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Two multiple strain lactic streptococcal starter concentrates were examined to determine whether or not elimination of one of the three component strains, by addition of its specific phage, resulted in disproportionate inhibition of acid production (strain dominance). Both starters showed dominance when tested three to four weeks after freezing and storage at -20° C. In one starter, dominance persisted throughout ten months of storage. In the second starter, dominance decreased over eight months of storage, and the lag phase of growth increased from one to four hours. Plate counts showed only a two-fold difference in cell numbers between uninfected and phage-infected dominant strain cultures. Differential freezing survival and recovery are suggested as factors in the production and alteration of strain dominance patterns.

The single strain components of the two starters were separately concentrated and frozen. They were then thawed at 30-day

intervals and survival, lactate sensitivity, acid-producing activity, sensitivity to lactate vs. sensitivity to pH, antibiotic production, and phage sensitivity were examined. Findings were compared to the results of tests done before freezing in an effort to determine the causes of the observed strain dominance.

Dominance by strain one in starter A was explained by its high activity when peptide fragments were supplied it by the proteolytic enzymes of the other starter A component strains, and by its ability to maintain its activity during frozen storage.

Occurrence of the dominant strain in Starter B was explained by its high survival, its tolerance to salt (indicating a lower degree of freezing damage), and its high activity after frozen storage.

It was concluded that there is no single factor identifiable as the cause of strain dominance. Phage sensitivity is suggested to be a good indicator.

The concept of strain dominance was shown to have practical value, and ways in which the techniques for its discovery might be improved are suggested.

Strain Dominance in Frozen Multiple Strain Lactic Starters

by

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STRAIN DOMINANCE IN FROZEN MULTIPLE STRAIN LACTIC STARTERS

INTRODUCTION

Lactic streptococcal starter cultures must be capable of consistent and rapid acid production for successful performance during cheese manufacture. The most common single cause of starter failure is bacteriophage infection. Christensen (10) noted that up to 80% of all starter failures may be imputed to phage infection.

Multiple strain starter cultures prepared as frozen concentrates are designed to minimize loss of activity due to phage infection by combining two or more strains of different phage sensitivities in a single starter. For the multiple strain starter to be least phage sensitive, however, it is essential that the strain balance be maintained until use in cheesemaking. Any imbalance in the frozen concentrate may be magnified both in the bulk starter and in the cheese vat.

The method and medium of propagation will determine the initial strain balance. Subsequent freezing, frozen storage, and thawing are all factors which could alter the strain balance prior to use in the bulk starter or cheese vat.

It has long been known that strain balance in laboratory cultures is altered by repeated subculturing. For example, in four different

studies (17, 37, 49, 67) milk cultures initially inoculated with equal volumes or numbers of cells from single strains were dominated by one of the strains after only a few subculturings. In each of these studies, imbalances were detected by phage infection of the multiple strain starter; the strain whose elimination caused the greatest inhibition of acid production was called the dominant strain.

Lundstedt (39) noted that there are factors which can alter strain balances when L. bulgaricus and S. thermophilus are grown together. Holzapfel et al. (31) observed that daily transfer of commercial mixed cultures in milk favored the predominance of S. cremoris. Gilliland (26) found differences in strain balance between two sets of starter cultures raised in milk or in broth, and attributed the observation to growth variation in the different media.

Multiple strain frozen starter concentrates are prepared by separately growing and concentrating the desired component single strains, mixing them in the desired proportions, and then freezing (20). Subculturing is not a factor.

Freezing and cold storage can also alter the strain balance. Maqur (40) observed that the survival of bacteria in freezing was dependent upon the rate of cooling, holding temperature, and the rate of thawing. Freezing and freeze-drying of mixed cultures is known to favor the predominance of certain strains (25). Speckman found that some strains are better suited to freeze-drying than are

others, in terms of survival and activity (62).

Freezing injury has been found to occur in a wide variety of organisms, including E. coli (43, 64), Shigella sonnei (46) Pseudomonas fluorescens (64), Aerobacter aerogenes (53), and Streptococcus lactis (45). Clark et al. (11) discovered that in sublethally-heated cells of Streptococcus fecalis, there was a temporary but sharp decrease in salt tolerance. Morichi and Irie (44) exploited this discovery to demonstrate a similarly sensitive group of cells in frozen or freeze-dried cultures of S. fecalis. It was their conclusion that both populations, the heat-damaged and frozen, had suffered the same basic kind of damage.

The sites of cellular damage due to freezing are commonly identified as the permeability barrier and/or the cytoplasmic membrane (23, 44, 60). Bretz and Kocka (7) identified the cell wall as the site of the lesion in E. coli.

Several kinds of cells, upon thawing, reveal a population of reversibly freeze-injured cells. At least part of these cells can be restored to viability by the adjustment of the recovery environment, such as the recovery medium and temperature, or the resuspension medium and temperature, in the case of freeze-dried cells (23, 45, 55, 56, 60).

While the adjustment of the thawing environment allows recovery of a larger fraction of the cells, time is required for the repair

process, and a lag time usually follows. In an effort to assure immediate activity and growth in starters, prevention of freeze-damage by the use of cryoprotective agents during freezing has been implemented. Milk and its products (3, 9, 36, 59), serum (68), glycerol (1, 28, 30, 47, 58), Peptone (4), carbohydrates (22, 57, 71), basal medium for vitamin B_{12} assay (61), Tween 80 (8), dimethyl-sulfoxide (21, 54), yeast extract (33), dextran and sodium glutamate (24), and malt extract (32) have all been used as cryoprotective agents.

Manipulation of the conditions of freezing has been used to minimize freeze-damage, and to maximize the post-thaw activity. Moss and Speck (45) have shown that different strains of S. lactis differ in their abilities to withstand exposure to cold temperatures. Nei (48) and Litvan (38) have suggested hypotheses to account for freezing damage, and have presented evidence that survival is greater when cells are frozen slowly than when they are frozen quickly. However, it has been shown repeatedly that this is not the case with the lactic streptococci. Baumann and Reinbold (5) reported that fast freezing followed by fast thawing gave the best survival and activity in cultures stored at -20 C and -196 C. Lattey (35) subjected single strain cultures of S. lactis to a variety of freezing rates and storage temperatures, and demonstrated that survival and activity were best in the cultures frozen and thawed

most rapidly.

There are many reports (5, 14, 62) which demonstrate that cells frozen in liquid nitrogen at -196 C show higher survival and activity upon thawing than do cultures stored at -20 C, the temperature commonly used in dairy plants for the storage of frozen starters. The use of liquid nitrogen, however, requires not only a source of liquid nitrogen and the facilities for its use, but technicians trained in its use. None of these is common nor economical in the average dairy plant. The common alternative is the use of conventional freezers, and storage of the frozen starters at temperatures of -20 C to -40 C.

In recent years, such frozen multiple strain starters have been used routinely by an increasing number of cheesemakers. Their use provides greater protection against loss of activity due to phage infection, and eliminates problems that arise during the maintenance and subculturing of starters in the plant.

The effectiveness of frozen multiple strain starters is largely dependent upon their ability to maintain strain balance during frozen storage. It is assumed that no interaction among the strains occurs during frozen storage, although experiments testing this possibility have not been described in the literature. In part I of this report, evidence is presented of strain dominance in commercially prepared starters, and of the alteration of strain balance during frozen

storage. Examination of the component single strains of the frozen concentrates to determine the bases for the observed strain dominance, also unreported in the literature to date, is described in the second part.

MATERIALS AND METHODS

Part I

Media

Nonfat milk (NFM) at 11% solids was made from Matrix Mother Culture Medium (Galloway-West, Fond du Lac, Wisconsin) according to the manufacturer's directions. For strain dominance tests, 500 ml quantities were steamed for 30 min and then refrigerated at 4 C until needed, or for a maximum of three days.

Single strain cultures were maintained in NFM to which 25 ml/l of 5% aqueous litmus had been added. The sterile (121 C for 10 min) litmus milk was stored, 10 ml/tube, at room temperature (25 C) until needed.

Plate counts (pour plates) were made on lactic agar (19) to which 0.5 ml/l of Tween 80 had been added. Phage assays initially were made on lactic agar, which was replaced by M17 agar when the report by Terzaghi and Sandine appeared (67). Cells were diluted in 1% tryptone (Difco) and phages in 1% tryptone plus 0.01 M $CaCl_2$.

Titratable Acidity

Titrations were performed with 0.1 N NaOH standardized with potassium acid phthalate primary standard to the phenolphthalein

endpoint. Results were reported as percent total titratable acidity (TTA) calculated as lactic acid.

Cultures

Commercially prepared multiple strain starter concentrates, each containing three different strains of Streptococcus cremoris, were obtained from a commercial source. The starters were shipped from the manufacturer in styrofoam containers with dry ice, and placed in a freezer (-20 C) immediately upon arrival. Single strain cultures used for phage propagation and assay were subcultured weekly to fresh litmus milk. The cultures were held at 4 C for six days, and incubated at 30 C for 15 h before the next transfer.

Bacteriophages

Phages were obtained from Dr. Joanne Nyiendo and were prepared and stored as described earlier (50). The phage stocks had titers ranging from 10^7 to 10^9 pfu/ml, and phages from each were capable of lysing their host single strain cultures at titers used in the strain dominance tests; the phages were host specific and none would infect the other strains used in the multiple strain starter cultures. Plaque assays were made by the overlay technique of Adams (2).

Strain Dominance Tests

Five flasks, each containing 500 ml of steamed NFM were temperature-equilibrated in a water bath at 30 C. One flask was kept as an uninoculated control. Culture concentrate was thawed in chlorinated water (100 μg/ml) at 47-50 C, and 0.5 ml added to each of the remaining flasks. Three of these were then infected, each with 0.5 ml of a phage specific for a different component strain in the starter. No phage was isolated during repeated trials for one of the component strains of starter B. The remaining flask was kept as an uninoculated control.

Immediately following infection (T_0), 9.0 ml samples were aseptically withdrawn from each of the flasks to determine the TTA. Additional samples were withdrawn at 60 min intervals thereafter. Samples for plate counts were taken at T_0 from the concentrate, and from each of the flasks after 330 min of incubation (T_{330}). All plate counts were made in duplicate and results reported as averages of the two counts in each case.

Part II

Media

The medium for the propagation of the single strain concentrates was similar to that described in the patent of Farr (20). It

contained: skim milk (Matrix, Galloway-West) 10 g/l; lactose, 20 g/l; yeast extract (Amberex, a gift of Amber laboratories) 5 g/l, Na_2HPO_4, 6.8 g/l; KH_2PO_4, 10 g/l. In some experiments, 19 g/l of beta-glycerophosphate (Sigma, grade II) was added; all such preparations were indicated by a final "3" in their designation, as A1-3.

Plate counts were done in salt agar in addition to plain M17 agar. Salt agar was M17 prepared as described in Part I, but containing final concentrations of 1.0%, 1.15%, or 1.25% NaCl.

Other media were prepared as described in Part I.

Cultures

The single strains used for propagation of the cell concentrates were isolated from the stock cultures described in Part I. Each of the single strains was plated on the differential agar of Reddy et al. (57) and three or four typical colonies of S. cremoris were picked into M17 broth. Each isolate was then used to plate its homologous phage. The isolate giving the clearest, most uniform plaques and/or the highest titer was the one selected for use as the stock culture.

Stock cultures of each strain were so isolated, and were carried in M17 broth, with weekly transfer, as described in Part I.

Phages

Each of the phages used in Part I was plated with its newly-isolated homologous host. From a plate with 30-100 plaques, several (3-4) of the clearest and most uniform plaques were picked with sterile applicator sticks into 0. 5 ml aliquots of M17 broth. The phages were then propagated according to the procedures of Terzaghi and Sandine (67).

After propagation, the phage suspensions were tested for their ability to lyse a growing culture of their homologous hosts. Each host was inoculated (1% v/v) from a 16-hr broth culture into 100 ml of broth in a side-arm flask. Growth was measured in a Klett-Summerson Photoelectric Colorimeter. After 1 h of growth at 30 C, 0. 1 ml of the homologous phage suspension was added to the flask. Thereafter, hourly readings were taken and recorded. The results appear in Table 1.

The stock phage suspensions were periodically titered throughout the experiment. Titers did not drop, but rose slightly in some cases. The results of the last titer are recorded in Table 1, also.

Propagation of the Single Strain Cell Concentrates

The single strains were propagated in a 14-liter Fermentation Design fermenter. The pH was monitored and controlled by a

Table 1. Titers of phages specific for component single strains of starters A and B, and measurement of the ability of each to lyse its homologous host. Each of two side-arm flasks containing 100 ml (each) of sterile broth was inoculated with 3 ml of a 16 h culture of host cells, and incubated at 30 C. After 60 min of incubation, one flask was infected with 0.1 ml of its homologous phage. Readings were made at T_0 and hourly intervals thereafter on a Klett-Summerson photoelectric colorimeter.

hrs	A1	A1+a1	A2	A2+a2	A3	A3+a3
0	--	--	21	22	6	6
1	1		21	23	8	8
2			27	31	12	12
3			37	37	12	12
4			51	33	13	7
5			63	21	17	5
6			75	18	23	5
7			--	--	36	6

hrs	B1	B1+b1	B2	B2+b2	B3	B3+b3
0	15	12	12	12	11	12
1	16	11	12	12	11	11
2	17	17	14	14	17	15
3	20	18	22	8	25	23
4	25	18	31	8	44	32
5	34	19	50	7	64	35
6	43	18	--	--	81	38
7	--	--	--	--	--	--

Titers of phage suspensions used, in pfu/ml

a1=[2]	b1=$8\text{-}9\times10^8$
a2=$1\text{-}2\times10^6$	b2=$2\text{-}3\times10^8$
a3=$7\text{-}8\times10^9$	b3=$1\text{-}2\times10^7$

[1] = not done

[2] = could not titer a1 on A1 after repeated attempts.

Fermentation Design pH control module with an Ingold pH electrode. Base, 10 N NH_4OH, was added automatically to the culture medium by a peristaltic pump controlled by the pH module.

The assembled fermenter, containing medium (71) was sterilized and allowed to cool to 30 C. It was then inoculated (1%, v/v) with the desired single strain. The inoculum was a 16 h culture, incubated at 30 C in the same medium as that in which the propagation was carried out.

Growth was allowed to continue for 15 h. At the end of that time, the pH was adjusted to 6.8, and the cells were collected in a Sharples Super Centrifuge.

After centrifugation, the cells were resuspended in 1/10th the original volume of refrigerated propagation medium. The concentrate was then pipeted into 18x125 mm sterile screw-cap tubes containing the various cryoprotective agents, to a final volume of 10 ml. A 10 ml sample containing no cryoprotective agent was included as a control.

The tubes containing the 10 ml of concentrate were immediately placed in a freezer at -20 C.

Cryoprotective Agents

The cryoprotective agents (CAs) used were made up and sterilized in stock solutions just before use. Glycerol (G) was used, to

give a final concentration of 10% (v/v). Beta-glycerophosphate (GP) was used from a stock solution of 95 g of GP dissolved in 100 ml distilled water. Stock solution (1. 0 ml) was added to each tube. Malt extract (ME - Difco) was made up as a 30% (w/v) stock solution, and enough was added to give a final concentration of 6% (2. 0 ml/ 10 ml).

Plate Counts

The concentrate was defrosted by holding the tube in a 45 C water bath so that the contents of the tube were below the surface. The tubes were briefly (5-10 sec) swirled in the water bath, then withdrawn and shaken vigorously, until cool to the touch. This was repeated until the tube contents were liquid, and the tube still cool to the touch. The concentrate was then placed in an ice bath, and a 1:10 dilution made. Both the dilution and the concentrate, used to inoculate the tests, were kept in the ice bath until all the tests had been inoculated, normally 60-90 min altogether.

Plate counts were performed as in part I, on all four tubes, in M17, 1. 0%, 1. 15%, and 1. 25% salt agar (pour plates).

In addition, to estimate the degree of chaining, a sample of the concentrate was blended before plating, as in Martley (41). A 1. 0 ml sample of the concentrate was pipeted into 100 ml of 0. 1% NFM, and blended for 40 sec in a semi-micro stainless steel

container. Subsequent dilutions and plating were as described earlier.

Phage Sensitivity

To determine whether or not the frozen single strains retained their sensitivity to their homologous phages, a test similar to the dominance test in Part I was used. Three flasks, each containing 500 ml of NFM, were placed in a 30 C water bath. The first was kept as an uninoculated control. The second and third were inoculated, each with 1. 0 ml of the concentrate. The third was also infected with 1. 0 ml of the appropriate phage suspension. At 0 elapsed time, T_0, and at 2 h intervals thereafter, TTA was determined for each of the flasks.

Activity Tests

Activity tests were done in NFM and in NFM supplemented with 1% Stimilac (Marschall). For each test, 0.1 ml of the 1:10 dilution of the concentrate to be tested was inoculated into 10 ml of NFM or NFM-stim. Duplicate tubes were inoculated. One set of duplicates was incubated at 30 C for 6 h, another set at 22 C for 14 h.

At the end of the incubation period, TTAs were determined for every tube.

Lactate Sensitivity

A series of dilutions of sodium lactate (Sigma) in M17 broth was prepared, ranging from 1% to 7% in 1% increments. Each of the solutions of sodium lactate prepared was pipeted into a sterile Spectronic 20 colorimetric tube, 5. 0 ml/tube. Two additional tubes were prepared, containing only M17 broth, one as a blank, one as an uninhibited growth control. The tubes were inoculated with 0. 1 ml of the 1:10 dilution of the concentrate to be tested, and incubated at 30 C. The absorbance of each tube was read immediately after inoculation and at hourly intervals thereafter. If little or no growth occurred in the control, the inoculum was doubled at the next test. Absorbance was read in a Bausch & Lomb Spectronic 20 colorimeter-spectrophotometer at 590 nm.

Sensitivity to pH vs. Sensitivity to Lactate

To compare the pH tolerance and lactate tolerance of each of the strains, samples were grown in NFM to which had been added 1. 0 N HCl (sterile) or 10% lactic acid (sterile), as in Collins (12). Enough acid was added in each case to bring the pH of the medium to 5. 5. After the 30 day tests were nearly completed, a second set of tests was initiated using half the amount of acid added (above) to bring the pH to 5. 5.

Six bottles containing 100 ml of NFM each were used in each test. The first was an uninoculated control. The second contained only cells. The remaining four contained added acid. Two contained HCl, at a higher and lower concentration, and two contained 10% lactic acid at the higher and lower concentrations. The bottles were inoculated with 1.0 ml of the 1:10 dilution of the concentrate to be tested, and incubated at 30 C for 6 h. At the end of the incubation period, the TTA of each of the bottles was determined, and compared to the TTA taken at T_0.

Tests for Antibiotic Production

The cultures were tested for antibiotic production by the method described in Collins (13), but using M17 agar. Both the concentrated and 16 h cultures of the unconcentrated single strains were tested.

The concentrate, after all tests had been inoculated, was centrifuged, and the supernatant spotted on a lawn of the strain to be tested for sensitivity to test the possibility of production of some inhibitory product during frozen storage.

RESULTS

Part I: Commercially Prepared Multiple Strain Frozen Starter Concentrates

Starters were said to show strain dominance when infection by the phage for one of the component strains resulted in greater inhibition of acid production than infection by phages for the other strains. The strain whose infection by the specific phage caused the greatest degree of inhibition of acid production was therefore referred to as the dominant strain.

Preliminary experiments with two different commercially-prepared multiple strain starter concentrates indicated that strain dominance occurred in each culture (Fig. 1). Of three different lots of one starter examined, one showed distinct strain dominance, and two did not. The lots differed in age; therefore it seemed worthwhile to study the effect of storage time on strain balance and two starters designated here as A and B were carefully examined in this regard. Young lots, 3-4 weeks old, of each starter showed a clear dominance pattern (Fig. 1). Considerable inhibition of acid production occurred, with infected dominant strain cultures lagging 1.5 h in A to 3 h in B behind the uninfected controls. Less inhibition occurred when strains other than the dominant strain were infected.

Older lots of culture A (Fig. 2) showed decreasing dominance

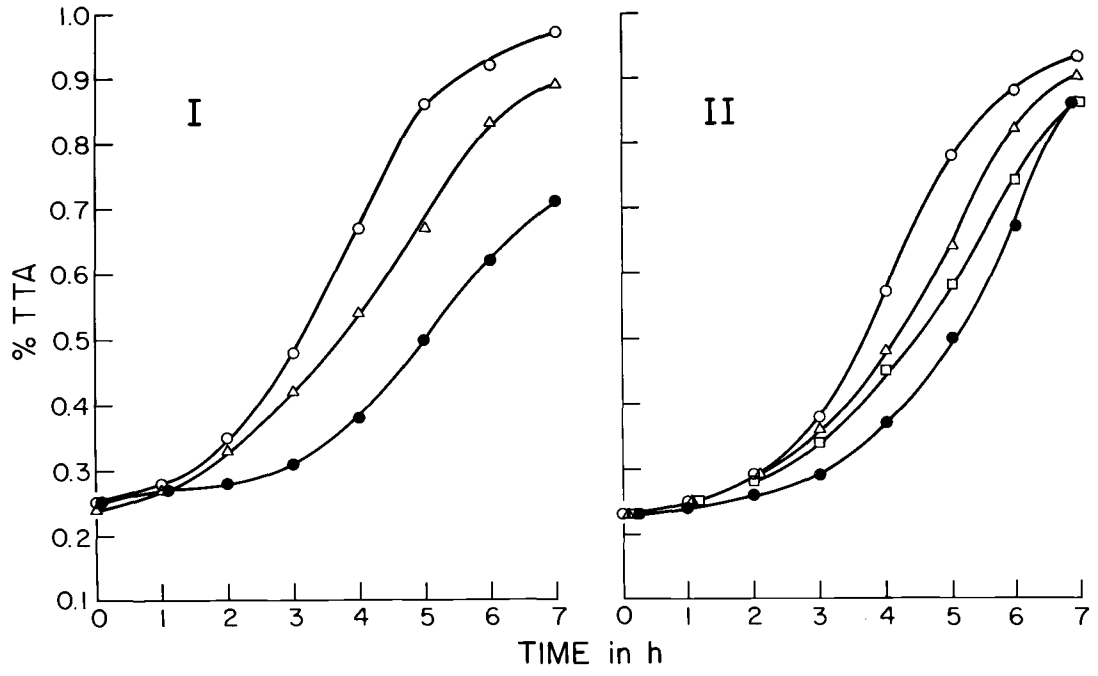

Fig. 1. TTA of 11% NFM cultures inoculated (1%) from 3-4 week old frozen (-20 C) concentrated multiple strain starter cultures and infected with phages homologous for one component strain. I. Starter B: O, uninfected control; △, infected with phage b1; ●, infected with phage b2. II. Starter A: O, uninfected control; □, infected with phage a1; △, infected with phage a2; ●, infected with phage a3. Data plotted were averaged from three experiments.

with increasing age. Inhibition of acid production was decreased to about 1 h in the lots aged 1-1. 5 months, and at 7-8 months the acid production in the cultures infected with any one of the component strain phages differed little from the uninfected control. Also, in the lots aged 7-8 months, the lag phase in all A cultures had increased from 1 to 3 h.

Starter B showed less alteration of its strain dominance pattern in older lots (Fig. 3); dominance was clear after three months and was still visible, though decreased, after ten months of storage. There was no appreciable change in the length of its lag phase.

Plate counts were run to determine if the decrease in acid production observed as a result of phage infection was accompanied by a corresponding decrease in cell numbers. The plate count data corresponding to the acid production data depicted in Fig. 1-3, appear in Table 2. The T_0 counts in culture B were fairly stable while those of culture A decreased during frozen storage. The T_{330} plate counts revealed that cell numbers in the uninfected controls were approximately twice those in which the dominant strain was infected by phage addition. This difference remained fairly constant in culture B, but decreased with increased storage time in culture A.

Approximately one log of growth (cfu/ml at T_{330} - cfu/ml at T_0) occurred in the uninfected controls. In the cultures infected with the dominant strain phage, growth was slightly reduced except in case

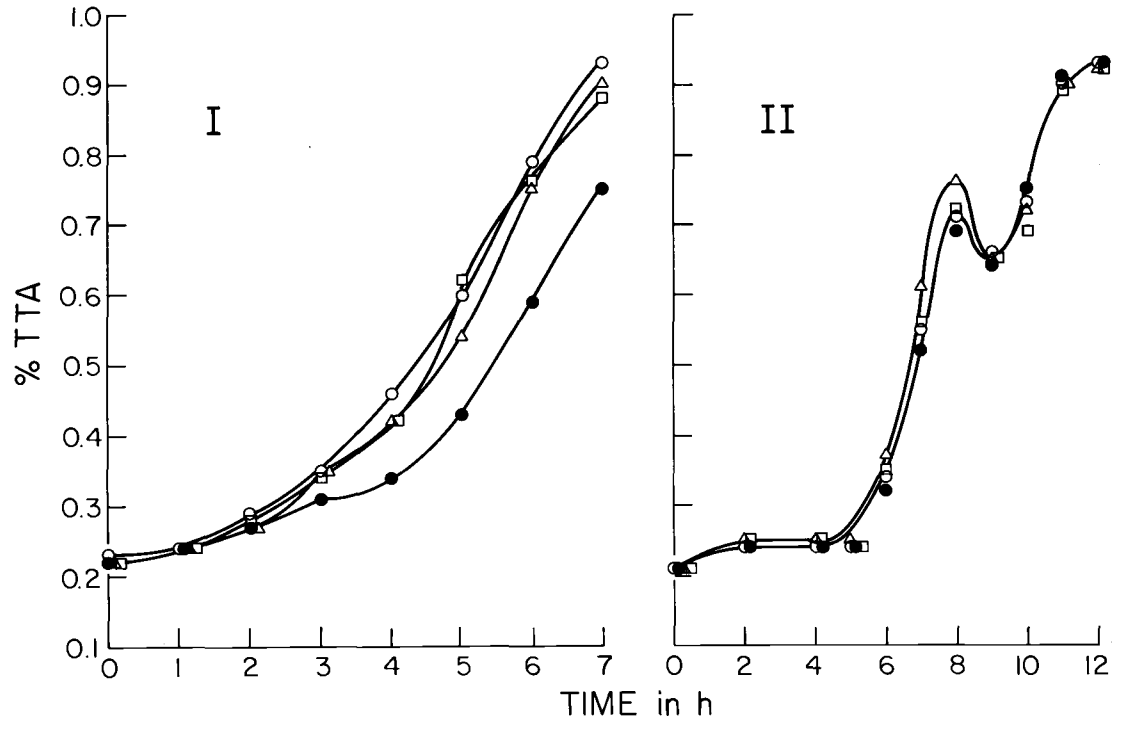

Fig. 2. TTA of 11% NFM cultures inoculated (1%) from 1.5 month old (I) or 7-8 month old (II) frozen (-20 C) concentrated multiple strain starter culture A infected with phages homologous for each component strain. O, uninfected control; □, infected with phage a1; △, infected with phage a2; ●, infected with phage a3. Data plotted were averaged from four experiments.

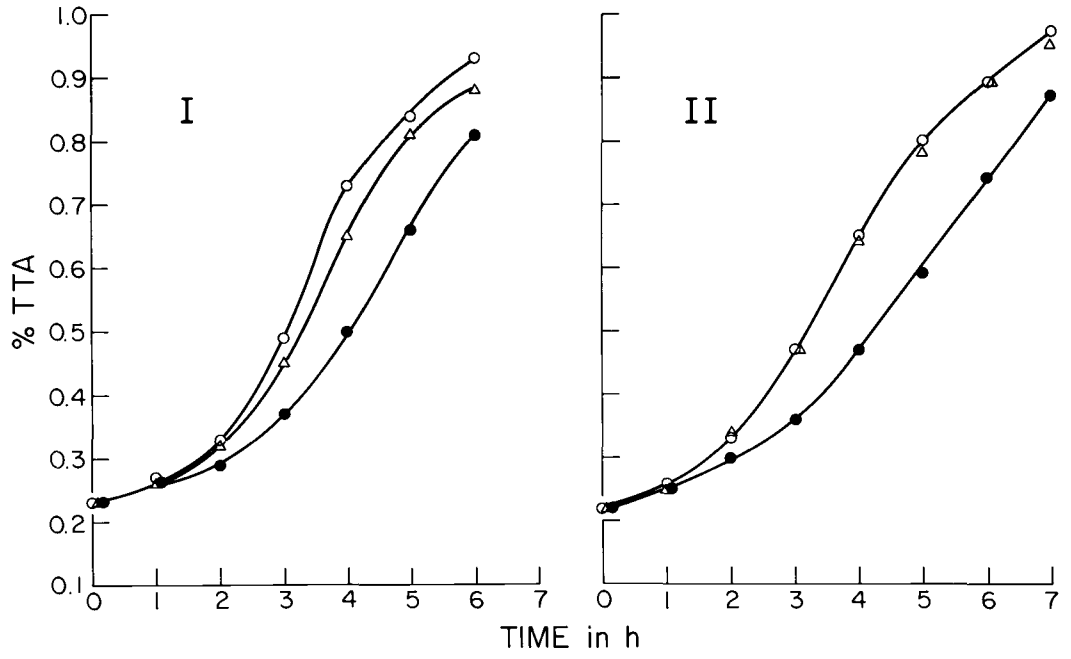

Fig. 3. TTA of 11% NFM cultures inoculated (1%) from 3 mo. old (I) or 10 mo. old (II) frozen (-20 C) concentrated multiple strain starter culture B infected with phages homologous for two of the three component strains. O, uninfected control; △, infected with phage b1; ○, infected phage b2. Data plotted were averaged from four experiments for I and five experiments in II.

Table 2. Plate count data (cfu/ml x 10^7) from repeated trials for starters A and B at T_0 and T_{330}, comparing uninfected (C) and phage (ϕ) infected (ϕa3 or b2) dominant strain cultures.

Starter A

3-4 weeks			1-1.5 months			7-8 months		
	T_{330}			T_{330}			T_{330}	
To	C	ϕb2	To	C	ϕb2	To	C	ϕb2
2.0	90	19	0.63	38	21	0.15	14	13
2.7	73	18	0.92	31	15	0.25	29	28
2.5	62	14	1.7	8.4	16	0.51	52	26
			0.18	25	18			
			0.54	18	25			

Starter B

3-4 weeks			3 months			10 months		
	T_{330}			T_{330}				
To	C	ϕb2	To	C	ϕb2	To	C	ϕb2
2.4	41	21	5.1	64	29	7.1	27	13
5.9	47	12	6.6	65	29	0.93	1.5	12
5.8	39	1.5	9.0	51	35	9.3	33	14
			8.4	84	26	11.5	12	15

of the ten month-old lots of culture B. Here much less growth was observed, although acid production was normal. Determination of average chain lengths (Table 3) by microscopic examination revealed that all lots of frozen starter concentrate contained only diplococci and single cells regardless of age, while chains averaging 7-15 cells in length were found in the cultures at T_{330}. The microscopic counts indicated that a decreasing chain length in starter B accounted for the increasing T_0 counts (Table 2) in this culture during frozen storage.

Part II. Single-strain Frozen Concentrates Prepared in the Laboratory

Plate Counts

The results of the plate counts for concentrates of the single strain components of starters A and B are shown in Tables 4 through 7. The first two tables show viable cell counts, the second two, percent survival.

In some cases, survival increased when the cells were plated on salt agar. Where survival increases or decreases are mentioned, it is in relation to survival on unsupplemented M17 agar. It should be pointed out, though, that survival is based on the plate counts at T_0 on the type of agar in question, i. e., percent survival on 1% salt agar is based on the T_0 count in 1% salt agar being set equal to 100% survival.

Table 3. Average number of cells per chain appearing in uninfected and phage infected dominant strain cultures for starter B at T_0 and T_{330} when stored for different periods.

T_{330}			3 months			10 months		
To	C[a]	φb2	To	C	φb2	To	C	φb2
2.3	16.	6.4	1.9	7.7	3.7	1.4[b]	14.1	7.6
2.1	14.7	--	1.9	9.1	4.5		14.6	15.2

[a] Uninfected

[b] 100 fields counted, no chain greater than 2.0 observed; many single cells observed

Table 4. Viable cell counts of component single strain cell concentrates of starter B in M17 agar and in M17 agar plus 1.0%, 1.15%, or 1.25% NaCl (final concentrations) after 0, 30, 60, and 90 days of frozen storage. Data shown are colony counts x 10^9. Average of duplicate plates, incubated 48 h at 30 C. Counts are corrected for volumes of CA added.

		M17				1% NaCl				1.15% NaCl				1.25% NaCl			
		G[1]	O	GP	ME	G	O	GP	ME	G	O	GP	ME	G	O	GP	ME
B1-1	0	35	--[2]	--	--	24	--	--	--	13	--	--	--	9	--	--	--
	30	23	18	10	15	17	12	9	11	13	11	6	10	7	5	3	5
	60	16	12	8	13	12	8	5	7	6	5	3	4	7	5	3	4
	90	21	10	8	15	10	5	5	6	7	4	4	5	8	5	4	7
B1-2	0	21	--	--	--	17	--	--	--	7	--	--	--	5	--	--	--
	30	15	17	12	19	8	10	6	9	3	4	2	3	1	3	1	2
	60	17	11	10	13	9	8	6	8	5	6	4	4	4	3	3	4
	90	13	11	12	13	10	9	9	9	5	6	3	5	3	2	2	2
B1-3	0	38	--	--	--	32	--	--	--	22	--	--	--	24	--	--	--
	30	34	22	21	30	24	15	14	24	15	11	11	17	9	8	10	14
	60	30	12	17	24	24	8	15	22	19	9	12	21	15	7	13	20
	90	33	16	21	23	24	13	13	18	16	14	14	17	17	9	13	16
B2-1	0	54	--	--	--	51	--	--	--	48	--	--	--	46	--	--	--
	30	41	47	52	49	40	47	50	49	39	48	53	45	35	45	43	38
	60	48	48	42	47	49	47	42	46	50	45	39	48	50	42	41	47
	90	46	38	31	33	45	37	34	33	52	39	36	34	41	38	34	34
B2-2	0	46	--	--	--	47	--	--	--	46	--	--	--	45	--	--	--
	30	44	30	35	44	48	31	41	50	42	30	34	42	41	31	36	44
	60	34	29	34	32	35	26	35	28	37	27	31	28	46	27	31	29
	90	40	27	34	38	40	27	32	34	43	25	31	36	43	25	32	36
B2-3	0	49	--	--	--	50	--	--	--	53	--	--	--	52	--	--	--
	30	54	47	54	39	56	52	48	39	48	34	44	43	46	37	42	35
	60	44	31	34	30	48	29	31	31	43	27	33	29	47	27	33	26
	90	39	27	39	38	38	26	38	37	39	25	41	40	39	24	39	36
B3-1	0	20	--	--	--	13	--	--	--	12	--	--	--	11	--	--	--
	30	7	8	7	8	--	--	--	--	5	5	4	7	4	3	2	4
	60	4	6	3	5	2	3	1	2	1	2	1	1	8	1	.7	.8
	90	6	3	3	4	3	1	1	3	3	1	1	2	2	1	1	1
B3-2	0	6	--	--	--	6	--	--	6	--	--	--	--	7	--	--	--
	30	5	3	2	2	4	3	1	2	3	2	1	1	2	1	1	1
	60	4	2	2	2	2	1	.5	.5	1	1	.5	.5	1	.5	.5	.5
	90	4	1	2	1	3	1	1	1	3	1	1	1	2	.5	1	1
B3-3	0	.65	--	--	--	.35	--	--	--	.45	--	--	--	.45	--	--	--
	30	.16	.02	.08	.05	.37	.05	.09	.09	.28	.02	.02	.08	.22	.04	.04	.08
	60	.2	.02	.1	.1	.03	.01	.01	.02	.02	.01	.01	.02	.01	.004	.01	.004
	90	.09	.02	.05	.04	.06	.02	.04	.05	.06	.02	.03	.04	.06	.02	.03	.04

[1] G, O, GP, and ME were the cryoprotective agents used; G = glycerol, O = no cyroprotective agent added, GP = beta-glycerophosphate, and ME = Malt Extract.

[2] --, no data.

Table 5. Viable cell counts of component single strain cell concentrates of starter A in M17 agar and in M17 agar plus 1.0%, 1.15%, or 1.25% NaCl (final concentration) after 0, 30, 60, and 90 days of frozen storage. Data shown are colony counts x 10^9. Average of duplicate plates, incubated at 30 C for 48 h. Counts are connected for volumes of CA added.

		G[1]	O	GP	ME	G	O	GP	ME	G	O	GP	ME	G	O	GP	ME
A1-1	0	19	--[2]	--	--	21	--	--	--	19	--	--	--	20	--	--	--
	30	18	13	20	20	19	16	18	19	17	12	19	18	21	14	18	16
	60	21	15	15	14	18	5	16	12	21	15	15	13	18	13	15	12
	90	16	21	13	14	17	9	15	16	15	10	14	16	17	0	14	15
A1-2	0	69	--	--	--	58	--	--	-	50	--	--	--	61	--	--	--
	30	56	40	48	43	55	38	49	47	48	38	44	43	52	34	42	38
	60	43	40	46	41	42	39	46	40	41	39	43	39	43	36	43	42
	90	49	38	38	43	49	30	31	39	46	26	30	39	46	26	31	39
A1-3	0	8[3]	--	--	--	8[3]	--	--	--	8[3]	--	--	--	8[3]	--	--	--
	30	7	5	6	10	8	5	6	10	7	4	6	9	7	4	5	8
	60	8	5	6	7	8	6	4	5	8	5	6	9	9	5	6	7
	90	8	5	6	7	8	6	4	5	6	5	4	6	6	4	4	8
A2-1	0	8	--	--	--	4	--	--	--	2	--	--	--	2	--	--	--
	30	5	5	4	4	2	1	.7	2	1	1	.8	.4	.6	.5	.4	.4
	60	4	3	3	4	2	1	1	2	2	1	1	2	1	1	1	1
	90	4	2	3	3	3	2	2	2	2	1	1	1	2	1	1	1
A2-2	0	12	--	--	--	5	--	--	--	4	--	--	--	2	--	--	--
	30	5	3	2	4	8	.3	.3	.3	.7	.6	.3	.8	.5	.5	.2	0
	60	5	4	4	6	4	3	3	5	3	2	2	3	3	2	1	2
	90	6	4	5	5	4	3	2	3	3	2	2	2	2	1	1	2
A2-3	0	37	--	--	--	28	--	--	--	19	--	--	--	20	--	--	--
	30	21	23	25	18	14	15	12	9	9	10	8	7	8	7	6	5
	60	23	12	21	20	22	11	22	20	19	11	19	18	14	8	15	17
	90	22	11	19	14	15	10	16	14	12	8	10	8	11	9	11	10
A3-1	0	19	--	--	--	17	--	--	--	19	--	--	--	20	--	--	--
	30	23	14	18	25	22	19	17	18	20	15	14	17	16	11	13	16
	60	21	12	19	20	19	11	18	19	17	8	15	17	16	7	12	11
	90	22	15	16	18	23	12	17	15	20	11	13	15	20	10	12	13
A3-2	0	7	--	--	--	6	--	--	--	6	--	--	--	6	--	--	--
	30	3	1	1	3	4	1	1	3	5	2	1	2	4	1	1	2
	60	5	1	1	3	5	1	1	3	5	2	1	2	4	1	1	2
	90	4	1	2	2	3	1	2	2	4	1	1	2	3	1	1	2
A3-3	0	12	--	--	--	12	--	--	--	12	--	--	--	12	--	--	--
	30	10	7	9	8	--	--	--	--	9	6	15	7	6	4	11	6
	60	9	--	--	9	8	--	--	9	9	--	--	7	7	--	--	7
	90	7	5	6	7	7	4	6	9	5	5	5	5	7	4	5	6

[1] G, O, GP, and ME were the cryoprotective agents used; G = glycerol, O = no cryoprotective agent added, GP = beta-glycerophosphate, and ME = Malt Extract.

[2] --, no data.

[3] Less than 30 colonies per plate.

Table 6. Percent survival of component single strain cell concentrates of starter A in M17 agar and in M17 agar plus 1.0%, 1.15%, or 1.25% NaCl (final concentrations) after 0, 30, 60, and 90 days of frozen storage. Percent survival was calculated as cell counts at T_x/cell counts at T_0 x 100 for a given strain in a given medium. Percent survival was calculated using viable counts corrected for the volumes of the added CAs.

	M17 agar				1.0% salt agar				1.15% salt agar				1.25% salt agar			
	G[1]	O	GP	ME	G	O	GP	ME	G	O	GP	ME	G	O	GP	ME
A1-1																
30	102	66	105	105	94	76	87	90	91	63	99	92	104	72	88	80
60	109	78	77	70	88	71	77	58	110	80	78	65	90	67	73	62
90	85	106	65	74	81	41	72	79	81	53	75	83	87	47	72	76
A1-2																
30	81	58	70	63	95	66	84	80	90	75	88	86	85	55	69	62
60	62	57	67	59	72	67	80	69	82	77	86	78	71	58	70	69
90	71	54	55	62	85	51	54	67	93	52	66	79	75	43	50	63
A1-3																
30	92	61	73	123	94	58	76	120	85	52	71	118	81	51	66	101
60	104	69	83	111	101	63	86	89	95	58	79	106	109	63	71	89
90	100	68	73	81	95	69	50	64	75	68	49	71	79	55	49	95
A2-1																
30	56	26	50	53	50[2]	29[2]	18[2]	43[2]	59[2]	56[2]	46[2]	22[2]	29[2]	26[2]	23[2]	20[2]
60	43	34	33	47	40	31	30	38	81	68	54	78	55	59	47	59
90	52	29	34	41	84	42	47	55	130	71	65	76	95	53	39	51
A2-2																
30	33	26	20	33	14[2]	6[2]	6[2]	5[2]	19[2]	16[2]	9[2]	20[2]	21[2]	19[2]	9[2]	0[2]
60	38	29	31	44	80	47	51	92	84	58	43	84	111	83	60	98
90	47	34	38	40	76	46	39	54	78	41	43	65	100	54	50	63
A2-3																
30	77	85	90	64	51	54	43	32	49	51	44	38	38	33	29	23
60	84	45	77	73	78	41	78	71	97	55	100	92	69	37	72	82
90	81	41	68	53	55	34	58	48	61	42	51	41	56	43	54	47
A3-1																
30	121	77	94	130	130	111	100	105	106	77	76	92	79	55	76	77
60	113	66	101	106	114	66	106	111	80	45	81	90	80	36	58	52
90	115	81	87	96	135	73	102	93	107	58	69	78	99	47	58	64
A3-2																
30	48	182	15[2]	15	61	16[2]	21[2]	45	42	20[2]	18[2]	36[2]	58	16[2]	23[2]	26
60	79	20	18	45	80	19	16	45	89	18	22	49	74	13	16	40
90	61	21	22	33	47	20	24	31	69	23	23	34	58	18	19	28
A3-3																
30	83	57	76	67	--[3]	--	--	--	77	50	125	56	50	30	88	49
60	71	--	--	75	67	--	--	69	73	--	--	61	59	--	--	60
90	55	40	50	52	58	32	50	73	38	39	41	38	54	30	43	48

[1] as in Table 5; [2] less than 30 colonies per plate; [3] --, = no data.

Table 7. Percent survival of component single strain concentrates of starter B in M17 agar, and in M17 agar plus 1.0%, 1.15%, 1.25% NaCl (final concentration) after 0, 30, 60, and 90 days of frozen storage. Percent survival was calculated as (cell counts at T_x / cell counts at T_0 x 100 for a given strain in a given medium. Percent survival was calculated using viable counts corrected for the volumes of the added CAs.

	M17 agar				1.0% salt agar				1.15% salt agar				1.25% salt agar			
B1-1	G[1]	O	GP	ME	G	O	GP	ME	G	O	GP	ME	G	O	GP	ME
30	66	51	30	44	81	57	43	51	99	80	47	76	75	49	35	56
60	45	34	24	38	60	41	22	36	44	44	26	32	70	48	34	43
90	60	28	24	45	43	20	20	26	50	29	25	38	78	94	74	77
B1-2																
30	72	79	56	89	47	58	35	53	47	64	30*	50	26*	53	28*	45*
60	84	51	48	60	55	47	36	44	67	86	56	54	81	55	57	25
90	61	51	59	62	61	52	55	55	75	87	47	72	95	81	85	74
B1-3																
30	90	56	58	79	77	47	44	79	69	48	49	76	39	32	42	58
60	79	31	46	62	77	26	49	71	88	39	57	95	62	29	55	82
90	88	42	56	60	75	42	42	57	75	63	63	75	73	37	55	67
B2-1																
30	75	87	96	90	78	92	98	85	81	101	111	94	87	111	108	95
60	88	89	78	88	97	92	82	90	95	95	81	101	125	106	101	118
90	85	70	58	61	88	73	68	65	109	82	77	72	101	94	85	85
B2-2																
30	96	65	77	98	97	62	87	107	91	65	74	91	91	69	80	99
60	74	64	75	70	71	55	70	57	81	58	68	60	90	60	69	65
90	87	59	76	84	82	54	67	70	73	55	68	78	96	61	71	80
B2-3																
30	112	96	112	99	111	103	97	154	91	65	85	81	89	71	81	67
60	9	63	71	63	96	58	62	61	78	52	62	55	91	52	63	50
90	79	56	80	78	75	51	76	74	74	48	79	75	74	64	75	69
B3-1																
30	36	38	35	39	--[2]	--	--	--	44	37	3[illegible]	57	33	25	15	33
60	20	28	14	26	14	20	8	18	10	14	6	11	8	11	7	7
90	27	11	12	20	26	11	8	19	23	11	7	17	16	7	6	13
B3-2																
30	76	49	30	37	74	47	19	32	49	33	17	25	32	22	11	17
60	62	38	24	32	26	16	6	8	18	11	5	6	9	6	4	4
90	63	21	30	20	49	13	16	14	48	14	17	12	32	10	10	9
B3-3																
30	25	3[3]	14[3]	8[3]	105	14[3]	26[3]	26[3]	62	4[3]	4[3]	18[3]	49	9[3]	9[3]	18[3]
60	31	3	15	15	9	1.4	4	4	.04	.6	1.3	4	.02	.8	1.3	1
90	14	3	7	7	17	6	12	15	13	4	7	10	13	4	6[illegible]	7

[1] G, O, GP, and ME were the cryoprotective agents used; G = glycerol, O = no cryoprotective agent added, GP = beta-glycerophosphate, and ME = Malt Extract.

[2] --, no data.

[3] Less than 30 colonies per plate.

Tables 8 and 9 show the ratios of growth on salt agar to growth on M17 agar for each CA at each test period. The ratio shows that only a fraction of the cells growing on M17 can grow on salt agar, and thus indicates the size of the population of cells injured by freezing.

Plate counts were also performed on 16-h cultures of the unconcentrated single strains. These cells were grown in M17 broth, or in NFM, and plated in M17 agar and/or M17 agar containing added salt. These data are presented in Table 10.

Plate counts performed on samples of blended concentrate were compared to those done on samples not blended. The data are presented in Table 11, as the ratio, blended counts: unblended counts. The data show clearly that there was no significant chain formation in any case.

Each strain showed differences in survival when stored in different cryoprotective agent (CAs). The cells of a given strain, when grown in medium containing GP, usually showed different responses to the CA's than did cells of the same strain grown in medium without GP.

Where comparisons are made, the norms are the counts made on M17 agar, or of cells stored in Glycerol. Differences in percent survival refer to differences greater than or equal to 10% survival. This figure is more or less arbitrary, but Standard Methods for the

Table 8. Ratio of growth on salt agar to growth on M17 agar for component single strain cell concentrates of starter A, after 0, 30, 60, and 90 days of frozen storage. Ratio calculated as viable counts in x% salt/viable counts in M17 for a given strain, in a given CA at a particular test time.

		30				60				90			
	To	G[1]	O	GP	ME	G	O	GP	ME	GO	O	GP	ME
A1-1													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	1.06	.97	1.21	.88	.91	.86	.96	1.05	.87	1.01	.41	1.16	1.13
1.15	.98	.88	.93	.93	.87	1.0	1.01	1.0	.97	.94	.49	1.13	1.1
1.25	1.03	1.04	1.12	.87	.79	.86	.89	.98	.90	1.05	.45	1.13	1.06
A1-2													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.84	.99	.95	1.01	1.17	1.02	.99	1.0	.98	1.01	.78	.83	.90
1.15	.72	.86	.94	.91	.99	.98	.97	.94	.95	.95	.69	.88	.91
1.25	.88	.93	.83	.87	.88	1.03	.90	.92	1.02	.94	.70	.82	.90
A1-3													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	1-1.14	1.03	.95	1.05	.98	.98	.91	1.05	.80	.95	1.02	.69	.78
1.15	.86-1.14	.93	.89	.98	.96	.92	.84	.95	.96	.75	1.0	.67	.88
1.25	.86-1.14	.88	.84	.91	.83	1.05	.91	.86	.80	.79	.81	.67	1.17
A2-1													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.46	.41	.73	.16	.37	.33	.42	.47	.47	.74	.67	.64	.62
1.15	.21	.22	.20	.21	.09	.46	.41	.38	.43	.51	.50	.39	.38
1.25	.23	.12	.11	.12	.09	.34	.39	.36	.37	.42	.42	.26	.28
A2-2													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.45	.17	.10	.14	.06	.80	.62	.61	.76	.73	.61	.47	.60
1.15	.31	.15	.19	.14	.19	.57	.52	.36	.48	.52	.37	.36	.50
1.25	.20	.11	.15	.09	0	.48	.49	.32	.36	.43	.32	.27	.31
A2-3													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	1.02	.68	.64	.49	.51	.96	.92	1.03	1.0	.69	.85	.86	.94
1.15	.71	.45	.42	.34	.41	.82	.86	.91	.89	.53	.72	.53	.55
1.25	.75	.37	.28	.24	.26	.62	.61	.69	.84	.52	.79	.59	.66
A3-1													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.9	.97	1.3	.97	.73	.91	.9	.95	.95	1.06	.81	1.05	.88
1.15	1.0	.88	.77	.81	.71	.79	.68	.80	.86	.93	.71	.79	.81
1.25	1.07	.70	.77	.76	.64	.76	.89	.62	.53	.92	.62	.71	.72
A3-2													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.93	1.18	.83	1.3	.97	.95	.95	.82	.94	.71	.89	1.0	.86
1.15	.84	.73	.92	1.0	.69	.94	.90	.98	.92	.93	.92	.87	.86
1.25	.85	1.04	.75	1.3	.52	.80	.65	.76	.76	.81	.74	.73	.73
A3-3													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	1.0	--[2]	--	--	--	.94	--	--	.92	1.06	.82	.60	1.14
1.15	.98	.9	.86	.81	.81	1.0	--	--	.8	.68	.96	.81	.71
1.25	.98	.59	.81	.57	.77	.82	--	--	.78	.97	.73	.85	.91

1 - as in Table 4; 2 - -, no data.

Table 9. Ratio of growth on salt agar to growth on M17 agar for component single strain cell concentrates of starter B, after 0, 30, 60, and 90 days of frozen storage. Ratio calculated as viable counts in x% salt/viable counts in M17, for a given strain, in a given CA, at a particular test time

B1-1													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.68	.73	.67	.85	.69	.78	.70	.54	.57	.96	.47	1.1	.80
1.15	.38	.58	.61	.61	.67	.37	.43	.41	.33	64	.37	.79	.67
1.25	.27	.31	.26	.32	.35	.42	.38	.39	.31	.71	.43	.83	.94
B1-2													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.82	.54	.61	.52	.49	.53	.76	.62	.60	.82	.84	.76	.73
1.15	.32	.24	.26	.17	.18	.52	.54	.38	.58	.40	.56	.26	.38
1.25	.23	.09	.15	.11	.11	.22	.24	.27	.34	.70	.72	.65	.54
B1-3													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.84	.72	.71	.63	.84	.82	.70	.89	.95	.72	.85	.63	.79
1.15	.58	.45	.50	.49	.56	.64	.73	.73	.89	.49	.88	.65	.73
1.25	.63	.27	.36	.46	.46	.49	.58	.77	.83	.52	.57	.61	.70
B2-1													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.94	.98	.99	.97	1.01	1.04	.97	.99	.97	.98	1.02	1.1	1.0
1.15	.88	.95	1.02	1.02	.92	1.05	.94	.92	1.02	1.14	1.06	1.17	1.04
1.25	.74	1.1	.95	.84	.78	1.05	.88	.96	1.0	.89	1.02	1.09	1.04
B2-2													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	1.03	1.09	1.04	1.17	1.13	1.03	.89	1.01	.89	1.02	.99	.92	.90
1.15	1.01	.95	.99	.97	.94	1.09	.92	.91	.87	1.08	.93	.90	.94
1.25	.98	.93	1.03	1.02	1.0	1.19	.97	.90	.92	1.09	.94	.92	.94
B2-3													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	1.04	1.02	1.11	.90	1.98	1.09	.95	.91	1.01	.97	.95	.99	.97
1.15	1.08	.98	.72	.82	1.09	.97	.89	.94	.95	1.01	.92	1.07	1.04
1.25	1.07	.85	.72	.78	.90	1.07	.88	.96	.85	1.01	.87	1.01	.94
B3-1													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.65	--	--	--	--	.47	.47	.36	.46	.62	.54	.40	.64
1.15	.60	.74	.59	.53	.87	.30	.31	.27	.24	.49	.52	.35	.51
1.25	.54	.50	.37	.23	.46	.22	.21	.26	.15	.31	.29	.27	.35
B3-2													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.90	.88	.87	.58	.78	.37	.38	.23	.24	.70	.55	.50	.64
1.15	.87	.56	.58	.49	.61	.22	.24	.18	.17	.67	.58	.51	.53
1.25	1.04	.44	.47	.37	.48	.15	.16	.18	.12	.53	.49	.37	.50
B3-3													
M17	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0	.54	2.06	2.5	1.0	1.8	.15	.25	.13	.15	.67-1.2	1.11	.84	1.23
1.15	.69	1.25	1.0	.22	1.6	.10	.15	.06	.16	"	.89	.65	1.02
1.25	.45	1.4	2.0	.44	1.6	.05	.20	.06	.04	"	.95	.89	.93

Table 10. Viable cell counts on 16 h cultures of single strains of starters A and B, in M17 agar and in M17 agar plus 1.0%, 1.15%, and 1.25% NaCl (final concentrations); viable counts of 16 h cultures from NFM. All cultures were inoculated 1% (v/v) from overnight broth cultures of the single strain, and incubated 16 h at 30 C.

I	ratio, salt agar:M17[1]					
M17	1.0	1.0	1.0	1.0	1.0	1.0
1.0%	1.17	.86	1.22	1.08	1.0	1.05
1.15%	1.08	.55	.63	.86	1.05	1.06
1.25%	1.19	.49	.12	.61	1.01	1.09

II	[2]Plate counts x 10^7					
	A1	A2	A3	B1	B2	B3
M17	70	126	67	118	127	75
NFM	248	222	38	65	266	65

[1] 5 replications averaged

[2] 7 replications averaged

Table 11. Ratio of colony counts from blended samples to colony counts of unblended samples of component single strain cell concentrates of starters A and B, after 0, 30, 60, and 90 days of frozen storage. Samples were blended 40 seconds, and plated. The ratio was calculated for a given strain, at a given time, in G, as (blended counts/unblended counts) x 100.

	A1-1	A1-2	A1-3	A2-1	A2-2	A2-3	A3-1	A3-2	A3-3
0	.96	.97	--[1]	1.15	.96	--	1.24	--	.88
30	1.05	.88	1.18	1.16	.74	1.1	--	1.0	.88
60	.83	.95	.95	1.57	1.5-23	1.1	1.39	1.7	.42
90	.91	.98	.86	.79	.89	1.0	1.02	1.3	.95

	B1-1	B1-2	B1-3	B2-1	B2-2	B2-3	B3-1	B3-2	B3-3
0	1.00	1.35	1.08	.89	1.02	.99	.61	1.05	1.08
30	.72	--	--	--	--	--	--	.89	1.0
60	--	--	.91	1.01	1.22	.96	--	--	--
90	1.00	1.58	.94	.98	.83	.93	.69	1.0	.02

[1] -- = no data

Examination of Dairy Products (p. 86) states, ". . . workers who cannot duplicate their own counts on the same plate within 5%, and the counts of other analysts within 10% should discover the cause(s) and correct such disagreements." This indicates that plate count differences greater than 10% would be significant.

Starter A. With one exception, G was the best CA as far as survival was concerned. Usually, but not always, ME was the next best. When no CA was used, (0), survival was much lower.

Survival was often lower after 30 days of frozen storage than after 60 days, but was always lowest after 90 days.

For strain A3, the CA's were ranked according to their effectiveness (in preserving the viable count): G, ME, GP, 0.

It can be seen in Table 6 that A3-1 survived freezing best. In strain A3, there was a positive correlation between the numbers of cells and their survival, i.e., there was something about the sheer mass of cells that helped them to survive. The three preparations of A3 were ranked by their viable counts A3-1, A3-3, A3-2.

Exceptions to this correlation were found in G at 60 days, for example. Here, the survival of A3-2 was higher than that of A3-3 (80% vs. 67% in 1% NaCl; 89% vs. 73% in 1.15% NaCl; 74% vs. 59% in 1.25% NaCl). However, the magnitude of the difference in count between A3-2 and A3-3 (5.3 vs. 8.8×10^9) was much less than that between A3-1 and A3-3 (21.2 vs. 8.8×10^9).

Strain A2 was most inhibited by NaCl at a concentration of 1. 25%. Percent survival was nearly always lower on 1. 25% agar than on M17. Stored without any CA, Strain A3 was quite susceptible to freezing damage. Survival was especially low at NaCl concentrations of 1. 15% and 1. 25% (13-23% in A3-2; 36-77% in A3-1).

Strain A2 survived best when frozen in G, ME, GP, or 0, noted in the order of their effectiveness. The GP-grown cells, A2-3, survived best in G, GP, 0 or ME. With one or two exceptions, the viable counts ranked A2-3, A2-2, A2-1. Here, however, there was no clear relationship between the numbers of cells and their survival, as was found with A3. The survival of A2-1 exceeded that of A2-3 in G at 1. 0%, 1. 15%, and 1. 25% NaCl at 90 days. At 90 days, the viable count of A2-3 was five to seven times greater than that of A2-1. Similar differences were found for viable counts in 0 at 60 days, and in GP at 60 days.

Preparations A2-1 and A2-2 generally showed better survival at 60 and 90 days than at 30 days when plated in salt agar. The plate counts at 30 days, however, were quite low and based on a sample which was, statistically, not representative of the population (less than 30 colonies per plate). Survival on salt agar was normally lower than on M17 for A2-3.

As with the other strains, A1 survived least well when stored without any CA . Both A1-1 and A1-2 survived best when stored with

G, GP, ME, or O, listed in the order of their effectiveness. Preparation A2-3 survived best when in ME, G, GP or O. Strain A1 generally showed high survival at 30 days, and declined to a low at 90 days, lacking the increased survival at 60 days seen with A2 and A3.

Viable counts for the three preparations rank them A1-2, A1-1, and A1-3. There was no apparent correlation between viable count and survival. For example, A1-1 in GP showed the highest survival after 30 days in frozen storage.

Where added NaCl enhanced survival, the concentration preferred was 1.0% or 1.15%. Increased survival was shown by A1-2 in all CA's in 1.0% and 1.15% salt agar at 30 and 60 days.

In general, the cell preparations grew least well in agar to which 1.25% NaCl had been added. The ratio of growth in salt agar to growth in M17 illustrates this quite clearly.

There was a great deal of variation among the preparations as to which agar gave the best growth. The three preparations grown in medium containing GP were quite similar, however, growing best in plain M17, and worst in M17 containing 1.25% NaCl.

Starter B. In starter B, as with starter A, the best CA was G, with one exception. With no CA, survival was generally much lower. In O, strain B2 showed the best survival, followed by B1, then B3.

The best CA for B2 (in all three preparations) was G. The CA's, ranked in the order of their effectiveness, for each preparation, were:

B2-1, G, O, GP=ME; B2-2, G, ME, GP, O; B2-3, G, ME, GP, O. This is an average. If one looks at the effectiveness of the CA's at each test period, 30, 60, and 90 days, there was considerable variation.

The highest survival was often found in B2-1. The viable counts for the three preparations of B2 were the highest of the concentrates prepared from the single strain components of starter B. Survival does not correlate with viable count. However, the counts for all preparations were high, and the magnitude of the differences was so small that distinct differences in survival would not be expected.

Preparation B2-1 survived better when plated in salt agar, preferring 1.25% salt, particularly after 90 days of frozen storage; B2-2 appeared to have no preference, and B2-3 grew best in M17 with no added NaCl, second best in M17 with 1.0% NaCl.

Strain B1, all three preparations, survived best when stored in G. For the individual preparations, the CA's were ranked: B1-1, G, ME=0, GP; B1-2, G, O, ME, GP; B1-3, G, ME, GP, O.

Preparations B1-2 and B1-3 gave the highest survival among the preparations of B1; the viable counts ranked the three preparations B1-3, B1-1, B1-2. The difference in survival between B1-3 and B1-2 was always two- to threefold.

Preparation B1-1 had a higher survival in G in salt agar; after 90 days of frozen storage, survival in 1.15% and 1.25% salt agar was

better for all CA's. Preparation B1-2, in all CA's, survived best in 1.25% NaCl after 90 days.

Strain B3 had the lowest viable counts and the lowest survival of any of the strains examined. The CA's, ranked by their effectiveness, for B3 were: B3-1, ME, G, O, GP; B3-2, G, O, ME, GP; B3-3, G, ME, GP, O. In B3-1 there was considerable variation in these preferences at the different test times; in B3-2, slight variation, and in B3-3, none.

Preparations B3-1 and B3-2 survived best in M17, and worst in 1.25% NaCl agar. Counts in B3-3 were quite low, and survival rarely exceeded 10-20%.

In general, the preparations of B2 showed the highest survival, with B1 occasionally the better survivor, usually in the 90 day, sometimes in the 60 day tests. The B3 preparations always had the lowest survival.

Phage Sensitivity

Inhibition of a host by its homologous phage was calculated by the expression:

$$\% \text{ Inhibition} = \frac{\text{net TTA}_{\text{host}} - \text{net TTA}_{\text{host + phage}}}{\text{net TTA}_{\text{host}}} \times 100$$

Starter A. Of the strains in starter A, A3 was the one most inhibited by addition of its homologous phage (Table 12). For A3,

the average inhibition is about 90%. In A3-1, the TTA's for the infected cultures were unusually high at 30 days. If this unusual value is omitted, the average for A3-1 was 88%, which is close to the 96% seen in A3-2, and the 92% for A3-3.

Preparations A2-1 and A2-2 showed 32 and 44% inhibition respectively. However, A2-3 showed 83% inhibition, about twice that of the cells not raised in BGP.

Preparation A1-1 and A1-3 were 59 and 56% inhibited by their phages, respectively. Phage inhibition in A1-2 was only 29%, the TTAs for the infected cultures always higher than those seen in A1-1 and A1-3.

Phage sensitivity of the host strains did not show consistent and predictable changes throughout the test period.

Starter B. Strain B2 was inhibited by its phage 89-93%. The respective values for B2-1, B2-2, and B2-3 were 89, 90, and 93% inhibition (Table 12).

Strain B1 gave variable results, and showed little inhibition. The values for B1-1, B1-2, and B1-3 were, respectively, 8, -7, and -17%.

The values for B3-1 and B3-2 were, respectively 35 and 33% inhibition. The activity of B3-3 was generally very low, so that only one test gave a figure: 44% inhibition at T_0, in G.

Table 12. Percent inhibition of component single strain cell concentrates of starters A and B by their homologous phages after 0, 30, 60, and 90 days of frozen storage. Cells were inoculated, 1 ml of concentrate into 500 ml of NFM, and incubated at 30 C for 6 h. TTAs were determined at T_0 and at 2 h intervals thereafter. Inhibition was calculated as

$$(\text{net TTA}_{\text{host}} - \text{net TTA}_{\text{host + phage}} / \text{net TTA}_{\text{host}}) \times 100$$

	A1-1	A1-2	A1-3	A2-1	A2-2	A2-3	A3-1	A3-2	A3-3
0	46	35	--	8	90	96	90	94	88
30	66	38	38	61	-11	64	-18	86	100
60	63	32	38	-19	95	94	79	82	87
90	0	22	8	91	-2	90	100	89	93
AVERAGE	60	29	42	32	44	83	64	90	92
	B1-1	B1-2	B1-3	B2-1	B2-2	B2-3	B3-1	B3-2	B3-3
0	-17	-4	-5	91	93	93	41	19	44
30	8	-13	-58	89	89	96	45	58	-100
60	-9	-21	-7	87	93	93	4	9	0
90	0	11	-6	88	89	93	14	33	0
AVERAGE	8	-7	-17	89	90	93	35	33	--

[1] Average computed as $\dfrac{\Sigma \text{net TTA}_{\text{host}} - \Sigma \text{net TTA}_{\text{host + phage}}}{\Sigma \text{net TTA}_{\text{host}}}$

Activity Tests

The results for the activity tests for the strains in starters A and B are shown in Tables 13 through 16. By activity is meant acid-producing activity, calculated as percent lactic acid.

Remarks made later in reference to inoculum (page 52) are applicable here also.

Starter A, 6 h activity. The activity of A3 in NFM was low, ranging from 0.04-0.11%, and is similar for all three preparations in G (Table 13). Differences among the preparations were found among the other CAs.

The inoculum in A3-1 was large enough to have given good activity in all the CAs. The TTAs were quite similar in G and ME, 0.07-.10%, and in GP, and O, where they were slightly lower, 0.06-0.09%.

The inocula from cells stored in GP, O, and ME in A3-2 were apparently too small to give good activity; no values greater than 0.04% were found.

Of noteworthy interest was the observation that the activity of A3-1 and A3-2 in NFM-stim was about four times greater than that in NFM. The increase in NFM-stim was only slightly less in A3-3.

If one calculates the ratio of the net TTA to the number of cells in the inoculum, this ratio is found to be similar for cells at 30 and

Table 13. Acid producing activity of component single strain cell concentrates of starter A after 0, 30, 60, and 90 days of frozen storage in various cryoprotective media. Activity was measured as TTA in NFM and NFM-stim after 6 h at 30 C.

		G[1]					O				GP					ME				
		I[2]	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S
A1-1	0	19	.27	14	.40	21	--[3]	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	18	.31	17	.40	23	13	.30	23	.40	24	18	.31	17	.40		16	.24	15	.32	
	60	19	.28	15	.28	15	15	.16	11	.19	13	13	.16	11	.20	15	11	.12	11	.13	12
	90	15	.19	13	.32	22	21	.10	4	.16	7.8	11	.15	12	.21	18	11	.11	9.6	.18	16
A1-2	0	0	69	.48	6.9	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	50	.47	9.4	.59	12	40	.43	11	--		.43	.43	9.9	--	--	35	.44	13	--	--
	60	39	.26	6.7	.36	9.2	40	.26	6.6	.38	9.6	42	.26	6.2	.30	7.2	33	.20	6.0	.25	7.6
	90	44	.29	6.6	.49	11	38	.19	5.0	.33	8.8	34	.17	5.0	.29	8.6	34	.15	4.4	.27	7.8
A1-3	0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	6.6	.31	47	.60	91	4.9	.30	61	--	--	5.3	.31	59	--	--	7.9	.24	30	--	
	60	7.5	.27	36	.28	37	5.5	.16	29	.19	35	5.9	.16	27	.20	34	7.1	.12	17	.13	18
	90	7.2	.19	26	.32	44	5.4	.09	17	.16	30	5.2	.14	27	.20	39	5.2	.11	21	.18	35
A2-1	0	8.2	.07	8.5	.21	26	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	4.1	.07	17	.11	27	4.7	.03	6.4	.05	11	3.7	.005	1.3	0	--	3.5	.01	2.9	.02	5.7
	60	3.2	.09	28	.12	38	2.8	.04	14	.07	25	2.4	.05	21	.06	25	3.1	.03	9.7	.03	9.7
	90	3.9	.08	21	.05	13	24	.03	13	0.0	0	2.5	.05	20	.02	8	2.7	.05	19	.01	3.7
A2-2	0	12	.26	22	.37	31	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	4.2	.22	52	.22	52	3.1	.09	29	.15	48	2.2	.08	36	.07	32	3.1	.08	26	.11	36
	60	4.9	.17	35	.15	31	4.1	.11	27	.12	29	3.9	.07	18	.04	10	5.1	.11	22	.12	24
	90	5.0	.14	28	.08	16	4.1	.08	20	.01	2.4	4.1	.03	7.3	--	--	3.8	.07	18	.03	7.9
A2-3	0	27	.27	9.9	.42	15	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	19	.15	7.9	.18	9.5	23	.11	4.7	.15	6.4	22	.15	6.8	.18	8.1	14	.14	9.9	.19	14
	60	21	.20	9.8	.22	11	12	.12	9.7	.15	12	19	.10	5.3	.14	7.4	16	.14	8.8	.16	10
	90	20	.17	8.5	.21	11	11	.09	7.1	.07	6.3	17	.10	5.9	.10	5.9	12	12	10	.12	10
A3-1	0	19	.08	43	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	20	.09	4.4	.35	17.1	14.1	.09	5.6	.34	24	16	.07	4.4	.29	18	20	.09	4.6	.34	17
	60	19	.10	5.2	.40	211	12.1	.06	4.8	.32	26	17	.07	4.1	.31	18	16	.08	5.0	.35	22
	90	19	.08	4.1	.29	151	15.1	.06	3.9	.21	14	15	.07	4.7	.24	16	14	.07	4.9	.29	20
A3-2	0	6.7	.11	16	.18	27	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	2.9	.07	24	.18	62	1.2	.04	33	.06	50	0.9	.04	44	(--)	--	2.3	.04	17	.08	27
	60	4.7	.09	19	.11	23	1.3	.03	23	.02	15	1.1	.03	27	.01	9.1	2.4	.03	13	.02	31
	90	3.7	.07	19	.12	32	1.4	.03	21	.02	14	1.4	.02	14	.03	21	1.9	.03	16	.03	12
A3-3	0	12	.10	8.1	.40	32	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	9.3	.07	7.5	.23	25	7.1	.07	9.8	.24	34	8.5	.08	9.4	.20	24	6.6	.07	11	.18	27
	60	7.9	.07	8.9	.23	30	--	.07	--	.25	--	--	.08	--	.24	--	7.4	.09	12	.23	31
	90	6.1	.04	6.6	.07	12	4.9	.05	10	.08	16	5.6	.03	5.4	.06	11	5.2	.05	10	.06	12

[1] as in Table 4; [2] I = inoculum, viable cells x 10^7; NFM = TTA in NFM; S = TTA in NFM-stim; R_n = ratio of TTA in NFM to I, x 10^{-10}; R_s = ratio of TTA in NFM-stim to I, x 10^{-10}; [3] -- = no data.

Table 14. Acid producing activity of component single strain cell concentrates of starter B after 0, 30, 60, and 90 days of frozen storage in various cryoprotective media. Activity was measured as TTA in NFM and NFM-stim after 6 h incubation at 30 C.

		G[1]					O					GP					ME				
		I	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S
B1-1	0	35	.16	4.6	.38	11		--[2]	--	--	--		--	--	--	--	--	--	--	--	--
	30	20	.07	3.4	.23	11	18	.06	3.4	.14	8.0	9.3	.05	5.4	.14	15	12	.07	5.8	.20	17
	60	14	.05	3.5	.10	7.1	12	.02	1.7	.05	42	7.5	.01	1.3	.02	2.7	10	.04	3.8	.08	7.7
	90	19	.06	3.2	.18	9.7	9.7	.04	4.1	.10	10	7.6	.03	3.9	.07	9.2	12	.05	4.1	.14	11
B1-2	0	21	.17	8.2	.39	19	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	14	.06	4.4	.27	20	17	.05	3.0	.22	13	10	.04	3.8	.17	16	15	.06	4.0	.24	16
	60	16	.06	3.8	.16	10	11	.05	4.7	.14	13	8.9	.03	3.4	.06	6.7	10	.06	6.0	--	--
	90	11	.05	44	.17	15	11	.08	7.5	.20	19	11	.07	6.3	.14	13	10	.07	6.9	.15	15
B1-3	0	38	.11	2.9	.50	13	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	31	.11	3.6	.31	10	21	.08	3.8	.18	8.5	20	.09	4.6	.25	13	24	.09	3.8	.24	.10
	60	27	.14	5.2	.29	11	12	.08	6.8	.10	8.5	16	.07	4.5	.16	10	19	.08	4.3	.19	10
	90	30	.08	2.7	.03	1.0	16	.06	3.8	.17	11.0	19	.05	2.6	.16	8.3	18	.05	2.8	.18	9.9
B2-1	0	54	.21	3.9	.54	9.9	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	37	.34	9.3	.37	10	47	.31	6.5	.35	7.4	47	.33	7.1	.36	7.7	39	.18	4.6	.31	8.0
	60	43	.18	4.2	.28	6.5	48	.13	2.7	.24	4.9	38	.15	3.9	.26	6.8	38	.15	3.9	.25	6.6
	90	41	.14	3.4	.13	3.1	38	.10	2.6	.09	2.4	28	.10	3.5	.13	4.6	27	.08	3.0	.12	4.5
B2-2	0	46	.49	11	.53	12	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	39	.47	13	.51	13	30	.43	15	.49	17	32	.43	14	.47	15	36	.42	12	.42	12
	60	30	.35	12	.36	12	29	.25	8.6	.30	10	31	.27	8.7	.32	10	25	.24	9.4	.34	13
	90	36	.25	7.0	.30	8.4	27	.20	7.4	.23	8.5	31	.21	6.8	.25	8.1	31	.20	6.5	.23	7.5
B2-3	0	49	.51	11	.59	12	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	49	.32	6.5	.35	7.1	47	.20	4.2	.25	5.4	49	.23	4.7	.33	6.7	77	.23	2.9	.31	4.0
	60	40	.54	14	.61	15	31	.44	14	.49	16	31	.45	15	.53	17	24	.45	19	.55	2.3
	90	35	.23	6.6	.37	11	27	.14	4.8	.11	4.0	35	.13	3.7	.13	3.7	31	.14	4.6	.16	5.2
B3-1	0	20	.12	5.9	.29	14	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	6.5	.11	17	.25	39	7.6	.09	12	.20	26	6.3	.10	16	.20	32	6.3	.11	18	.22	35
	60	3.6	.09	25	.16	44	5.6	.09	16	.19	34	2.5	.08	32	.12	48	4.3	.09	21	.18	42
	90	4.9	.06	12	.18	36	2.7	.04	15	.10	38	2.3	.04	18	.06	26	3.2	.05	16	.09	28
B3-2	0	6.3	.08	13	.28	44	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	4.3	.08	19	.10	23	3.1	.08	26	.16	52	1.7	.07	40	.09	52	1.8	.06	33	.09	49
	60	3.5	.04	11	.05	14	2.4	.05	21	.05	21	1.4	.04	29	.04	29	1.6	.04	25	.03	19
	90	3.6	.06	17	.09	25	1.3	.01	7.6	.005	3.8	1.7	.01	50	.01	5.9	1.0	0.0	--	0	--
B3-3	0	.65	.02	31	.03	46	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	.14	.02	143	0	--	.02	.01	500	.01	500	.08	.02	250	.01	125	.04	.02	500	.01	125
	60[3]																				
	90[3]																				

[1] as in Table 4; [2] --- no data; [3] no activity measurable; [4] I, NFM, R_n, S, and R_s as defined in Table 13.

Table 15. Acid producing activity of component single strain cell concentrates of starter A after 0, 30, 60, 90 days of frozen storage in various cryoprotective media. Activity was measured as TTA in NFM and NFM-stim after 14 h at 22 C.

		G[1]					O					GP					ME				
		I[2]	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S
A1-1	0	19	.50	26	.55	28	--	--[3]	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	18	.54	30	.47	26	13	.48	37	.37	29	18	.52	28	.40	22	16	.43	27	.33	20
	60	19	.46	24	.36	19	15	.36	24	.28	19	13	.37	28	.22	16	11	.24	22	.17	15
	90	15	.40	27	.32	22	21	.21	10	.18	8.8	11	.53	47	.06	5.3	11	.20	18	.19	17
A1-2	0	69	.67	9.7	.56	8.1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	50	.55	11	.72	14	40	.53	13	.66	16	43	.61	14	.64	15	35	.59	17	.58	17
	60	39	.52	13	.46	12	40	.46	12	--	--	42	.44	11	.34	8.2	33	.44	13	.41	13
	90	44	.50	11	.57	13	38	.47	13	.49	13	34	.45	13	.42	12	34	.37	11	.39	11
A1-3	0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	6.6	.22	33	.16	24	4.9	.25	51	.19	39	5.3	.17	32	.09	17	7.9	.27	34	.17	22
	60	7.5	.12	16	.06	8.0	5.5	.13	24	.08	15	5.9	.13	22	.18	31	7.1	.19	27	.11	16
	90	7.2	.12	17	.09	13	5.4	--	--	--	--	5.2	--	--	--		5.2	.12	23	.08	15
A2-1	0	8.2	.33	40	.43	52	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	60	4.1	.25	79	.25	79	2.8	.18	64	.12	43	2.4	.07	29	.07	29	3.1	.14	45	.10	32
	90	3.2	.21	54	.20	51	2.4	.11	46	.09	38	2.5	.09	36	.08	32	2.7	.06	22	.03	11
A2-2	0	12	.40	33	.53	44	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	4.2	.44	25	.47	112	3.1	.28	90	.33	107	2.2	.19	86	.21	96	3.1	.23	74	.30	97
	60	4.9	.43	88	.30	61	4.1	.19	46	.13	32	3.9	.12	30	.08	20	5.1	.25	49	.20	39
	90	5.0	.30	60	.25	50	4.1	.14	34	.10	24	4.1	.06	15	.02	4.9	3.8	.10	26	.10	26
A2-3	0	27	.49	18	.59	22	--	--	--	--	--		--	--	--	--		--	--	--	--
	30	19	.37	20	.50	27	23	.22	12	.38	16	22	.36	16	.40	18	14	.42	30	.42	30
	60	21	.40	20	.36	18	12	.20	16	.24	19	19	.16	8.4	.23	12	16	.22	14	.30	19
	90	20	.28	14	.33	17	11	.17	15	.18	16	17	.19	10	.20	11	12	.28	24	.27	24
A3-1	0	19	.09	4.8	.65	35	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	20	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	60	19	.11	5.8	.56	29	12	.09	7.2	.44	36	17	.09	5.3	.47	28	16	.08	5.0	.52	33
	90	19	.28	14	.46	24	15	.08	5.2	.47	31	15	.07	4.7	.32	22	14	.07	4.9	.39	27
A3-2	0	6.7	.04	5.9	.34	51	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	2.9	.07	24	.24	83	1.2	.04	33	.08	67	0.9	.04	44	.09	100	23	.05	22	.15	65
	60	4.7	.10	21	.26	55	1.3	.06	45	.08	61	1.1	.03	27	.04	36	2.4	.03	13	.09	38
	90	3.7	.08	22	.25	68	1.4	.05	36	.11	78	1.4	.04	30	.09	67	1.9	.05	26	.11	56
A3-3	0	12	.03	2.4	.58	47	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	9.3	.07	7.5	.34	37	7.1	.07	9.9	.34	48	8.5	.07	4.1	.33	20	6.6	.07	11	.30	46
	60	7.9	.06	7.6	.30	38	--	.08	--	.37	--	--	.05	--	.24	--	7.4	.07	9.5	.27	37
	90	6.1	.07	12	.28	46	4.9	.07	14	.27	55	5.6	.07	13	.21	38	5.2	.07	14	.21	40

[1] as in Table 4; [2] NFM, R_n, S, R_s, and I are as defined in Table 13; [3] --, = no data.

60 days, regardless of CA, in NFM for A3-1 and A3-2. The ratios are similar for the same times and preparations in NFM-stim, also.

Strain A2 gave mixed results. Both A2-1 and A2-2 had inocula comparable in size to those of A3-2, when stored in G. However, A2-1 had activity about the same as A3-2, while A2-2 had better activity. In the other CAs, both A2 preparations had larger inocula than did A3-2. In A2-1, activities were the same or slightly higher than A3-2. In A2-2, the activities, while low, were still about twice as high as those of A3-2.

The activities of the three A2 preparations in NFM-stim were much the same as the activities in NFM, except in G at T_0, when the activity in NFM-stim was higher. Strain A2-1 had activity as low or lower than A3-2 (.01%-.08%), while A2-2 showed activity considerably higher than either of them (.04-.37% at T_0 at 60 days dropping off to .01%-.08% at 90 days).

The inocula were higher in A2-3. Activity in NFM and NFM-stim was higher than in A2-2, except at 90 days, in which case the cells in A2-3 were much more active (.07%-.21%, cf. .01%-.08%).

Activities of A2-2 and A2-3 in O were quite similar. With cells stored in ME, activities of A2-3 were considerably higher than those of A2-2. This was more pronounced in cells of A2-3 stored in GP, compared to those of A2-2.

The activity of A1 was high, and obviously greater than that of

A3. The inocula of A1-1 and A3-1 were of comparable size. However, the activity with A1-1 was greater than with A3-1, regardless of the CA in which the cells had been stored.

The activities of A3 in NFM-stim, while slightly less than those of A1-1 at 30 days, were always higher at 60 and 90 days. It is also noteworthy that the differences between activity of A1-1 in NFM and in NFM-stim were not as great as those in A3. The differences in A1 at T_0 or 90 days only approached twofold in some cases. Compare this with the two- to fourfold differences observed with A3. The activities of A1-3 were remarkably similar to those of A1-1, though the inocula of A1-3 were considerably smaller than those of A1-1.

Starter B, 6 h activity. The activity tests of the strains in starter B are difficult to compare because of the large disparity in inoculum sizes.

Strain B2 was the most active (Table 14). The ratios, TTA: inoculum size, described earlier, were found to be similar for B2-1 and B1-1 in G, though the inocula were of very different sizes. In this case, the differences in activity varied directly with the numbers of cells in the inoculum, i. e., if the inoculum of cells in B2-1 contained twice as many cells as that of B1-1, activity was about twice as great. Making the same comparison for B2-2 and B1-2, this was not found to be the case, and B2-2 was the more active.

Preparation B2-3, in G, showed activity equivalent to that of

B2-2 (about . 2%-. 5%). In the other CAs, a smaller inoculum of B2-2 produced higher activities than were found in B2-3.

The activity of B2 in NFM-stim was higher than in NFM, but the differences were not great. The ratios of TTA to inoculum size for cells stored in G were quite close for both activity media. In the other CAs, there was much more disparity, the TTAs in NFM decreasing with time in frozen storage much faster than the TTAs in NFM-stim.

In B1, activity declined much more rapidly than in B2 (e. g. . 21% to . 14%, cf, . 17% to . 04%, over the 90 day period). After the T_0 activity in G, the TTAs were rather low in all CAs. The ratios of TTA:inoculum size were similar for activity in NFM and NFM-stim.

Preparation B1-3, while its inoculum was larger than those of B1-1 or B1-2, maintained its activity during frozen storage much better in NFM and in NFM-stim.

Of the B3 preparations, only B3-1 had sufficiently high viable counts to give good activity test results. The inocula for B3-1 were smaller than any of the inocula for the B1 preparations, but the activity was comparable to that of B1 in G and O, and perhaps a little higher in GP and ME. The activity in NFM-stim was usually comparable to that of B1 for the 30 day and 60 day tests, but tended to decrease in the 90 day tests.

Starter A, 15 hours. The TTAs of all three preparations of A3 (Table 15) were quite similar to those at 6 h, in NFM. In NFM-stim, the TTAs were slightly higher than those at 6 h. At 90 days, they were quite a bit higher (. 25% and . 28%, cf. . 12% and . 07% in G, for A3-2 and A3-3).

The ratios, TTA:inoculum size, were, in NFM-stim, higher at 15 h than at 6 h. They ranged from three to eight times higher for cells stored in G, in NFM, two to six times higher for cells frozen in other CAs.

For strain A2, all the activities at 15 h were higher than those at 6 h. The best activity was seen in G. At 60 and 90 days, in CAs other than G, the activity in NFM-stim was often less than that in NFM. This was not found to be true in A2-3.

The ratios for A2, TTA:inoculum size, for activity in NFM-stim, were less than two times greater than for activity in NFM, and were often the same as for NFM.

The TTAs for A1-1 and A1-2 were all higher than those for 6 h, whether in NFM or NFM-stim. Activity in NFM-stim was almost always lower than in NFM at 60 and 90 days, except for cells stored in G. The ratios of TTA:inoculum size were similar or lower in NFM-stim than in NFM.

Preparation A1-3 showed activities at 15 h lower than those at 6 h. The only exception to this was in the NFM activity test of cells

stored in ME.

Starter B, 15 h. The activity of B2 at 15 h (Table 16) was higher in almost every case than at 6 h, whether in NFM or NFM-stim. The TTAs for cells in NFM and NFM-stim were quite similar, activity in NFM-stim often being less than in NFM.

In all the preparations of B2, the ratio of TTA:inoculum size was similar in NFM and in NFM-stim, and there was no great difference among the three preparations.

For B1-1 and B1-2, activity in NFM was only slightly higher after 15 h than it was after 6 h. The 15 h activity in NFM-stim was higher than in NFM at 30 and 60 days, but at 90 days it was about the same as in NFM, in GP and O. However, in G and ME, it was still higher than the 6 h value.

The activity of B3 was higher in every case, NFM and NFM-stim, in G, for B3-1 and B3-2, at 15 h than at 6 h. In other CAs, the activity in NFM was only slightly higher than at 6 h. The activity in NFM-stim was considerably higher at 30 days and at 60 days, but in many cases had fallen to near the value for 6 h by 90 days.

The NFM activity of B3-2, in CAs other than G, was higher than the 6 h TTA only at 30 days. In NFM-stim, both the 30 and 60 day tests tended to be higher.

Table 16. Acid producing activity of component single strains cell concentrates of starter B after 0, 30, 60, and 90 days of frozen storage in various cryoprotective media. Activity was measured as TTA in NFM and NFM-stim after 14 h at 22 C.

		I[1]	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S	I	NFM	R_N	S	R_S
B1-1	0	35	.10	2.9	.43	12	--[2]	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	20	.09	4.4	.34	17	18	.08	4.6	.18	10	9.3	.08	8.6	.21	23	12	.09	7.4	.28	23
	60	14	.11	7.8	.29	21	12	.06	5.0	.15	13	7.5	.04	5.3	.05	6.7	10	.06	5.8	.18	17
	90	19	.08	4.3	.19	10	9.7	.05	5.2	.09	9.3	7.6	.04	5.3	.07	9.2	12	.05	4.1	.16	13
B1-2	0	21	.09	4.3	.43	21	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	14	.08	5.9	.32	24	17	.06	3.6	.25	15	10	.07	6.7	.22	21.1	15	.08	5.4	.30	20
	60	16	.09	5.7	.30	19	11	.07	8.6	.26	32	8.9	.03	3.4	--	--	10	.07	2.0	--	--
	90	11	.07	6.1	.26	23	11	.05	4.7	.20	19	11	.05	45	.14	13	10	.06	5.9	.20	20
B1-3	0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	31	.11	3.6	.31	10	21	.10	4.7	.23	11	20	.09	4.6	.42	21	24	.15	6.3	.20	8.4
	60	27	.19	7.1	.44	16	12	.09	7.7	.12	10	16	.07	4.5	.15	9.7	19	.08	4.3	.23	12
	90	30	.09	3.0	.37	12	16	.08	5.1	.19	12	19	.07	3.6	.18	9.4	18	.08	4.4	.25	14
B2-1	0	54	.62	12	.56	10	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	37	.56	15	.47	13	47	.53	11	.45	9.5	47	.56	12	.48	10	39	.53	14	.39	10
	60	43	.41	9.6	.42	9.8	48	.31	6.4	.39	8.1	38	.34	8.9	.39	10	38	.35	9.2	.40	11
	90	41	.27	6.6	.26	6.3	38	.24	63	.25	6.6	28	.28	9.9	.29	10	27	.20	7.5	.19	7.2
B2-2	0	46	.61	14	.19	4.2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	39	.65	17	.58	15	30	.58	20	.57	19	32	.61	19	.46	15	36	.61	17	.53	15
	60	30	.53	17	.52	17	29	.38	13	.39	14	31	.39	13	.40	13	25	.40	16	.41	16
	90	36	.48	13	.39	11	27	.32	12	.33	12	31	.34	11	.29	9.4	31	.32	11	.27	8.9
B2-3	0	49	.64	13	.58	12	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	49	.61	12	.57	12	47	.55	12	.51	55	49	.62	13	.59	12	77	.56	7.3	.60	7.8
	60	40	.69	17	.72	18	31	.60	20	.70	23	31	.67	22	.64	21	24	59	24	.69	28
	90	35	.52	15	.37	11	27	.29	11	.23	8.4	35	.34	9.8	.30	8.6	31	.37	12	.32	11
B3-1	0	20	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	6.5	.13	20	.40	62	7.6	.16	21	.37	49	6.3	.14	22	.36	57	6.3	.16	25	.38	60
	60	3.6	.09	25	.26	72	5.6	.11	20	.39	70	2.5	.09	36	.21	83	4.3	.11	26	.28	66
	90	4.9	.15	30	.44	88	2.7	.06	23	.24	90	2.3	.05	22	.15	66	3.2	.05	16	.20	63
B3-2	0	6.3	.15	24	.48	76	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	4.3	.08	19	.23	54	3.1	.12	39	.33	106	1.7	.10	58	.73	132	1.8	.11	60	.25	137
	60	3.5	.08	23	.13	37	2.4	.08	33	.26	108	1.4	.04	28	.06	43	1.6	.05	32	.09	57
	90	3.6	.05	14	.16	45	1.3	.02	1.5	.04	30	1.7	.02	12	.04	24	1.0	.02	20	.23	234
B3-3	0	.65	.02	31	0	0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	30	.14	.01	56	0	0	.02	.01	50	.005	25	.08	.009	112	.005	63	.04	.002	50	.005	125
	60[3]																				
	90[3]																				

[1] I, NFM, P_s, S, and R_s are defined in Table 13; [2] --, = no data; [3] = no activity measurable.

Lactate Sensitivity

Lactate sensitivity tests for the 30 day test period were done in broth medium containing 1% to 7% sodium lactate, as described by Osborne and Chubb (51). In these initial tests, no growth was observed in lactate concentrations greater than three percent, so the remaining tests were done in medium containing one to five percent lactate, the 4% and 5% concentrations being included in case any increases in lactate tolerance might emerge during storage.

Starter A. The results of the lactate sensitivity test for the strains of starter A appear in Table 17. Plotting absorbance against time, it was found that any culture showing net growth less than 0.4 units in 6 h did not enter the log phase of growth and was therefore assumed to be inhibited. The other categories of inhibition, a = good growth, b = moderate growth, c = severe inhibition, and d = no growth, were arbitrarily set (see Table 17 for further explanation of categories).

The comparisons drawn here are made on the basis of normal growth in M17 broth containing no added lactate.

A priori, it was not known what the range of survival and activity encountered in the frozen and thawed single strain concentrates might be. For this reason, a standard volume was selected arbitrarily for the test inoculum. A 1:10 dilution of the concentrate was used

Table 17. Growth of component single strain cell concentrates of starters A and B in solutions of M17 broth plus sodium lactate, 0-7% v/v) in 1% increments, after 0, 30, 60, and 90 days of frozen storage. Solutions were inoculated 2% (v/v), incubated at 30 C, and absorbance read hourly on a Bausch & Lomb Spectronic 20 colorimeter-spectrophotometer. a = good growth, net increase in absorbance (net absorbance) greater than .40; b = moderate growth net absorbance .21-.40; c = severe inhibition, net absorbance .11-.20; d = no growth, net absorbance 0.0-.15.

	Percent lactate:	A1-1 0 1 2 3 4 5	I[1]	A1-2 0 1 2 3 4 5	I	A1-3 0 1 2 3 4 5	I
days in	0	a a a e d d	19.4	a a a c d d	69	- - - - - -	-
frozen	30	a a b c d d	19.8	a a a c d d	56	c c d d d d	6.6
storage	60	a a b d d d	21.1	a a b d d d	39	[2]b c d d d d	15
	90	a b d d d d	16.4	a a c d d d	44	[2]a c d d d d	14
		A2-1		A2-2		A2-3	
"	0	a a c d d d	8.2	a a c d d d	12	a a a c d d	27
	30	a b d d d d	4.6	a b d d d d	9.1	a a a c d d	19
	60	a b d d d d	4.7	b c d d d d	4.7	a a b d d d	21
	90	b c d d d d	5.4	[2]a b d d d d	11	a a c d d d	20
		A3-1		A3-2		A3-3	
"	0	a a b d d d	19	a a a c d d	6.7	a a a b d d	12.4
	30	a a a b d d	23	a b c d d d	3.2	a a b d d d	10.3
	60	a a a c d d	21	b c d d d d	5.3	b c d d d d	8.8
	90	a a a d d d	22	[2]a b c d d d	8.2	a a c d d d	6.8
		B1-1		B1-2		B1-3	
	0	a a b d d d	35	a a c d d d	21	a a c d d d	38
	30	a b c d d d	20	a b c d d d	14	a b c d d d	31
	60	a - c d d d	14	a - c d d d	16	a - c d d d	27
	90	b c d d d d	19	a c d d d d	11	a b d d d d	30
		B2-1		B2-2		B2-3	
	0	a a c d d d	58	a a b d d d	46	a a a c d d	66
	30	a a c d d d	37	a a b c d d	39	a a a c d d	61
	60	a a c d d d	43	a a b d d d	30	a a a c d d	40
	90	a a c d d d	41	a a b d d d	36	a a b d d d	35
						B3-3	
	0	a a d d d d	20	a a d d d d	6		.7
	30	a b c d d d	7	a c d d d d	4		[2].1
	60	d d d d d d	4	d d d d d d	4		[2].2
	90	c d d d d d	5	b d d d d d	4		[3].08

[1] I = inoculum size x 10^7; [2] 0.2 ml inoculum; [3] 0.1 ml of concentrate used as inoculum; [4] all d

to make the results more nearly comparable to the unconcentrated single strains.

Strains A3 and A1 were less sensitive to lactate than A2. In those two strains, complete inhibition of growth occurred at 4% lactate, while in A2, complete inhibition occurred at 3%.

Comparing the growth of A3-1 and A1-1, in which the inocula contained approximately equal numbers of cells, it is clear that A3 showed less sensitivity to lactate, particularly in the later tests. Preparation A1-2, which was inoculated with more cells than was A3-1, was more sensitive to lactate than was A3-1.

The numbers of cells in the inoculum for A2-1 and A2-2 compare well with those in A3-2. Here again, it is apparent that A2 is the more sensitive to lactate.

Note that in A3-2, and in A3-3, the inoculum at 90 days was larger than that at T_0, and yet growth was less. Sensitivity to lactate appears to have increased with time in frozen storage.

It is hard to assess the effect of propagation of cells in medium containing GP upon sensitivity to lactate. The effect on A3 appears to be minimal. Comparing the lactate sensitivity of A3-3 to A3-2, where the inocula are of comparable sizes, there appears to be little effect. Growth in GP-containing medium seems to have helped A2, but the number of cells in the inoculum of A2-3 was considerably larger than that of A2-1 or A2-2, so it is impossible to separate the

effects of the larger inoculum from the effects of growth in GP.

The effects of growth in GP upon A1, in terms of lactate sensitivity, appeared damaging rather than protective. The inocula at 60 and 90 days were about the same as for A1-1 at 90 days. In A1-1 it would be expected that the 90 day-old cells would be less hardy than the 60 day-old cells of A1-3, but their growth was better than that of the younger cells raised in BGP.

Starter B. Results of the lactate sensitivity tests for the strains of starter B are shown in Table 17.

Strain B2 was as sensitive to lactate as any of the other components of starter B. With considerably higher numbers of cells in the inoculum, B2-1 grew no better in lactate than did B1-1 or B1-2 in the 30 day test. However, the lactate sensitivity of B2 did not change over the 90 day period, while in strain B1, sensitivity increased. The viable count also dropped considerably over this period, in B1, making the comparison to B2 more difficult.

The viable counts in B3 were so low as to make comparisons impossible. However, at T_0, B1-2 and B3-1 were comparable, and B3-1 appeared to be the more sensitive to lactate.

Growth in GP apparently reduced the sensitivity of B2 to lactate. At 60 and 90 days, the viable counts of B2-3 were close to those of B2-2 at T_0 and 30 days. It would be expected that the younger cells be more tolerant of lactate, but the 90 day-old cells of B2-3

grew as well as did the younger cells, and the 60 day-old cells grew better.

Propagation in GP did not appear to have affected the lactate sensitivity of B1. In almost every case, the cell counts of B1-3 were higher than those of B1-1 and B1-2, and yet growth in lactate was no better. Growth in GP had, therefore, either no effect, or a slightly deleterious effect on the lactate sensitivity of B1.

Sensitivity to pH vs. Sensitivity to Lactate

In order to try to separate the expected inhibitory effects of pH from those of lactate ion, activity tests were carried out in NFM which had been brought to the same pH with either a mineral acid, HCl, or with lactic acid.

These acid activity tests gave mixed results, and the differences in net TTA were small. The only data available for comparison are those of Collins (12) who also observed that the differences are small. The results are presented in Table 18.

Starter A. Differences in activity between cells inoculated into HCl and into lactic acid were most often found in A2 and A1. Generally, the activities at the lower concentrations of the two acids were similar, and any differences were found at the higher concentrations. Other than this, there did not seem to be any systematic differences.

Table 18. Activity of component single strain cell concentrates of starters A and B in milk acidified with HCl or lactic acid, after 0, 30, 60, and 90 days of frozen storage. Cells were inoculated into 100 ml of NFM acidified with sufficient HCl or lactic acid to bring the milk to pH 5.5. A second set of bottles had half as much added acid. Activity, after 6 h, is here recorded as TTA calculated as lactic acid. Bottles were incubated 6 h at 30 C.

	A1-1				A1-2				A1-3			
	0	30	60	90	0	30	60	90	0	30	60	90
CELLS	.24	.28	.34	.40	.44	.47	.28	.35	--	.23	.09	.19
HCl-1	.08	.19	.17	.17	0	.28	.27	.20	--	.10	.02	.07
HCl-2	--	.23	.26	.31	--	.44	.36	.33	--	.14	.05	.09
LAC-1	0	.15	.11	.08	0	.20	.15	.09	--	.04	0	.05
LAC-2	--	.26	.24	.25	--	.33	.31	.32	--	.11	.02	.10
	A2-1				A2-2				A2-3			
CELLS	.20	.23	.24	.15	.21	.17	.19	.17	.34	.37	.25	.20
HCl-1	.15	.15	.11	.14	.13	.08	.14	.16	.07	.20	.21	.13
HCl-2	--	--	.15	.14	--	.22	0	.17	--	.26	.24	.19
LAC-1	.07	.14	.06	.07	.05	.01	.18	.20	0	.11	.19	.06
LAC-2	--	--	.13	.13	--	.15	.13	.21	--	.73	.20	.15
	A3-1				A3-2				A3-3			
CELLS	.10	--	.09	.04	.09	.07	.07	.06	.14	.10	.08	.08
HCl-1	.03	--	.10	0	.07	.06	.04	.06	.03	.14	.07	.11
HCl-2	--	--	.08	.01	--	.09	.05	.06	--	.12	.08	.08
LAC-1	.06	--	.08	.01	.05	.05	.05	.05	.02	.10	.07	.07
LAC-2	--	--	.08	.02	--	.08	.07	.07	--	.10	.08	.05
	B1-1				B1-2				B1-3			
CELLS	.08	.09	.10	.05	.07	.09	.09	.07	.11	.12	.13	.08
HCl-1	.04	.08	.10	.06	.09	.07	.08	.08	.09	.07	.13	.11
HCl-2	--	.08	.09	.09	--	--	.12	.07	--	--	.14	.09
LAC-1	.03	.02	.07	.05	.08	.03	.09	.07	.04	.06	.16	.08
LAC-2	--	.08	.08	.08	--	--	.13	.07	--	--	.13	.08
	B2-1				B2-2				B2-3			
	0	30	60	90	0	30	60	90	0	30	60	90
CELLS	.22	.17	.34	.20	.34	.27	.45	.39	.45	.46	.51	.41
HCl-1	.01	.06	.20	.02	.19	.11	.32	.09	.24	.14	.30	.25
HCl-2	--	--	.36	.16	--	--	.40	.25	--	--	.39	.40
LAC-1	0	.02	.12	.10	0	0	.18	.16	.10	.12	.18	.22
LAC-2	--	--	.30	.20	--	--	.35	.20	--	--	.30	.35
	B3-1				B3-2				B3-3			
CELLS	.10	.15	.14	.15	.09	.07	.03	.10	.02	.02	0	0
HCl-1	0	.10	.14	.07	.08	.04	.03	.04	.01	.01	"	"
HCl-2	0	.14	.15	.11	--	.07	.03	.08	0	.02	"	"
LAC-1	0	.03	0.0	.05	.09	.02	.07	0	.01	.09	"	"
LAC-2	0	.11	0.0	.09	--	.03	.02	.06	--	.01	"	"

In A3-3, the activity in HCl exceeded that in lactic acid at both concentrations in the 30 and 90 days tests, but not in the 60 day test.

The activity of A2-1 in HCl at the higher concentration was greater than in lactic acid at T_0, 60, and 90 days, but not at 30 days. In A2-2, activity in HCl was greater at the higher concentration at T_0, and at both concentrations at 30 days.

In A2-3 the activity in HCl was higher at both concentrations at T_0, 30, and 90 days.

Preparation A1-1 shows slightly more activity in lactic acid at the lower concentration at 30 days, and slightly more activity in HCl at 60 and 90 days. Preparations A1-1 and A1-2 showed greater activity in HCl than in lactic acid at the higher concentration at 30, 60 and 90 days; in A1-3, differences were very small.

There is also evidence for sensitivity to pH without regard for the acid type. Activity in acidified milk is often lower than activity in unacidified milk, for example, in the higher concentrations of acids, activity is lower than that in unacidified milk for A3-1 at 0 and 90 days, for A3-2 at 0, 30, and 60 days. With A3-3, the activity in acidified milk was about the same as in unacidified milk. With A1, all preparations were less activie in acidified than in unacidified milk at the higher concentration of acid. Preparation A2-3, all tests were lower in acidified than in unacidified milk.

Starter B. Preparations B2-1 and B2-2, at 30 and 60 days, showed greater activity in HCl at the higher concentration. At 90 days, the activity in lactic acid was higher in both concentrations. At the lower concentration, activity in HCl was slightly higher except at 90 days, as mentioned.

In B2-3, activity in HCl was always higher than activity in lactic acid; the differences were fairly large only at T_0 and 60 days.

In B-1, activity in HCl was greater at T_0, 30, and 90 days at the higher concentration. The same was true for B1-2 and B1-3, except at 60 days, when the activity in lactic acid was slightly higher than in HCl.

Preparation B3-1 gave higher activity in HCl at 30, 60 and 90 days. In B3-2, at the higher concentration, activity in lactic acid was slightly higher at T_0 and 60 days. The activity in B3-3 was too low to give dependable results.

The sensitivity to pH is quite noticeable in B2. The activity in acidified milk at the higher concentration is always lower than that of the unacidified milk. Activity in acidified milk is similar to unacidified milk with B1, with a few exceptions, though activity is always low. The activity of B3, similarly, is low, but is usually close to that of unacidified milk.

Antibiotic Tests

No antibiotic activity was detected in any strain at any time under the conditions described.

DISCUSSION

When one strain dominates the population in a multiple strain starter, bacteriophage infection of that strain can cause failure or severe inhibition of acid production. The commercially-prepared frozen starters examined here showed dominance in the young lots, but differed in their responses to frozen storage. Starter B maintained essentially the same dominance pattern throughout the test period, while starter A showed progressively less dominance and, in the oldest lots, a significantly longer lag phase.

It is noteworthy that none of the strain specific phages added to culture A influenced acid production in the 7-8 month-old frozen lots. These data illustrate that in non-dominant multiple strain starters, the infection by phages for only one strain has little effect on acid production since other strains in the mixture cause normal acid production. These findings justify the use of multiple strain starters as a means of minimizing the consequence of phage infection under commercial conditions and point to the need for further studies to find ways to minimize dominance in frozen cultures.

The decline in acid production at eight to nine hours in frozen starter A stored seven to eight months (Fig. 3) occurred in each of the four trials with each culture whether or not a phage was added; therefore the data were plotted as shown though an explanation for

the effect is lacking.

The initial (T_0) plate counts decreased steadily in A, which would account for the extended lag phase. The corresponding counts for B showed numbers of cells increasing with storage time, apparently because of decreasing chain length. In general, the final counts after 5.5 h showed cell numbers in the uninfected controls to be about twice those of the cultures in which the dominant strain had been infected with phage.

Acid production in cultures in which the dominant strain was infected lagged about 2-3 h behind controls in B, and about 2 h in A (Fig. 2). If this is equated to 2-3 generation times, on the average, then a 4-8 fold difference in the cell numbers might be expected. However, only a twofold difference was observed which might be due to a number of factors:

(a) an underestimation of the cell numbers in the controls due to chaining which is diminished in phage-infected cultures,

(b) a greater amount of acid produced per cell in the dominant strain; such data have appeared in another report (65),

(c) production of inhibitory substances; lysis of the dominant strain may release substances which inhibit acid production by the surviving cells,

(d) removal of the dominant strain, causing an inhibitory interaction between the remaining strains, or removal of a stimulatory factor ordinarily contributed by the dominant strain.

At the time of testing, the frozen starters had been exposed to three physical treatments, any of which might selectively affect different component strains: freezing, storage, and thawing. It has been shown (5) that different cells have different sensitivities to freezing and thawing, and that the speed of freezing and thawing affects survival. Similarly, reports (16, 18, 34) affirm that the temperature of storage commonly used, and used in these experiments (-20 C), is not the optimal temperature for maintaining frozen concentrates. Yet, this temperature is one used by many dairy plants for storing frozen cultures. Under these conditions, strain dominance might emerge from differences in survival and recovery from freezing. In a recently thawed culture, there are dead, injured, and healthy cells. The speed with which the injured cells can recover may account for differences in the survival and activity of various strains.

Implicit in these experiments are two assumptions. One, that the component strains in the original starter are present in a ratio not significantly different from 1:1:1, though the results of Nichols and Ineson show that the normally dominant strain predominates even

if it is inoculated at a much lower concentration than the other strains (49).

Assumption two is that the phage infection removes the sensitive strain from the culture effectively and selectively. So far, techniques are not available to conclusively prove this. In this regard, Wentworth and Romig (71) have reported that some staphylococcal strains contain a mixture of sensitive and resistant cells which could be an important consideration in the present study if freezing and storage alter the proportion of such cells.

While this study was carried out with a limited number of multiple strain frozen concentrates, the data show undesirable strain dominance in frozen starters occurs, and that it is affected by time of storage in the frozen state. There is some evidence this is due to numerical predominance as opposed to physiological. Since subculturing is not a factor it is suggested differential freezing survival and recovery may be responsible for the production and alteration of the dominance pattern.

In addition to differential freezing survival and recovery or antibiotic production, the strain dominance observed in this study might have resulted from differences in the sensitivities of individual strains to lactate, pH, or to differences in acid-producing activity. These factors were examined for each of the three component strains in both starter included in the Part I study.

The dominant strain A3 in starter A, was less able to survive frozen storage than strain A1; the survival of strain A2 was less than survival of A3. These results indicate that some factor other than freezing survival was responsible for the dominance observed in starter A.

It is logical to expect that during freezing a portion of the starter cells will be sublethally damaged, and that these cells will be sensitive to added salt in the growth medium. With the passage of time in frozen storage, the number of damaged cells would be expected to increase, accompanied by cell death, especially in the case of the injured bacteria. Some recovery would also be expected after the initial freezing damage had occurred, as well as chain disruption; both of these occurrences would allow increases, real or apparent, in survival.

The ratios of blended viable counts to unblended counts were low, always less than two. Chain formation was minimal, therefore, and chain disruption can be discounted in this case.

The expected presence of damaged cells was most apparent when the ratios of cell growth in 1.25% salt agar and M17 agar were compared. The least affected by salt was strain A1, followed by A3, and the most affected was A2. The sensitivity to salt was much more pronounced in strain A2 than it was in either strain A3 or A1. After an initial decrease, the ratio increased, as expected if injured cells

recovered sufficiently to be reclaimed on salt plates.

It is interesting that the salt sensitivity noted in the control experiments with the unconcentrated single strains (Table 10) was altered by the propagation and concentration procedure. This may have been due to the inclusion of NFM in the laboratory propagation medium. In this respect, it can be seen that there was considerable difference in the growth performance of the single strains in milk and broth (Table 10).

In starter B, B2 showed the best survival, and the proportion of cells growing on salt agar was the highest for this strain. The initial drop in the number of cells recoverable on salt agar was greater for B1 than for B3, but the two approached equality in this respect by the time of the final test. Insofar as survival and salt sensitivity are concerned, B2 was the superior strain.

The relative phage sensitivities of the starter component strains merits consideration because dominance has been defined with respect to infection of these individual strains. If phage sensitivity of strains should change with time in frozen storage such that the degree of inhibition of the host by its homologous phage would increase or decrease, changing dominance patterns would be apparent, as in Part I. However, inhibition of hosts by their phages did not show the consistent decreases during storage which would have explained the failure of phages to significantly affect acid production in older starter lots, as

observed in Part I. The data varies somewhat, but not consistently enough to conclude that inhibition changes with frozen storage. There are cases in which the percent inhibition is quite consistent (A1-2, A3, B2). It therefore appears that phage infection experiments support conclusions made regarding dominant strains in the stored frozen concentrate.

The activity tests with starter A component strains revealed that the dominant strain, A3, was not the best acid-producer in NFM; the non-dominant strain, A1, was the most active in this regard, with A2 next, and A3 the least active. The activity of each of the strains in NFM-stim was more revealing, however; strain A3 showed a much greater increase in activity when inoculated into NFM-stim than did either of the other strains.

Stimilac provides a source of utilizable peptides in the medium. Cowman and Speck (15) have shown that the permanent loss of acid-producing activity by the lactic streptococci during frozen storage is paralleled by a loss of proteinase activity. The proteinase activity of A3 apparently was deficient, and therefore its activity in NFM-stim gave indication of its activity in NFM when the necessary peptides were supplied by the proteolytic activity of the other component strains. The activities of A1 and A2, assumed to result from more normal proteinase systems, also were enhanced in NFM-stim; therefore the added Stimilac supplied utilizable peptides in addition to those

liberated by their own proteolytic enzymes.

This indicates that during growth in mixed culture, A3 was supplied with the peptides necessary for acid production by the proteolytic activity of the other component strains. It is likely, for this reason, that A3 was able to contribute a major portion of the acid-producing capacity of the starter. That it does so is clear, because its removal caused the greatest loss of activity in the mixed starter.

The activity of the starter B component strains showed no great increases over activity in NFM when inoculated into NFM-stim. This indicates that any low activity observed was attributable to low numbers of cells in the concentrate, rather than to defective proteinase systems. The dominant strain in starter B, B2, was the most active in NFM, followed by B1 and B3. Since the cell populations of B3 were so small, it is concluded that the test results with this strain were not reasonable estimates of its activity as a concentrate compared to those of the other two strains.

Lactate sensitivity has been shown to affect the activity of some strains by several researchers (27, 52, 51). For example, Osborne and Chubb (51) showed that lactate was inhibitory, and that the effect varied a great deal among strains. They reported that as much as 7% ammonium lactate was required to inhibit some strains.

Results of the present study show that lactate sensitivity was

not greatly different for any of the strains examined. One of the non-dominant strains in starter A, A2, was distinctly less tolerant of lactate than were the other two. In starter B, lactate sensitivity was about the same for all three component strains.

The medium used for the propagation of these single strain concentrates contained 20 g/l of lactose (2%). If this were stoichiometrically converted to lactic acid, the final concentration of the acid would also be 2%. Milk contains almost 5% lactose, about 20% of which the lactic streptococci utilize to produce about 1% lactate. The strains examined here would not normally grow in greater than 3% lactate. From this it appears that lactate sensitivity would not be a dominance factor, but the possiblity that it contributes at least slightly to strain dominance with some strains should be kept in mind. For example, growth in mixed cultures under commercial conditions may well be influenced by the small differences observed here. Yet these data indicate that in the individual strain preparations, the lactate sensitivity or tolerance is not a contributor to dominance.

Collins (12) suggested that pH tolerance might be related to dominance, but noted only small differences between strains in this regard; the differences noted here are also small. Activity was measured in lactic and hydrochloric acids to separate the effects of pH and lactate senstitivity.

The differences in activity in lactic acid and in HCl were small

and inconsistent, but occurred more often in some strains than in others. For example, in starter A, the two nondominant strains were affected more often than the dominant strain.

The sensitivity of the strains to low pH was quite noticeable. In starter A, A3 was slightly less inhibited by low pH than were the other two components; that is, activity in acidified NFM was similar to activity in nonacidified NFM. In starter B, B2 was nearly always less active in acidified milk than in the unacidified control. While activity was lower with the other two strains, they were generally closer to the activity shown in the unacidified NFM.

Starter cell sensitivity to low pH is of considerable practical interest. Thomas and Batt (68) have estimated that the lactic streptococci may produce as much as 1.2 μM/mg dry weight bacteria/min of lactic acid, which is approximately 10% of the dry weight. Where starter concentrate or bulk starter is prepared and held in the acidified state acid sensitivity of the cells may play a significant role in the viability of the concentrate or starter.

Antibiotic production has received much attention in strain dominance work. Numerous workers have found that the production of antibiotics (13, 37) or inhibitory substances (42, 72) was responsible for the emergence of dominant strains. There are also reports in which antibiotic production was not found to be the cause of dominance (12). The present study concurs with the latter, in that no

evidence was found for the production of antibiotics or inhibitory substances by any strain.

The foregoing discussion relates to cultures in which glycerol was used as the cryoprotective agent (CA), since this was the CA recommended in the original patent (20) which has served as the basis for the now wide-spread commercial use of frozen concentrates. The data gathered here on survival, salt sensitivity, and activity indicate that of the CAs tested, glycerol is the one of choice. Malt extract was also effective, but often ranking second to glycerol.

Beta-glycerophosphate was expected to be a valuable CA from the work of Terzaghi and Sandine (67), also. Terzaghi has reported (personal communication) that some lactic streptococcal cultures stored at refrigeration temperatures (2 to 5 C) remained viable after several months of storage in GP-containing medium. In the present study, it was found to only slightly improve the preservation of cells only slightly over freezing without CA. It may be that the cells maintained in a buffered medium are more variable, since the normal accumulation of acid in a non-buffered medium will select for a more homogeneously acid-resistant cell population.

The effect of including GP in the propagation medium varied with the strain. Generally the cells propagated in GP revealed different preferences for CAs and different survival in salt than did cells of the same strain raised in medium not containing GP. In starter A

strain A3 became more tolerant of lactate as a result of growth in the presence of GP; the lactate tolerance of A1 however, seems to have been reduced. In starter B, strain B1 appeared less salt sensitive as a result of having been raised in the buffered medium.

It should be mentioned that the present work does not deal with strain interactions that might have occurred in mixed culture. When growing together in bulk culture or in the cheese vat, there may be interactions among the competent strains of the starter which cause the production of stimulatory or inhibitory substances. The possibility of such interactions is real, but the data here indicate that freezing damage is the main dominance factor. In view of this, involved studies to discover interactive effects do not seem justified.

The measurements that were made on the single strains are such that any reasonably well-equipped dairy microbiological laboratory could perform them. Without some means of monitoring interactions in mixed strain growth, the tests used by such laboratories would have to be similar to those used here. It is suggested that activity, survival, and degree of inhibition by phage would be most helpful in identifying mixed strain startes which are susceptible to dominance.

Summarizing the salient aspects of dominance in starter A, it initially appeared that A3, the dominant strain, was producing a stimulatory factor; its removal from the mixed culture by phage

infection resulted in severe inhibition of activity, though it showed much lower activity than did the other strains in single strain cultures. Its superior growth in NFM-stim indicated that it was probably very active in mixed culture, obtaining the peptides necessary for acid production from the proteolytic activity of the other component strains; in this way it became the most active, and even the dominant strain.

In starter B, it is clear that the dominant strain was the best survivor, the most active, and the least salt sensitive. The activity of the other component strains, in single strain cultures, was initially comparable to that of B2, but B2 remained the dominant strain by virtue of its ability to survive and remain active during frozen storage.

Previous work using phage infection as a dominance test makes no mention of the titer of the phages used, nor of controls to indicate the degree to which they eliminated their hosts. The present study takes this into consideration. Control tests were conducted to determine the relative sensitivities of each host to its phage in broth culture by measuring absorbance decreases after infection. Data indicated that all hosts were effectively eliminated by phage addition.

It is conceivable that the use of different phages might have revealed a different pattern of dominance. The data on activity and survival argue against this; prior to the present work, all the single

strains used were tested for sensitivity to the 67 different phage suspensions available in our laboratory. In a simple spot test, the phages to which each single strain was sensitive were selected. Alternative virulent phages for the two dominant strains were found, but the two used in this study were the ones which gave clear plaques and highest titers. The titers of the alternate phages were lower by a factor of at least 10^3.

When the undiluted phage suspensions were used for the spot tests, zones of lysis could sometimes be found on the non-dominant strains B1, B3, A1 and A2. When a 10^{-2} dilution of the suspensions was made and used in the spot test, only one phage was found to give a zone of lysis on the strains A2, B1, and B3. Several propagations were then required to prepare a phage stock of sufficient titer to lyse their respective hosts.

Interestingly, none of the 67 phages would form plaques on strain A1. However, Nyiendo stated (personal communication) that A1 was sensitive to phage a1, so it was tried. Repeated attempts yielded no phage stock of a1 on strain A1, though it titered easily on an alternate host (Scl 95). Even so, when inoculated into the mixed strain starter or into the single strain concentrate, a1 inhibited acid production, though it also did not plaque on any of the other component strains. Therefore, it may also be necessary to perform activity tests as well as spot tests to determine the phage sensitivities of

some host strains. One wonders from this how often unexplained cheese manufacturing vat failures in industry may have been caused by phages which could not be detected by the overlay assay method or by spot tests.

The present work did not indicate consistent or predictable change in the degree of inhibition of hosts by their phages with time in frozen storage. The infection of the mixed strain starters in Part I showed that the removal of the non-dominant strains by their phages caused progressively less inhibition of acid production as time in frozen storage increased. This indicates that their contribution to the activity of the starter progressively decreased with time in frozen storage. The tests performed here offer no explanation of why this occurred, but do show that the dominant strains retained their activity. It can be concluded, therefore, that the dominant strain is the one which, for one reason or another, maintains high activity throughout the period of frozen storage.

The commercially prepared starters used in this study achieved TTAs which exceeded .75% at six hours in all cases (uninfected controls). The single strain concentrates prepared by the author had activities considerably lower, ranging from .78% (B2-3) to .25% (B3-3) at T_0, to .67% (B2-3) to .23% (B3-3) after 90 days of frozen storage. However, this does not take into account the size of the inoculum, which varied because of differences in survival. It is

probable that the medium and method of propagation used here, adapted from the 1969 patent of Farr (20), differed from the methods now used by manufacturers who supply the frozen starters.

The concept of strain dominance demands continued attention from the dairy fermentation industry. Multiple strain lactic starters are used to minimize the risk of diminished acid production due to phage infection. Knowledge of the dominance relationships among the strains in certain combinations will allow improved multiple strain starters to be compounded. Dominant strains of similar phage relationships should not be spaced closely in culture rotations.

The development of nondominant starters may not be a realistic goal. It will require investigation of the phages able to infect a given strain to determine which can completely inhibit the activity of the strain, and under what conditions this may occur. Some indication of the dominance in a starter may be made clear if the mixed strain starter is infected with amounts of phage predetermined to cause the same degree of inhibition in each of the components over the time period and conditions imposed by the test.

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