to genetic stability, Ashwood-Smith (1) has presented evidence for E. coli and Pseudomonas showing that genetic change is not induced by repeated freeze-thaw cycles. The extent to which a bacterium is damaged by freezing is determined in part by its genetic make-up, (1) but is also influenced by the growth medium used. Goldberg and Eschar (10) correlated the sensitivity of lactic acid bacteria to damage from freezing with specific alterations in cellular fatty acids. They found that the viability of S. lactis and Lactobacillus was better preserved against freeze-damage when cells were grown in medium supplemented with oleic acid or Tween 80. Work of Morichi and Irie (19) suggested that cellular injury caused by freezing or freeze-drying is similar to that caused by sublethal heating, i.e. membrane damage. Future research should investigate possible strain differences in cell wall structure and composition as related to freezing.

Internal pH-control medium (PHASE 4) has the unique property of continually neutralizing acid produced by actively metabolizing bacteria, thus maintaining the pH of the growing culture sufficiently high to prevent acid damage to the bacteria. Neutralization of growing cultures, before the stationary growth phase is reached, results in higher bacterial cell concentrations

(11). According to Lamprech and Foster (14) lactic streptococci survive freezing better at pH 7.0 than at pH 5.0. Added glycerol protects bacterial cells from freeze-injury at pH 5.0, but offers no benefit at neutral pH. In the absence of added glycerol, PHASE 4 cultures may be protected from freeze-injury by soluble and insoluble buffer salts in the medium acting as cryoprotectants. Storage temperatures, however, play an apparently greater role in maintaining starter activity than does pH (2).

In light of the activity results obtained with individual cultures transported transcontinentally, it is feasible to prepare cultures in internal-pH-control medium, transport such cultures without freezing or refrigeration and maintain them under refrigeration for up to one month without appreciable loss of activity. The commercial application of such technology would result in substantial savings both in energy costs for freezing and storing, and in reduced shipping costs due to the exclusion of dry ice. For longer storage, cultures can be shipped prefrozen with dry ice or transported unfrozen, to be then frozen upon arrival.

Frozen bulk starters prepared with internal-pH-control medium have already been successfully used in Pacific Northwest cheese plants for more than one year for both Cheddar and cottage cheese manufacture. Such cultures have been successfully transported to cheese plants by each of the treatment methods employed in this study, as well as unfrozen and packed in wet ice.

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CHAPTER 5

ECONOMIC COMPARISONS OF CHEDDAR CHEESE MANUFACTURED WITH DEFINED-STRAIN AND COMMERCIAL CULTURES[a]

ABSTRACT

The exclusive use of defined-strain cultures has resulted in manufacturing improvements of significant economic importance for five Pacific Northwest cheese plants. Starter failure from bacteriophage infection was eliminated. Exclusive use of defined strains in cheesemaking resulted in greater cheese uniformity, day-to-day consistency, and upgraded cheese quality. The percentage of total cheese graded "A" was increased by almost 10 percent. Because of predictable starter activity, manufacturing procedures were standardized in factories. Ripening times were eliminated, make times shortened and culture rotations were replaced with a single culture-blend. Some factories have increased cheese yields by adding whey cream to cheese milk. The above improvements resulted in a revenue increase of over $1 million for a cheese factory producing 25 million lb of cheese/yr. The combination of these improvements, all based on defined-strain technology, enabled the factories to increase production--some by nearly 50%. To date, more than 128 million lb of Cheddar cheese were manufactured with the defined-strain cultures.

[a]Technical Paper No. 6403, Oregon Agricultural Experiment Station.

INTRODUCTION

Successful cheesemaking relies on the unimpaired fermentation of milk sugars to acid by lactic acid bacteria. These bacteria, however, like many other biological entities, are subject to viral infection (bacteriophage). Such infection impairs bacterial processes which ultimately results in decreased or total cessation of acid production. Cheese produced under low-acid conditions represents a public health hazard due to potential outgrowth of pathogenic organisms and, due to decreased quality, receives lower sales prices in the market place. This cheese also requires special handling and reprocessing.

In an effort to minimize phage infection, most cheesemakers have resorted to rotating several different commercial blends of starter cultures. The theory behind this practice suggests that each commercial culture contains different bacterial strains which are theoretically insensitive to the same infecting virus. Thus, by rotating the cultures in cheesemaking, a host bacterial strain (indicator strain) is hopefully not present long enough to support phage growth with its impaired acid development. Commercial cultures, for the most part, are unknown blends of starter strains which vary in activity (acid production), flavor characteristics, and phage sensitivity (2,4,15,19,23). Use of these cultures results in finished cheeses of varying quality and flavor. The more cultures used in rotation, the more variable the characteristics and quality of the resulting cheeses. Because activity can vary from culture to culture, the cheesemaking process often must also be altered accordingly to accommodate the activity and properties of each culture used.

Research conducted both in New Zealand and in the U. S. has shown the acid-producing bacteria themselves to be the major source of phage (1,4,8,10,20,) and sensitivity to a single phage can be shown by several supposedly unrelated strains (2,4,7,8,9,20).

Therefore, the more cultures used in rotation, the greater the likelihood of similar strains being present which could be infected by a common phage, and greater the chance for release of temperate phages.

DEFINED-STRAIN CULTURE SYSTEM

The concept of using known single-strains of lactic streptococci for cheesemaking originated in New Zealand nearly 50 years ago (14). Since their introduction, defined strains have been used as single-strain, paired-strain, and multiple-strain starters (3,11,13,14,16,18). Cheesemaking, using defined-strains, offers better control over phage and culture activity, and results in cheese that is characteristically more consistent in grade and uniformity (6,13,17,18,21).

Defined-strains were used in this study as a multiple-strain starter which consisted of six, defined, phage-unrelated S. cremoris strains (13). The characterized blend of strains was used exclusively in daily cheesemaking, i.e. every vat of cheese was made with the same culture. There were no culture rotations. As single strains in the blend became infected with phage, virus-insensitive mutants of the infected strain were cloned to replace it, thus essentially maintaining the same blend of strains for all vats.

Manufacturing improvements from the use of this defined culture-blend resulted in major economical and technological advances in the following areas of cheese making in the Pacific Northwest:

1. Elimination of starter failure
2. Upgraded cheese quality
3. Standardized cheese manufacture
4. No culture rotations
5. Yield increases from added whey cream
6. Increased cheese production per plant

ECONOMIC EVALUATION OF INDIVIDUAL IMPROVEMENTS

1. ELIMINATION OF STARTER FAILURE.

Plant A, a medium-sized Cheddar cheese plant processed approximately 800,000 lb (363,636 kg) of milk per day. It was equipped with five 31,000-lb (14,091 kg) open-vats which were refilled five to six times in the production of 25 to 28 vats of cheese per day. Prior to introduction of the defined strain culture technology, this plant experienced starter failure at a rate of 7.2% using commercial cultures (Table 5.1). Although starter failure in Cheddar cheese does not represent a total product loss, it does represent a downgrading of product quality with a concomitant economic loss in terms of potential revenue from the cheese. Generally, downgraded cheese of "2nd grade" quality is sold as "economy cuts", while "B grade" cheese is used in the manufacture of processed cheese products.

Table 5.1 compares the cheese grading results for the same three-month period over four consecutive years. The first year represented the grades of cheese manufactured with commercial cultures. The latter three periods showed grades and percentages for cheese manufactured with a defined-strain culture. Starter failure was virtually eliminated using this system as evidenced in Table 5.1.

Similar elimination of starter failure has been reported by all five cheese plants currently using this starter system. A typical price differential between B and 2nd grade cheese is $0.15/lb. Assuming production of 25 million lbs of cheese per year, increased revenue due to the elimination of starter failure ("dead vats") equalled:

25,000,000 lbs cheese/yr X 7.2% B-grade X $.15/lb = $270,000

Table 5.1. Comparison of grades obtained using commercial and defined-strain cultures in the manufacture of Cheddar cheese for similar time periods over four years.

Grade	Dec. 1, 1978 to Feb. 28, 1979 (Commercial)	Dec. 1, 1979 to Feb. 28, 1980 (Defined-strain)	Dec. 1, 1980 to Feb. 28, 1981 (Defined-strain)	Dec. 1, 1981 to Feb. 28, 1982 (Defined-strain)
A	89.2%	90.8%	99.05%	99.13%
2nd *	3.6	9.2	.73	.87
B **	7.2	0	.22	0
Total	100%	100%	100%	100%

* Usually high acid flavor
**Starter failure

2. UPGRADED CHEESE QUALITY.

Table 5.2 summarizes calculations that reflect increased cheese value resulting from upgraded quality. Figures are based on an assumption of an average production of 25 million lbs of cheese per year. For comparative purposes, cheese price and total production volume data were held constant for the three-year period to demonstrate changes in net cheese worth resulting from changes in cheese grades alone.

As noted in the production SUMMARY, cheese produced for each year with the defined-strain culture system returned more than $0.5 million more than equivalent cheese produced with commercial cultures. This gain was due to an increased proportion of Grade A cheese (approximately 10%).

3. STANDARDIZED CHEESE MANUFACTURE.

Average make times (setting to salting), for all five cheese factories using defined-strain cultures were 3.5 to 4 h, with a corresponding milling acidity of 0.47%. As starter activity was consistent and predictable, the total make time for each vat of cheese fell within a specific time frame, which allowed production activities, personnel, materials and equipment to be efficiently scheduled.

Figures 5.1 and 5.2 show pH distributions of finished cheeses manufactured with commercial and defined-strain cultures over a four-year period. Observations were made during the same three-month period for each of the four years. Figure 5.1 indicates that cheese manufactured with commercial cultures varied widely in final pH (4.90 to 5.4). The majority of cheese made in 1981 and 1982 with defined-strain cultures (Figure 5.2) showed pH values within a more narrow range (5.0-5.2).

Decreases in overall make time resulted from the elimination of the ripening time before setting. A ripening time of .5 to 1 h is usually required in Cheddar cheese

Table 5.2. Increased value achieved from improved cheese quality as based on an average production of 25 million lb/yr.[a]

Year	Cheese Grade	% Production	Price/lb		Total $ Value
1978	A	89.2	1.41	=	$32,443,000
	2nd	3.6	1.29	=	1,161,000
	B	7.2	1.145	=	2,061,000
					$34,665,000
1979	A	90.8	1.41	=	$32,007,000
	2nd	9.2	1.29	=	2,967,000
	B	0	1.145	=	--
					$34,974,000
1980	A	99.05	1.41	=	$34,915,125
	2nd	0.73	1.29	=	235,425
	B	0.22	1.145	=	62,975
					$35,213,525

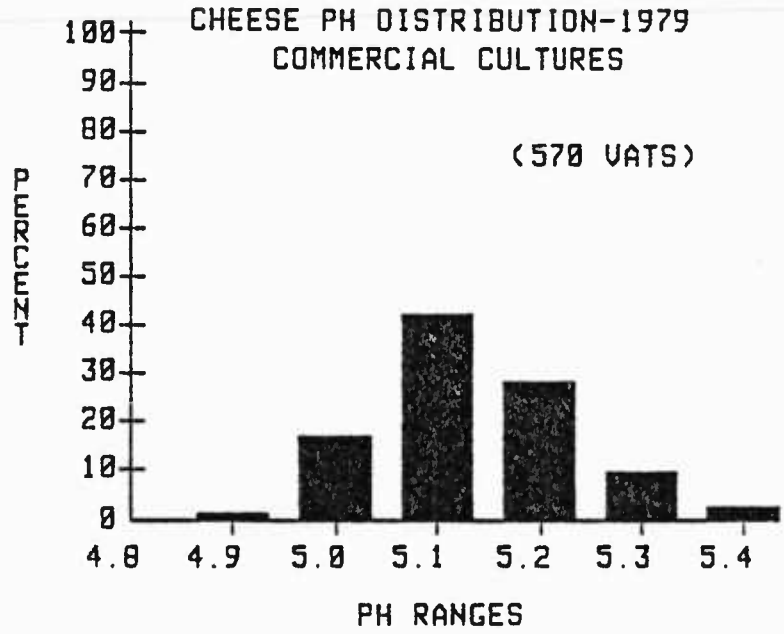
SUMMARY:

1980 Total Value = $35,213,525

1978 Total Value = $34,665,000

$ 548,525 Increased value from upgraded quality.
(1.58% increase over 1978)

[a]Cheese price and production volume data held constant for all three time periods.



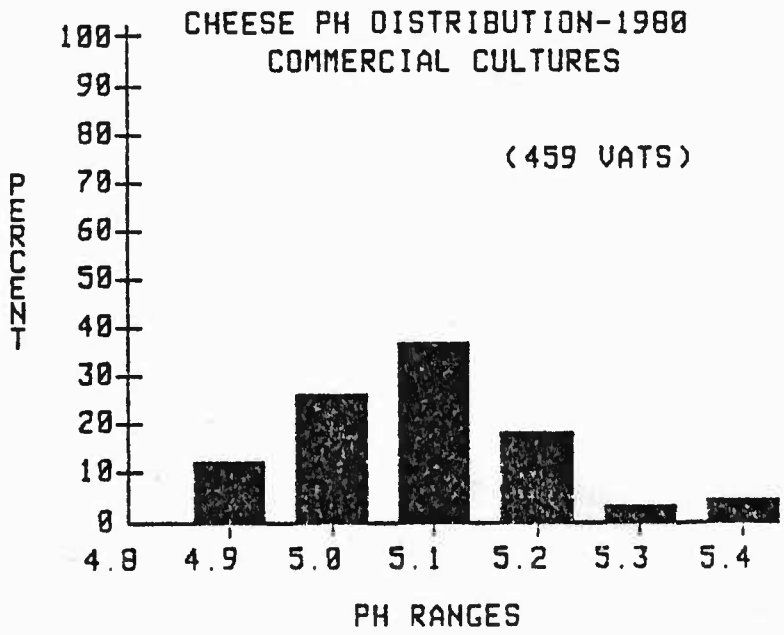


Figure 5.1. pH distribution ranges for Cheddar cheese manufactured with commercial cultures (corresponding time periods for consecutive years).

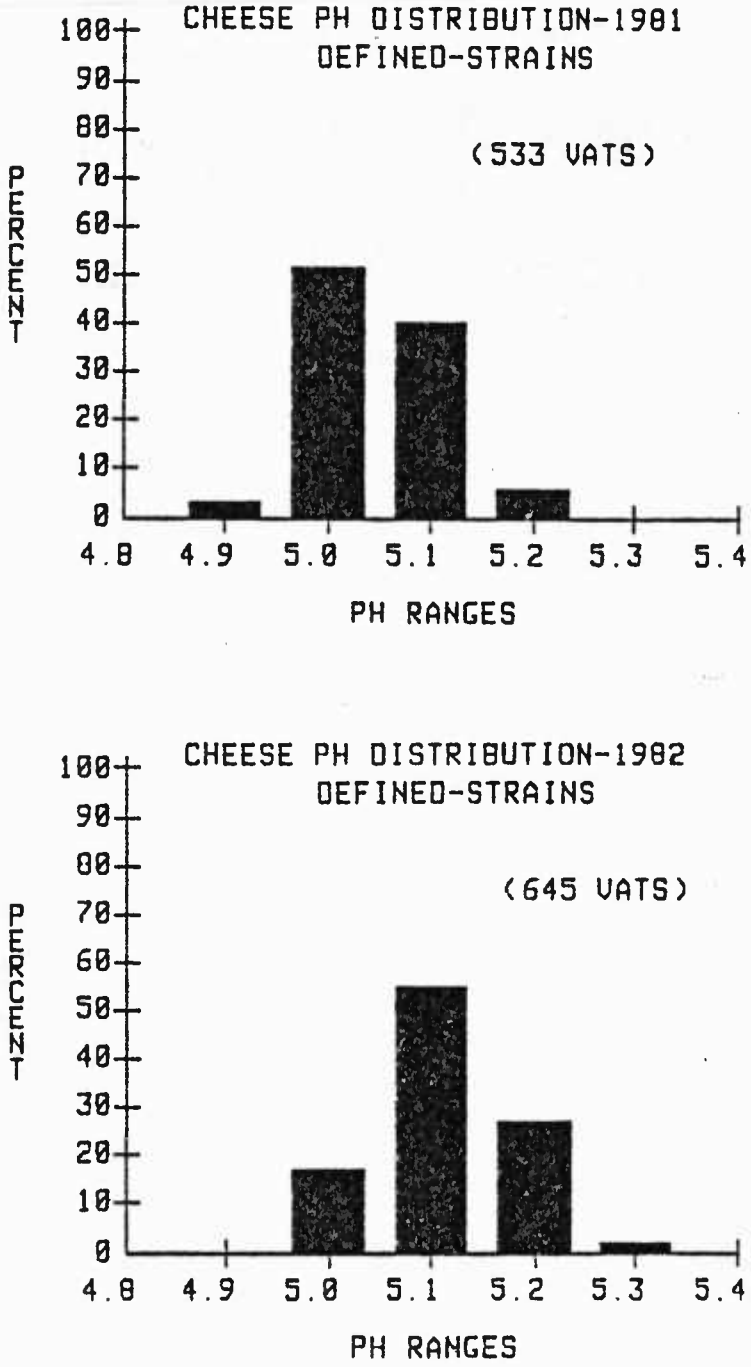


Figure 5.2. pH distribution ranges for Cheddar cheese manufactured with defined-strains (corresponding time periods for consecutive years).

manufacture to encourage bacterial propagation to sufficiently high numbers thus facilitating cell survival through the cooking and cheddaring steps. The omission of the ripening time lowers phage-stress on the culture system by shortening the period of time in which the fluid milk and culture are exposed to outside phage contamination (14) and reduces the overall time in which bacteriophage can propagate.

Cheese milk inoculated with defined-strain cultures required an average of 0.75% starter. For hold-over starter, this inoculation amount was usually increased by less than 0.1%. Hence, the amount of bulk starter needed daily could readily be predicted in advance.

4. NO CULTURE ROTATIONS.

The following two examples illustrate the economic advantages of eliminating starter rotations, which are commonly employed in an effort to overcome phage proliferation. In theory, a culture would be replaced by the next culture in the rotation before phage levels rise sufficiently to inhibit acid production. It is not uncommon to find 10 to 15 different commercial cultures used in rotation in a cheese plant (12). Often such rotations include cultures from different commercial suppliers, which may be identical or closely related in strain composition (12).

Example one is a Cheddar cheese plant which processes approximately 600,000 lb (272,700 kg) of milk per day. The plant is equipped with 5 open vats which are filled four times daily to manufacture a total of 20 vats of cheese. Approximately 2,000 liters of starter are needed for each day's production. Each consecutive group of five vats is inoculated with a different culture in a four-day rotation involving 16 cultures, as shown in Table 5.3.

To successfully prepare starter in an 8-h period using the four-day rotation plan outlined above, Plant A must use a

Table 5.3. A typical four-day culture rotation plan involving 16 cultures, as practiced in many commercial cheese plants.*

Day 1		Day 2		Day 3		Day 4	
Starter Tank	Bulk Cult.	Starter Tank	Bulk Cult.	Starter Tank	Bulk Cult.	Starter Tank	Bulk Cult.
1	A	1	E	1	I	1	M
2	B	2	F	2	J	2	N
3	C	3	G	3	K	3	O
4	D	4	H	4	L	4	P
Prepare new bulk cultures		Prepare new bulk cultures		Prepare new bulk cultures		Prepare new bulk cultures	
	E		I		M		A
	F		J		N		B
	G		K		O		C
	H		L		P		D

* Cultures are prepared one day and used the next day.

combination of eight starter and holding tanks when making starter once per day. An alternative combination of six starter and holding tanks could be used to make starter twice daily (as soon as two holding or starter tanks are emptied they must be immediately prepared for the next day's starter). This method, however, would require a second shift of personnel.

By contrast, exclusive use of a defined-culture blend requires preparation as illustrated in Table 5.4. Where the culture rotation plan necessitated preparing four separate cultures at Plant A daily, starter preparation with the defined-strain blend was reduced to one 600 gal or two 300 gal (2,268 l) tanks per day which could be prepared in 4 to 6 h.

The likelihood of phage cross-relationships between cultures is much higher in the four-day rotation involving 16 mixed-strain cultures than in the defined-strain blend containing four to six single strains.

Example two illustrates the adoption of defined-strain technology into the planning and construction of a new Cheddar cheese plant. Plant C, a newly constructed cheese plant, processes 300,000 lb (136,350 kg) milk per day using 6 vats. Each vat is filled three-times per day. A four-day culture-rotation system would have required a different culture for each of the three daily fills. The above culture rotation plan would require a combination of 6 starter and holding tanks. Three new cultures must be prepared every day, requiring a full-time starter person.

Because defined-strain technology was to be used from the beginning, Plant C was constructed with two 800 gal (3,024 l) starter tanks only. Cheese is presently made four days a week and requires 270 gal (1,021 l) total starter per day.

270 gal day X 4 days = 1,080 gal of starter needed.

$$\text{Must make starter } \frac{1,080}{800} = 1.35 \text{ times/wk}$$

Table 5.4. Simplified starter preparation using a single blend of defined-strain cultures.

Day 1		Day 2		Day 3		Day 4	
Starter Tank	Bulk Cult.	Starter Tank	Bulk Cult.	Starter Tank	Bulk Cult.	Starter Tank	Bulk Cult.
1	DCB	2	DCB	1	DCB	2	DCB
Prepare new bulk culture		Prepare new bulk culture		Prepare new bulk culture		Prepare new bulk culture	
2	DCB	1	DCB	2	DCB	1	DCB

Using the defined-strain system, bulk culture is prepared 1.35 times per week as opposed to 4 times per week (three different cultures per time) for the rotation system. The elimination of rotations allows starter personnel to prepare bulk starter with one half-day's work, thus allowing them to perform other activities elsewhere for the remainder of the week. Because a full-time starter person is no longer required, a savings in labor is realized. This is approximated below:

Full-time person:	5 days X 8 h X $12.5/h =	$500.00/wk
Part-time person:	1 day X 8 h X $12.50/h =	$100.00/wk
	Savings in labor =	$400.00/wk

Salary equivalent = $400/wk X 52 wk = $20,800/yr

5. YIELD INCREASE FROM ADDED WHEY CREAM.

During cheesemaking, some milkfat is lost into the whey instead of being incorporated into cheese curd. "Whey cream" is obtained by separating the whey, and the recovered milk fat is usually churned into butter. Currently, cheese has higher value per lb than butter. Therefore, whey cream has more value when incorporated into cheese, than when made into butter. Traditionally, whey cream has not been added to cheese milk because phage may be carried over which can infect other cultures in a rotation. This does not present a problem with defined-strain cultures unless whey cream is supplied from an outside source.

For every pound of milk fat added back to cheese milk, a yield of 1.5 lb (.68 kg) of cheese is realized (C.A. Ernstrom, personal communication). There is, however, a limit to the maximum percentage of fat, relative to the casein content, that cheese can hold without altering physical characteristics or reducing the moisture content (5,22). The casein-to-fat (C/F) ratio must be properly balanced. The addition of too much fat results in an imbalance and a higher loss of fat in the whey.

An increased yield from added whey cream can also be shown using the Modified Van Slyke and Price Cheese Yield Formula (5):

$$Y = \frac{[0.93\ F + (0.78\ P - 0.1)]\ 1.09}{1.00 - W}$$

Y = Cheese yield; lb/100 lb milk
F = lb fat/100 lb milk
P = lb protein/100 lb milk
W = lb moisture/lb cheese

The following calculations are based on 100 lb of cheese milk with 3.7% milk fat, 3.2% protein and moisture content of the cheese at 37%.

Percent fat lost in the whey = 3 to 5%

3% X 3.7 lb fat = 0.111 lb fat lost in whey (whey cream)

Addition of whey cream to cheese milk

3.7 lb + 0.111 lb whey cream = 3.811 lb fat total

Table 5.5. Increased cheese yields from the addition of whey cream to cheese milk, as calculated by the Modified Van Slyke and Price Cheese Yield Formula.

lb fat	fat + whey cream	% H_2O	% Protein	lb Yield/ 100 lb milk
3.7	---	37	3.2	10.137
3.7	3.811	37	3.2	10.315
			Increased lb yield/100 lb milk =	.178

$$\text{Increased yield/lb fat} = \frac{1\text{ lb fat X }.178\text{ lb cheese}}{0.111} = 1.60\text{ lb cheese}$$

A yield of 1.5 lb cheese/lb added fat has been reported in actual cheesemaking (Gary Burningham, personal communication).

The following example illustrates the potential economic advantages of adding whey cream to cheese milk. Assuming that a dairy processor can realize the following product yields from milkfat in Cheddar cheese and butter:

1 lb. fat from whey cream can yield 1.50 lb cheese or 1.235 lb butter.

Applying typical West Coast wholesale prices of $1.51/lb cheese and $1.51/lb butter, then comparable values of 1 lb of fat incorporated into Cheddar cheese or butter are:

$1.51/lb cheese x 1.50 lb cheese = $2.26/lb fat as cheese

$1.51/lb butter x 1.235 lb. butter = $1.86/lb fat as butter.

Assuming a typical West Coast cheese price of $1.51/lb cheese:

$1.51/lb cheese X 1.5 lb cheese = $2.26/lb fat (as cheese)

As noted from the above discussion, milk fat has significantly greater economic value when incorporated into cheese. The net value per lb of fat as cheese vs. butter is $2.26 - $1.86 = $0.40.

In a typical cheese plant, milk fat recovered from whey cream per vat is approximately 0.3% of the total lb of milk in the vat. A cheese factory using 31,000 lb (14,091 kg) vats should recover 34.4 lb (15.64 kg) of milk fat as whey cream from each cheese vat. Assuming a maximum of 28 vats of cheese per day:

34.4 lb milk fat/vat X 28 vats/day = 963.2 lb (437.8 kg) milk fat/day.

963.2 lb/day X 365 days = 351,568 lb (15,980 kg) milk fat/yr.

351,568 lb milk fat X $0.40 = $140,627.20/yr increased return on fat as cheese.

Adding whey cream to cheese milk requires less labor and handling of cream than churning for butter. Energy costs for cooling and holding are also minimized or eliminated.

6. INCREASED CHEESE PRODUCTION.

Primarily due to manufacturing improvements listed above, it has been possible for Plant A to increase production from 19 to 28 vats per day. This represents a 47.38% increase in production over 2 years and a concomitant revenue increase of more than $15 million/yr.

28 vats/day X 3,100 lb cheese/vat X 365 days = 31,682,000 lb cheese/yr

19 vats/day X 3,100 lb cheese/vat X 365 days = 21,498,500 lb cheese/yr

increased production = 10,183,500 lb/yr

10,183,500 lb X $1.51/lb cheese = $15,377,085/yr

SUMMARY

The use of defined-strain technology in Cheddar cheesemaking has resulted in manufacturing improvements of major economic significance. As of April 1, 1982, total Cheddar cheese produced with defined-strain cultures exceeded 128 million lb (58 million kg).

Starter failure, due to phage infection, was virtually eliminated in all cheese plants using defined strains. Exclusive use of the same starter culture to ferment all cheese milk resulted in greater cheese uniformity from day to day and upgraded cheese quality. The percentage of cheese graded "A" was increased by approximately 10 percent, thus increasing the net worth of the total cheese manufactured by over 1.5%. Because of a consistent and predictable starter activity, cheese factories were capable of standardizing their manufacturing procedures. Ripening times were eliminated, thus reducing phage stress on the system, and cheese make-times were shortened. A single culture-blend was used daily, hence culture rotations, which are costly in terms of time, labor, equipment, and personnel, were eliminated. Several cheese factories, using defined-strain cultures, increased cheese yields by adding whey cream to cheese milk, a practice virtually impossible when culture rotations are used.

The improvements described above generated a revenue increase of more than $1 million for one cheese plant. The combination of these improvements, all based on defined-strain culture technology, has allowed all factories to increase production--some by nearly 50%.

Defined-strain culture technology has already resulted in substantial economic benefits for several Pacific Northwest cheese factories. This culture technology is certain to have a significant technological and economic impact on the future of the U.S. cheese industry.

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CONCLUSION

"The truth goes through three stages before it is recognized. In the first stage it is ridiculed, in the second it is opposed, and finally it is recognized as self-evident."

Author unknown

"It seems self-evident that starter activity can be controlled better if starters of known composition are used. Why then is almost all of the worlds' cheese made by traditional mixed starters of variable and often unknown composition?"

Lawrence et al, 1978

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