PREVENTION OF BACTERIOPHAGE LYSIS OF LACTIC ACID BACTERIA IN INDUSTRIAL FERMENTATIONS

by

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REVIEW OF LITERATURE

Bacteriophage in industrial fermentations. The earliest reports on the phenomenon of bacteriophage (phage) were those of the English investigator, Twort, in 1915 and the French bacteriologist, D'Herrelle, in 1917 (9, p.13). The latter author felt that he was working with an invisible living microbe which was a parasite upon bacteria. He first called this ultramicroscopic agent, Bacteriophagum. The term bacteriophage, meaning "bacteria-eating agent," has come into general use.

Destruction of lactic acid bacteria by bacteriophage is not a new discovery. Hadley and Dabney (12, p.17) recognized phage lysis of Streptococcus lactis as early as 1925. Whitehead and Cox in 1934 (33, p.59), observed an agent entering cheddar cheese starters by aeration, which eventually inhibited the lactic acid bacteria. A short time later they identified the agent as phage (33, p.61). Their observations were confirmed in the United States by Nelson, et al. (27, p.220), when studying slow acid production by butter cultures. At a later date, also in the United States, Babel (3, p.605) confirmed similar inhibitory agents as phage in his study of factors influencing acid production by cheese cultures. In Canada, confirmation of phage inhibition was

made by Johns and Katznelson in their studies on cheddar cheesemaking (18, p.56). Sutton (31, p.168) similarly reported lactic Streptococcus phage activity in Australia. Anderson and Meanwell (2, p.71) confirmed a similar agent as phage in investigations on slow acid production in cheesemaking. The importance of phage in the dairy industry now has been emphasized in all parts of the world. Inhibition of lactic acid bacteria has been shown to be harmful in the production of many varieties of cheese and cultured milk products.

Deane and Nelson (7, p.21) have recently reported the isolation of a race of bacteriophage active against Streptococcus thermophilus. This virus was sufficiently virulent as to cause almost complete disruption of production in some Swiss cheese plants. It has also been reported that certain lactobacilli important in commercial lactic acid production are affected by bacteriophage (23).

The importance of bacteriophage in the destruction of an acetone-butanol fermenting Clostridium species was pointed out by McCoy (24).

Katznelson (19, p.248) has similarly reported that bacteriophage markedly affected the production of 2, 3-butanediol by Bacillus polymyxa.

Another industrial fermentation affected by bacteriophage is the production of streptomycin. In 1947, Woodruff and co-workers (38, p.540), and Reilly et al. (29, p.465)

reported the isolation of a phage which affected strains of Streptomyces griseus. Reilly reported the lytic agent to be specific for streptomycin-producing strains of S. griseus. Strains not producing streptomycin were found to inactivate the phage.

Important factors, other than phage, which may influence the production of lactic acid by starter cultures, include contamination by: Quaternary ammonium compounds (QAC), certain naturally produced antibiotics, antibiotics used as therapeutic agents, and certain fatty acids.

Resistant or secondary growth following bacteriophage attack. A well known phenomenon of the bacteria-bacteriophage relationship is the development of secondary growth or resistant strains which arise from a lysed culture. Frequently, such secondary growth will differ morphologically or physiologically from the parent strain. Henry and Henry (15, p.536) in a study of virus-resistant and virus-sensitive strains of Staphylococcus aureus, reported that resistant variants had slower rates of growth, aerobic oxidation of sugars, and dismutation of pyruvate (anaerobic). The resistant variants did possess a higher rate of anaerobic glycolysis. There is some evidence that the mutants may differ from their parent strain in their type of biological output (24). D'Herrelle (9, p.188) suggested resistant forms may develop which had recovered from a virus infection. He further suggested that the exposure to the virus may have

brought about a hereditary immunity. D'Herrelle further stated, "It is indeed probable that all fixed mutations occurring in bacterial species are produced through the action of the bacteriophage."

Burnet (5, p.353) reported very little success in obtaining cultures of Staphylococcus aureus resistant to their phage types. However, he did isolate phage resistant mutants of a sensitive culture which had not been exposed to lysis by any bacteriophage. Luria and Delbrück (22, p.510) concluded that phage-resistance is due to a heritable change of the bacterial cell which occurs independently of the action of the virus. Demerec and Fano (8, p.135) in a study of phage-resistant mutants in Escherichia coli, compared the growth rates of 35 resistant mutants to the growth rate of the parent strain. On the whole, growth rates for the mutants were lower than for the parent. They reported that different resistant types appear to be independent and suggest they may be produced by changes comparable to gene mutations. Anderson (1, p.401) reported that exposure of an organism to phage will likely result in a secondary growth which will be resistant to the phage causing the lysis of the parent strain. In his study of metabolic changes of virus-resistant organisms he reported that phage-resistant mutants of Escherichia coli did not develop in a synthetic basal medium, whereas the parent or sensitive strain did. Parker and co-workers (28, p.78) report that the nature of

an organism may be more fastidious when mutation from phage sensitivity to phage-resistance occurs.

Attempts to develop and use bacteriophage-resistant lactic Streptococci. Whitehead and Cox (4, p.61) attempted to produce phage-resistant lactic streptococci but reported that immune strains generally lost their resistant properties and could not be controlled even in the laboratory. Aeration increased phage outbreaks. They concluded that phage exposure was not a permanent solution in obtaining a phage-resistant organism. Whitehead and Hunter (35, p.346) reported that a resistant culture prepared by the action of the primary phage alternately suffers attack by a further phage distinct from the original. They also reported the use of a phage-resistant culture in two different dairy plants, with the culture being lysed on first use in both plants. Phages isolated were quite different from the original phages and it is thought they originated within the culture. Whitehead and Hunter (34, p.79) also reported little trouble with a single strain culture for six months in one province in New Zealand, while another province had little trouble for two years with the same culture. Having more than one culture, and attempting to control them by rotation was not deemed desirable, their theory being that no two cultures had the same acid-producing characteristics. They further stated that the cheesemaker would probably have to change his manufacturing procedure if a rotational system

were used. Anderson and Meanwell (2, p.70) indicated that phage-resistant strains can be developed, but on reintroduction into factory use the cultures become susceptible to secondary races of phage. They suggested the use of multiple strain cultures, in the presence of phage, which results in a condition of "slowness" as against "total failure" in a single strain culture. Wagenaar and Prouty (32, p.866) reported that phage-resistant strains became susceptible to secondary races of phage upon reintroduction to a cheese plant. They found that it was impossible to predict how long resistant cultures would retain their resistance. The method of developing resistance was to expose sensitive cultures to phage for one, two, or three successive transfers. They indicated that the degree of retention of the phage-resistance varied between different strains of organisms.

An interesting phenomenon in developing phage-resistance is that of lysogenesis (the production and liberation of phage by a host cell without lysis of that host cell). According to one idea, lysogenic strains may actually be cells which mutate from phage-resistance to phage-sensitivity and which are affected by low concentrations of phage which are maintained by the small population of phage sensitive cells.

Excellent reviews of the problem of bacteriophage in cheese manufacture have been outlined by Elliker (11), Hammer and Babel (13) and Whitehead (36).

Quaternary ammonium compounds. Moore (26, p.63) reported that partial inhibition of commercial lactic acid starter was brought about by the presence of 25 ppm. of QAC in the milk. Complete inhibition was noticed in the presence of 50 to 75 ppm. QAC. Barber et al. (4, p.56), working with cultures of Streptococcus lactis, found that as little as 10 ppm. added QAC would cause some inhibition. Progressive decreases were observed with 25 to 50 ppm. and complete inhibition occurred when 100 ppm. of QAC was added to the milk. Miller and Elliker (25, p.285) reported a slight inhibition of both a mixed strain commercial lactic culture and a Swiss cheese culture by 5 ppm. QAC. Milling time of cheddar cheese was delayed 15 minutes by presence of 5 ppm. and 45 to 60 minutes by 10 ppm. QAC.

Non-acid milk. Occasionally certain "wild" strains of lactic streptococci will be found to produce an antibiotic which inhibits strains used in the manufacture of cheese and cultured milk. Milks containing such an agent are frequently termed "non-acid" milk. Hunter and Whitehead (17, p.125) reported wide variations in the susceptibility of starter cultures to non-acid milk. Single strain cultures in non-acid milk showed variations in acid production from 0.19 to 0.79 in a 17-hour incubation period. Two commercial mixed starters varied between 0.29 and 0.72. The acidity produced by both starters in normal milk was 0.72. In general, reports from various investigators agree that

the antibiotics of non-acid milk are quite heat stable. Names for the antibiotics have included nisin and diplococcin.

Commercial antibiotics. The use of antibiotics in the treatment of bovine mastitis has prompted a number of investigators to study the effect of these compounds on starter activity. Hood and Katznelson (16, p.32) found that as little as 0.05 units of penicillin in milk was inhibitory to S. cremoris. S. lactis was more resistant to the action of antibiotics. On the basis of later information Katznelson and Hood (20, p.967) were able to calculate that one treated quarter per cow of ten percent of a herd would leave sufficient penicillin in the milk to cause definite inhibition in starter activity. Hansen et al. (14, p.361) reported that pooled milk from four cows, each with one treated quarter, completely stopped acid production by starter organisms. They also found that the drug did not diffuse into untreated quarters. Penicillin was not destroyed by condensing or condensing and drying procedures. They subsequently observed that low levels of sulfa drugs and aureomycin dried with milk proved stimulative to starter action when the milk was reconstituted.

Recommendations for the number of milkings to be discarded following mastitis therapy with antibiotics have varied from three to ten when 200,000 units of penicillin were used. Although other antibiotics are inhibitory in smaller

concentrations they are also administered in smaller amounts. Doan (10, p.29) has stated that chloromycetin treatment produces the least effect on starter action. He recommended that at least three milkings be discarded after treatment with any antibiotic. Wilkowske and Krienke (37, p.1032) have reported that 0.3 unit per milliliter of penicillin did not retard the acid development of Lactobacillus acidophilus, Lactobacillus bulgaricus, and Lactobacillus casei. A concentration of 0.6 unit per milliliter resulted in less acid production, while 2.0 units per milliliter allowed no acid production after 48 hours at 35°C. Kosikowsky et al. (21, p.537) in analyzing 1794 milk samples in New York state found about one percent strongly inhibitory toward commercial lactic starter cultures. Approximately half of these samples were tested for the presence of sulfa drugs, with no trace of the chemical being present. Approximately 800 of these samples were tested for the presence of QAC. Amounts varying from trace quantities to three ppm. were found in about 4 percent of these samples. Stoltz and Hankinson (30, p.28) made a study of the effect of various antibiotics on lactic acid starter cultures. They reported that 0.1 unit of penicillin; 0.1 mg. of streptomycin; 5.0 mg. of streptomycin and 1.0 unit of penicillin in combination; 0.1 micro-gram of aureomycin; and 1.0 mg. of tyrothricin per ml. of milk would inhibit the lactic organisms. They concluded that the addition of 75,000 units of penicillin to a milk

supply would greatly inhibit lactic acid formation in approximately 17,000 pounds of milk.

Fatty acids. Inhibition of Streptococcus lactis by fatty acids found in milk also has been reported (6, p.1108). It was reported that oleic, butyric, caproic, linolenic, linoleic, arachidic, palmitic, and stearic acids have no effect upon S. lactis. However, caprylic, capric, and lauric acids were inhibitory to the growth of S. lactis.

PART I

IMPORTANCE OF BACTERIOPHAGE AS A CAUSE OF SLOW STARTERS

Starter activity is dependent on a number of factors, most of which are under the control of the operator. The importance of maintaining proper time and temperature of incubation and daily transfer are well known. Another important factor, the milk employed for growing mother and bulk starter cultures, also can be controlled easily by use of milk reconstituted from high quality, low heat, spray dried non fat milk solids. Preliminary trials on a batch of non fat milk solids will determine whether or not it satisfactorily grows the starter culture. If the reconstituted milk from the batch proves satisfactory, a uniform supply of starter milk is assured as long as powder from that batch is used.

It is more difficult, however, to maintain such uniformity in subsequent manufacturing steps in production of cultured milk products. A number of contaminating factors that retard activity of lactic acid bacteria may be present in the plant or in the mixed milk entering the plant. These include: germicides such as the quaternary ammonium compounds, antibiotics, and bacteriophage. Although low concentrations of quaternary compounds in milk are able to inhibit acid production by starter bacteria, they are not considered an important cause of slow starters. Antibiotics in milk are derived from two sources. One is the inhibitory

agents such as "nicin" produced by some lactic streptococci growing in milk. The other results from udder injection for treatment of mastitis and may result in contamination of milk with one or a mixture of such antibiotics as penicillin, aureomycin, terramycin, and streptomycin. Antibiotics entering milk through mastitis treatment have received a great deal of attention as a cause of starter failure in recent years. A number of workers have definitely established antibiotics to be responsible for some specific instances of slow starters. During the past two years at least 23 serious cases of starter failure in Oregon dairy plants have been investigated. Most of the plants experiencing the starter difficulties suspected antibiotics in the milk to be responsible. In two instances there was some indirect evidence of antibiotic in the milk. However, in the remaining 21 cases analysis of defective starters or cultured milk products has established bacteriophage as the source of the slow acid production. Application of plant sanitation procedures for phage elimination and use of strains of lactic acid bacteria resistant to phage isolated from the plant invariably improved rate of acid production. As the significance of phage as a primary cause of starter failure became apparent, the investigations on control methods were expanded somewhat to establish the general prevalence of the problem in various dairy products produced throughout the state. The data cited in this report were collected as part

of a study on isolation of lactic phages and development of phage resistance in lactic streptococci.

MATERIALS AND METHODS

Eighteen single strains of Streptococcus cremoris and Streptococcus lactis were used for these cultures. They represented both stock culture strains as well as isolants from commonly used commercial cultures. Samples examined for bacteriophage were collected at random from 32 different plants and represented occasional mother and bulk starter cultures, whey from cheddar, cottage and blue cheese plants, and cultured buttermilk. The samples were filtered through Selas candles and 0.5 ml. of whey added to respective 10 ml. quantities of sterile skim milk inoculated with the 18 different single strain cultures. Control tubes of each culture without filtrate also were prepared. Sterile resazurin solution was added for indicator before inoculation. The tubes were incubated at 30°C. and examined for resazurin reduction at 6 hours and at about half-hour intervals thereafter. Definite prolongation of reduction time beyond that of the control suggested inhibition and samples of inhibitory filtrate were subsequently heated at 100°C. for 5 minutes. Bacteriophage, if present, was destroyed by the heating. The effect of the heated filtrate on the test cultures then was repeated to establish whether or not the inhibitory substance was heat labile. This step plus the fact that filtrates were diluted about 20 times by inoculating 0.5 ml.

into 10 ml. of milk culture tended to minimize interference of results by antibiotics. When the inhibitory agent in any filtrates proved to be destroyed by the heating, some of the sample of filtrate was transferred to a fresh, sensitive culture which was incubated for 24 hours. Presence and concentration of any phage was determined by plaques and by increasing concentration of phage by successive passage. Attempts were made in questionable cases to build up the titer by transfer of the inhibitory agent.

RESULTS AND DISCUSSION

It was observed during the progress of the study that certain strains of the lactic streptococci employed for the survey were more sensitive to bacteriophage attack than others. Table 1 shows comparative sensitivities of the various strains. It is interesting to note that culture 144F was inhibited 25 times during the survey, while culture W was inhibited 31 times. Both of these were strains of S. cremoris from stock cultures. Of the 18 cultures employed only two were not inhibited during the entire survey. These were cultures K2 and R6. K2 (S. lactis) and R6 (S. cremoris) originated from the stock cultures. Equally interesting is the fact that over 50 percent (66 out of 115) of the total number of inhibitions during the entire survey included either cultures 144F, W, or AB.

TABLE 1

Frequency of bacteriophage inhibition of 18 single strain cultures of *S. lactis* and *S. cremoris*

Strains tested	Number of times each strain was inhibited
144	25
23	2
K2	0
R6	0
W	31
Oslo	3
104	3
H	9
HP	1
F	7
V	4
O	4
AA	2
AB	10
AC	3
AD	6
L1	2
L2	3

Nine of the 18 cultures employed in the survey were strains from stock cultures. Six of these were S. cremoris, while 3 were S. lactis. These 9 strains were inhibited a total of 74 times or 64 percent of the total number of trials. The 3 S. lactis strains were inhibited in only 2 out of a total of 74 trials.

As expected, S. lactis strains were attacked less frequently than S. cremoris. Nine strains were isolated from 3 different commercial cultures. Two of these were S. lactis while 7 were S. cremoris. The two S. lactis cultures were inhibited only 5 times, while the remaining 7 S. cremoris cultures were inhibited 36 times. These data show a total of only 7 inhibitions obtained from the 5 S. lactis cultures employed. Thirteen S. cremoris cultures were inhibited 108 times or 93.9 percent of the total inhibitions. These data are presented in Table 2. One interesting observation during the study was that certain purified races of bacteriophage isolated from filtrates were capable of attacking both S. lactis and S. cremoris.

Data of Table 3 show results from a total of 87 different random samples from 32 plants which were examined for presence of inhibitory materials. Filtrates of 49 of the samples contained heat labile substance that inhibited one or more of the 18 single strain starter cultures. Inhibition in 40 of these representing 27 plants was definitely established as due to bacteriophage. The inhibitory

materials in the other 9 samples were not identified, but it is significant that they constituted only a small proportion of the total number of samples showing inhibition. It is possible that some of these 9 samples also contained bacteriophage. Only one of the 87 filtrates contained an inhibitory substance that was not destroyed by boiling.

Of all the samples examined, inhibitory agents were observed in 30 of 56 of the buttermilk filtrates, 13 of 20 of the cottage cheese whey, and 7 of 11 filtrates of whey obtained from cheddar and blue cheese plants. Plant conditions of sanitation and methods of starter handling appeared more important than type of product in affecting incidence of bacteriophage.

In addition to the above samples 99 additional filtrates were prepared from various cheddar, blue and cottage cheese, and buttermilk samples in another study. Of these a total of 65 were found to contain heat labile inhibitory agents. In view of previous observations that most of such heat sensitive agents appeared to be bacteriophage, it must be presumed that most of the 65 samples also owed their inhibitory effect to bacteriophage. Results of the latter survey are summarized in Table 4.

TABLE 2

Frequency of bacteriophage inhibition encountered in selected stock cultures and in strains isolated from various commercial cultures

Cultures from which sensitive strains were selected	Number of strains	Number of times inhibited
Hansen	4 (*S. cremoris*)	21
Flav-O-lac	3 (*S. cremoris*)	15
Erickson	2 (*S. lactis*)	5
Stock	6 (*S. cremoris*) 3 (*S. lactis*)	72 2

TABLE 3

Incidence and identity of inhibitory substances from 32 Oregon dairy plants

Percent of plants with inhibitory substances	Percent of plants with inhibitory substances identified as phage	Total number of samples analyzed	Percent of inhibitory samples	Percent of inhibitory samples identified as phage
81.4	84.3	87	56.3	80

TABLE 4

Incidence of heat labile inhibitory substances in various dairy products

Product	Number of plants represented	Number of samples analyzed	Number and percent of samples containing inhibitory substances	
			(No.)	(Percent)
Buttermilk and starter	6	34	28	82.3
Cottage cheese	4	36	25	69.4
Cheddar and Blue cheese	4	29	12	41.4

PART II

PROCEDURE USED IN DEVELOPING BACTERIOPHAGE RESISTANT STRAINS

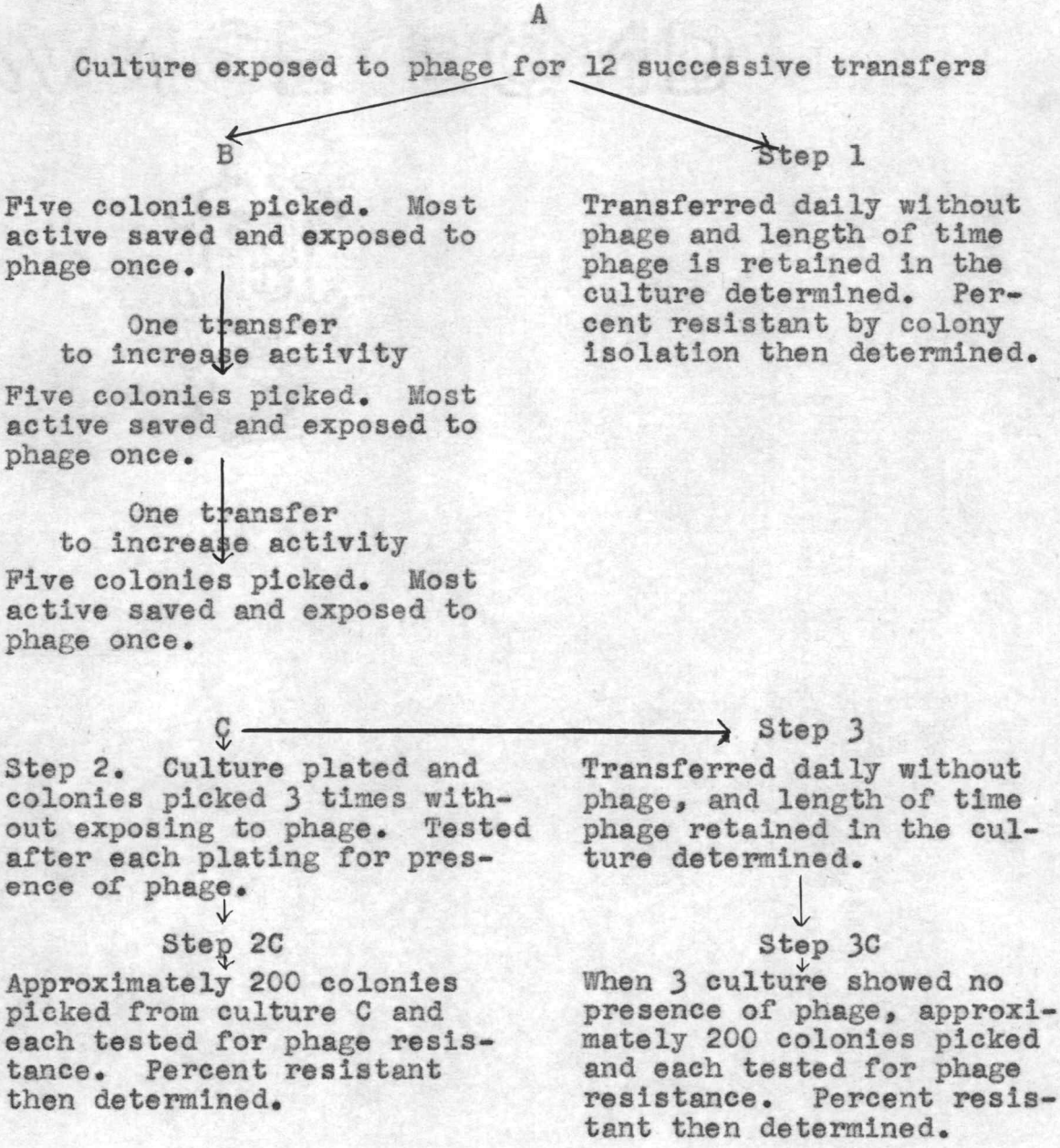
Two sensitive S. cremoris cultures, R6 and LH3, were employed in this study. Bacteriophage Strain D served as the inhibitory agent for both cultures. This phage was isolated from a large Oregon dairy plant. Lactobacillus agar was used for all plating and picking procedures. It had the following percent composition: Tryptone 2.0, yeast extract 0.5, gelatin 0.25, sucrose 0.5, dextrose 0.5, lactose 0.5, and agar 1.5 percent. All data were obtained at 30°C. The sensitive culture was exposed to the phage for 12 successive transfers. After such exposure, two phage-free transfers were made and the resulting culture A served as the starting point for three different procedures for selecting bacteriophage-resistant mutants. The three procedures are diagramed in the schematic outline in Figure 1. In procedure one no further treatment was given. In procedure B, culture A was plated, picked, and exposed to phage, the most active isolates retained, and the procedure repeated 3 times. The culture was then plated and isolates picked 3 additional times (procedure 2), without further addition of bacteriophage. Only the most active isolates were retained in this procedure. Approximately 200 colonies were picked at point 2C and checked for resistance.

Procedure 3 followed procedure 2 through plating and

isolating, in the presence of phage, to item C. At this point the culture was serially transferred without further addition of phage. When the culture was found free of phage (3C) approximately 200 colonies were picked and checked for resistance.

FIGURE 1

Schematic outline for developing bacteriophage resistance



After the above outlined procedures were completed, it was desirable to compare the results obtained from all three procedures. In order to make these comparisons it was necessary to determine the transfer, or plating, at which the phage was lost, and the percentage of phage resistance in the final culture. The cultures were transferred daily at 30°C. and the length of time that phage was retained in the culture was determined. Bacteria-free filtrates were obtained after each transfer or isolation and the original phage-sensitive culture exposed to this filtrate. Inhibition of the sensitive culture was easily determined by lack of acid production by the sensitive strain. After determining the length of time phage was retained in the culture, the percentage resistance was determined. Approximately 200 isolates were made from agar plates, subcultured in milk at 30°C., and transferred one additional time to assure activity. Developed resistance was determined by transferring in the presence of phage. Resistant strains coagulated milk within a 6 to 8 hour period, at 30°C., while sensitive strains did not develop under these conditions.

RESULTS AND DISCUSSION

Tables 5 and 6 indicate that the phage was lost the earliest in the plating technique (step 2), and retained the longest in serial transfer (step 1). Both cultures R6 and

LH3 lost the phage after the first plating (step 2). Evidently the plating and colony isolation procedure also effectively selected only resistant strains. It is considered that length of retention of bacteriophage is a more effective criterion of complete resistance than is percentage of resistance as determined on 200 isolates. This may be justified on the basis that even a few sensitive cells in several billions, may serve as a reservoir of phage.

It would appear that the phages apparently are carried only by sensitive cells, and the chance of picking a sensitive cell at random on a plate is quite remote. Thus effective elimination of phage in the plating and picking technique is probably due to pure mathematical chance.

Tables 5 and 6 suggest that 100 percent phage-resistance eventually can be obtained by all three procedures. However, they leave no doubt as to the superiority of a plating and isolation technique. Table 5 shows that it required 11 transfers to eliminate phage when using serial transfer (step 1), whereas using plating and isolation, phage was eliminated by the first plating in step 2 and the second transfer in step 3. Table 6 shows that seven transfers were required to eliminate detectable phage in serial transfer procedures, whereas phage was lost in the first plating using step 2, and the fourth transfer in step 3. The results would seem to indicate that step 2 is the most effective method of selecting active resistant starter

cultures.

TABLE 5

Effect of method of handling on retention of bacteriophage by culture R6 and percentage of resistant strains in subcultures

Method*	Transfer or plating at which phage was lost	Phage resistance of subcultures	
		No. of isolates	Percent resistant
Step 1	11th transfer	180	100
Step 2	1st plating	167	100
Step 3	2nd plating	193	100

* See Figure 1 for detailed procedure.

TABLE 6

Effect of method of handling on retention of bacteriophage by culture LH3 and percentage of resistant strains in subcultures

Method*	Transfer or plating at which phage was lost	Phage resistance of subcultures	
		No. of isolates	Percent resistant
Step 1	7th transfer	187	96.8
Step 2	1st plating	202	100
Step 3	4th transfer	178	100

* See Figure 1 for detailed procedure.

Relationship between bacteriophage resistance and starter activity. Since these data indicate that more complete resistance of lactic organisms to bacteriophage can be obtained, further investigation was necessary to determine the relationship between phage resistance and activity, or acid production of the organisms. Daily observations have indicated that cultures made resistant to phage by the above procedures are more durable cultures and less susceptible to other strains of phage when the culture is used in commercial dairy plants. In order to be of value, it is desired to have a starter culture which is not only phage-resistant but also one which possesses good, active, lactic acid-producing capacity. Generally speaking, it can be stated that on daily observations the phage-resistant multiple strain cultures appear more active than sensitive cultures. This was not always true as previous data has indicated that in certain instances aroma production as well as acid production may be impaired by exposing the organisms to bacteriophage.

It was then decided that actual comparison of a culture exposed and unexposed to phage was necessary in order to determine the effect of the phage on the activity of the cells. Four S. cremoris single strain cultures (W, 11E, R6, and 1144F), and one S. lactis (IS) were employed for these tests. The same method for developing phage resistance was followed as found in steps 1 and 2 of the previous data.

Each of the five single strain cultures with their respective phages were used for these observations. Each phage, other than IS, was purified by preparation and isolation from plaques previous to the selection procedure, and a single phage isolate used to develop resistance.

RESULTS AND DISCUSSION

A six hour activity test at 37.5°C. and a 16 hour activity test at 21°C. were used in comparing the acid production of phage-sensitive and phage-resistant lactic acid organisms. All sensitive cultures and those exposed to phage were incubated at 30°C. All cultures were allowed to coagulate twice at 21°C. after being incubated at 30°C., before the 21°C. activity test was made. On the third transfer at 21°C. the titratable acidity was recorded. Two successive trials were made with both activity tests. Tables 7, 8, 9, 10, and 11 represent results of these experiments.

The above data indicate that selected resistant strains may have significantly different rates of acid production than sensitive parent strains. Of exceptional interest are results which show resistant strains of 144F to be more active than sensitive strains in activity tests at 37.5°C., whereas the reverse was true at 21°C. Activity of resistant mutant strain IS was significantly lower than the sensitive parent at both temperatures. Activity tests at

37.5°C. indicate R6 sensitive and R6 resistant strains to be about the same, while 21°C. activity tests for the same strains show a great difference, the sensitive strain being far more active than the resistant mutant. There were no great differences in cultures 11E and W, by activity test of phage-sensitive and phage-resistant cultures at either 21° or 37.5°C.

There is very little apparent difference between the serially exposed (step 1) and the plated isolates (step 2) of all five cultures. Culture R6 shows the greatest difference at 21°C. In this instance, step 1, R6 had 0.09 of one percent less acid produced than in step 2.

One possible reason for the low activity in some of the resistant cultures may be that some sensitive cells are still present. With a rigorous procedure of plating, it is theoretically possible to eventually eliminate these sensitive cells and thus increase the activity of the culture.

TABLE 7

Effect of selection of bacteriophage resistant strains on activity of lactic streptococci (strain IS)

Procedure	6 hour activity test at 37.5°C.	16 hour activity test at 21°C.
	(Percent titratable acid)	(Percent titratable acid)
Sensitive	.42	.72
Step 1	.25	.62
Step 2, first plating	.24	.64
Sensitive	.39	.70
Step 1	.24	.61
Step 2, second plating	.24	.64

TABLE 8

Effect of selection of bacteriophage resistant strains on activity of lactic streptococci (strain W)

Procedure	6 hour activity test at 37.5°C.	16 hour activity test at 21°C.
	(Percent titratable acid)	(Percent titratable acid)
Sensitive	.22	.42
Step 1	.22	.38
Step 2, first plating	.22	.38
Sensitive	.23	.46
Step 1	.22	.41
Step 2, second plating	.23	.42

TABLE 9

Effect of selection of bacteriophage resistant strains on activity of lactic streptococci (strain 11E)

Procedure	6 hour activity test at 37.5°C.	16 hour activity test at 21°C.
	(Percent titratable acid)	(Percent titratable acid)
Sensitive	.23	.34
Step 1	.22	.30
Step 2, first plating	.22	.31
Sensitive	.23	.40
Step 1	.23	.35
Step 2, second plating	.21	.35

TABLE 10

Effect of selection of bacteriophage resistant strains on activity of lactic streptococci (strain R6)

Procedure	6 hour activity test at 37.5°C.	16 hour activity test at 21°C.
	(Percent titratable acid)	(Percent titratable acid)
Sensitive	.34	.73
Step 1	.32	.30
Step 2, first plating	.32	.39
Sensitive	.33	.74
Step 1	.32	.35
Step 2, second plating	.33	.36

TABLE 11

Effect of selection of bacteriophage resistant strains on activity of lactic streptococci (strain 144F)

Procedure	6 hour activity test at 37.5°C.	16 hour activity test at 21°C.
	(Percent titratable acid)	(Percent titratable acid)
Sensitive	.25	.71
Step 1	.33	.65
Step 2, first plating	.32	.66
Sensitive	.28	.73
Step 1	.33	.64
Step 2, second plating	.33	.66

PART III

PLANT UTILIZATION OF RESISTANT STRAINS AND ROTATIONAL METHODS

The primary purpose of this section of the study was to develop the practical application of bacteriophage-resistant strains of lactic streptococci, and rotational methods in maintaining active starters in dairy plants. The study included the use of phage-sensitive and phage-resistant starter cultures, both single and multiple strain which were prepared in the laboratory. A few commercial starter cultures were also utilized.

From previous work with starter cultures, it was thought that with suitable methods it would be possible to predict the usefulness of a culture in the plant. It was postulated that this might be accomplished by watching the "build-up" of phage with single sensitive indicator strains.

The bulk of work in this study was carried on with the cooperation of seven dairy plants in the state of Oregon. The types of products in which starter cultures were used included cottage cheese, cultured buttermilk, cheddar cheese, and blue vein cheese. All plants were personally visited before inception of the study and also during its progress.

The first step in this study was the preparation or isolation of active, single, and multiple strain cultures in the laboratory. These cultures consisted of either phage-

sensitive or phage-resistant strains. A total of 51 cultures were carried during the entire study. Twenty-seven of these were single strain, phage-sensitive cultures, while 6 were laboratory prepared phage-resistant cultures. Sixteen cultures (mostly S. lactis) were not affected by the laboratory stock phages. Of these 16 cultures, 7 were isolated from naturally soured cream (all S. lactis). Three of the 51 cultures were from commercial firms. Fourteen were multiple strain cultures consisting of various combinations of single strain phage-resistant organisms.

A total of 82 samples were analyzed during the study. Fifty-two of these samples were found to contain heat-labile inhibitory substances against one or more of the laboratory cultures. As stated before, in view of previous observations that most of such heat sensitive agents appeared to be bacteriophage, it must be presumed that most of the 52 samples also owed their inhibitory effect to bacteriophage.

A total of 111 active cultures were either mailed or delivered personally to the various dairy plants. Many of these were fresh transfers of cultures which had been used satisfactorily a previous time.

The next step consisted of obtaining whey samples for preparation of bacteria-free filtrates and the preparation of cultures consisting of strains not inhibited by this filtrate.

Before sending out new cultures to a plant, a whey

sample was obtained and analyzed for bacteriophage. In most instances cultures mailed contained only strains resistant to the particular phage indigenous to the plant. In order to further verify results, it was necessary to send starter cultures out at intervals for plant use when laboratory analysis indicated phage-susceptible strains in the cultures. This, of course, answered the question as to whether a phage build-up could be detected. Many times starter cultures were changed from one time to the next, even though no evidence of phage was indicated.

It must be pointed out that it is not always possible to obtain the desired plant cooperation with this type of project. The best cooperation, and in turn the most enlightening information, was obtained from plants 1 and 2 (Table 13). The mother cultures for plant 2 were carried in this laboratory. This permitted a close personal relationship with the plant and consequently more complete data.

RESULTS AND DISCUSSION

Single and multiple strain cultures used and results of phage studies in commercial plants are shown in Tables 12 to 19, inclusive.

The information obtained in working with plants 1 and 2 indicate that it may be possible to predict the success of a starter culture in a dairy plant before its actual use.

In plant 2, a phage build-up was noticed at various times. However, some cultures were not removed in order that reliability of predictions could be tested. In all such instances there were indications of phage attack of the starter culture within a few days. In two instances build-up of phage was shown by the indicator strains for cultures 26 and 301. However, these cultures were continued in service. Within two days after effects were shown by the indicator strains, the vat cultures were completely inactive. The value of indicator strains in detecting phage build-up and subsequent inactivity of the vat culture is thus demonstrated.

TABLE 12

Tabulation of single and multiple strain cultures employed

Strains of *S. lactis*	Strains of *S. cremoris*	Multiple strain starters
101	Oslo	Flav-O-lac (C)
101 Imm	Oslo Imm	Flavor-line (C)
104	11E	Danish (C)
23	11E Imm	10 (Oslo Imm, HP Imm, R6 Imm, L.d.)
E	4E	21 (Oslo Imm, 101 Imm, 11E)
L-1	144 Andy	26 (S, 5 Imm, 11E)
K3	W	27 (S, 5 Imm, 4E)
K4	W(S) Imm	29 (Oslo Imm, R6 Imm, HP Imm, 144 Andy)
K5	5	
K6	5 Imm	33 (S, 5 Imm, 11E Imm)
1 SC	R6	34 (Oslo Imm, S, 11E Imm)
2 SC	HP	35A (144Fco Imm, 11E Imm)
3 SC	144Fco Imm	35B (144co Imm, 11E Imm, L.d.)
4 SC	144F	36 (K3, 11E Imm, L.d.)
8 SC	HP Imm	37 (E, 11E Imm, L.d.)
9-1 SC	144Fco	38 (R6, 11E Imm, L.d.)
11 SC	FD20	39 (K3, E, L.d.)
	301	40 (101, E)
		41 (11E Imm, E, 144co, L.d.)
		42 (101, E, 144Fco, L.d.)
		43 (101, E, L.d.)

Imm--Indicates strain made resistant to both D and G phage races.

SC --Indicates strain isolated from raw, naturally soured cream.

C --Indicates commercial culture; all other multiple strain cultures were prepared from selected strains in the laboratory. One strain of *Leuconostoc dextranicum* (L.d.) employed for various multiple strain cultures.

TABLE 13

Effect of bacteriophage population on starter activity in plant 1

Starter used	Indicator strains inhibited	Starter activity in the plant
26	11E, 4E	slow
10	11E, 144F, W	good
35A	144F	flavor did not hold up
35B	W	excellent culture, liked acid and flavor
35B	11E Imm, 144Fco	slow
36	11E Imm, W, 144F	good
36	11E Imm, W, 144F	slow vat

TABLE 14a

Effect of bacteriophage population on starter activity in plant 2

Starter used	Indicator strains inhibited	Starter activity in the plant
10	11E, W, 301	good
Danish	26, 11E	good
26	26, 11E	"dead vat", complete loss
Flav-O-Lac	26, 301	good
Flav-O-Lac	not checked	good
Flav-O-Lac	W, 301	good
301	301, W, 144F	"dead vat", complete loss
33	11E	good
35A	W, 11E, 144F	good
35A	not checked	good
35A	not checked	good
35A	not checked	good
35A	not checked	good
35A	not checked	good
35B	not checked	noticed improved flavor in cheese
35B	not checked	good
35B	not checked	good
35B	not checked	good
35B	not checked	good

TABLE 14b

Effect of bacteriophage population on starter activity in plant 2

Starter used	Indicator strains inhibited	Starter activity in the plant
35B	not checked	good
35B	not checked	good
35B	144F	good
36	144F, 11E	good
36	not checked	good
36	not checked	good
36	not checked	good
36	not checked	good
36	144F	good
35B	144F	good
35B	not checked	good
35B	not checked	good
35B	not checked	good
35B	not checked	good
35B	not checked	good
35B	not checked	good
35B	not checked	good
35B	not checked	slow
35B	144F, 144Fco	slow
35B	144F, 144Fco, W	very slow
41	144F, 144Fco	good

TABLE 14c

Effect of bacteriophage population on starter activity in plant 2

Starter used	Indicator strains inhibited	Starter activity in the plant
41	not checked	good
41	not checked	slow
42	11E	good
42	not checked	good
42	not checked	good
42	144F, 144Fco, W	good
42	144F, W	slow
42	not checked	slow
42	11E, 144F, 144Fco, W	slow

TABLE 15

Effect of bacteriophage population on starter activity in plant 3

Starter used	Indicator strains inhibited	Starter activity in the plant
Flav-O-Lac	11E, 4E	no report
10	11E, 4E	no report
26	not checked	good
29	not checked	no report
35A	W	good
35B	not checked	good
35B	144F, W	no report
35B	not checked	no report
40 and 42	144 Andy, 144Fco	vats very slow
40 and 42	not checked	no report

TABLE 16

Effect of bacteriophage population on starter activity in plant 4

Starter used	Indicator strains inhibited	Starter activity in the plant
26	10, 11E, 4E	good
26	10, 11E	good
Flav-O-Lac	11E	no report
35A	not checked	no report
35B	not checked	good
35B	11E	good
35B	11E, 4E	slow
42	not checked	good
42	not checked	no report

TABLE 17

Effect of bacteriophage population on starter activity in plant 5

Starter used	Indicator strains inhibited	Starter activity in the plant
FD20	144F	slow
301	not checked	slow
35B	144F	good
S	144F, 144Fco	good
40	W, 144F, 144Fco	good

TABLE 18

Effect of bacteriophage population on starter activity in plant 6

Starter used	Indicator strains inhibited	Starter activity in the plant
10 and Flav-O-Lac	not checked	no report
26 and 29	not checked	26 very good
26 and Flav-O-Lac	not checked	no report
27 and Danish	not checked	no report
35A and Danish	not checked	no report
35B and 36	not checked	no report
35B and 36	not checked	no report
35B and 37	not checked	no report
40 and 42	144F, 11E, 35B, 36	good

TABLE 19

Effect of bacteriophage population on starter activity in plant 7

Starter used	Indicator strains inhibited	Starter activity in the plant
35A	not checked	good
40 and 42	not checked	40 best culture they have had in the plant
40 and 42	not checked	40 excellent

Phage build-up on cultures 35B, 41, and 42 was very obvious during their use in plant 2. Results were the same as those with cultures 26 and 301, and it can be assumed from these observations that "dead vats" would also have occurred in these instances if the cultures had not been changed.

It is interesting to note that when a single strain within a multiple strain culture was inhibited, another strain would produce acid and thus "carry the load". This appeared to be the case with starter 35B and 41. Starter 35B was used in plant 2 for 11 consecutive times and had become very slow. Starter 35B contained only two strains of S. cremoris, one of which, 144Fco, was definitely inhibited by previous whey filtrates. It was decided to use strain 144Fco in another culture, but to give the multiple strain culture an additional resistant strain. The culture was then employed in plant 2. Results indicated the same single strain, 144Fco, to be inhibited but the new culture (41) proved to be very active for the next few days. One of the reasons for phage difficulties in this plant was the apparent mutation of bacteriophage to affect otherwise resistant starters. This was suggested in the following series of observations. After mutant resistant strains had been employed for a short period, a rapid reduction in activity was noted. It could be established that the phage was different, rather than the organism having back-mutated to

sensitivity. When the affected resistant strain was tested against the original phage, it was found to remain resistant. However, the same strain of bacteria was sensitive to phage obtained at the later date.

Culture 40 was developed for a cheddar cheese plant which was having difficulty with "gassy" cheese. The culture appeared to eliminate their difficulty and also proved to be very active and durable.

It has been the writer's privilege to work with a commercial cheese plant for a period of almost three years. Specific data concerning starter cultures were accumulated and part of these data are presented to show the merits of a rotational system conducted without the benefits of phage tests. This plant was, and still is, highly infected with bacteriophage active against most lactic acid starter cultures. One of the primary objects of a rotational system is to keep ahead of the attacking phage, which in principle is the idea developed from phage tests.

Due to the high incidence of phage in this plant, it was found necessary to use more than one culture per day, as the use of one culture would often promote a slow vat of cheese. As no phage testing equipment was available, it was necessary to determine the length of time that a culture could be used before acid production began to show up and lysis occurred. This was determined by keeping daily records on the use of each culture. These data indicated

that three successive days represented the maximum that the average starter culture could be used and still maintain desirable characteristics. On the basis of these data, a regular rotational system such as found in Table 20 was instituted. This system was based on changing cultures every two days. The laboratory where mother cultures are carried in this plant was completely isolated from the manufacturing room.

Sixteen active mother cultures were carried at all times. These cultures represented 4 commercial sources indicated by the letters H, F, M, and D, and each source supplied 4 different cultures termed 1, 2, 3 and 4.

The bulk starter room also was isolated from the manufacturing sections of the plant. Five 10-gallon cans were used for incubating the bulk cultures. The procedure used in inoculating these cultures was to add each culture to a separate can, reserving sufficient inoculum to inoculate a fifth can with all 4 cultures. This system of inoculating provided an opportunity for examination of each culture individually, and the fifth can yielded evidence on how the cultures grew in association together. If one of the 4 cultures employed happened to produce inhibitory substances against another culture, it could be located with the fifth can. Knowing this, each culture could then be examined to find which one of the 4 was producing the inhibition. This situation was shown to occur, with wide variations in

inhibitions produced.

When cultures are obtained from commercial firms, it is possible that any 4 cultures for example, even at various intervals, may contain identical organisms. From the results obtained this did not appear to be the case. In order to eliminate this theoretical possibility of a phage build-up, a second rotational system was instituted for this plant. This system is illustrated in Table 21. Various types of rotational systems could be developed to meet the individual plant needs. The second system consists of nine cultures instead of sixteen. Cultures obtained from source D were definitely more phage-resistant than any other culture used. Thus, it was decided to rotate 3 of the D cultures in consecutive order, while one of the various six other strains was employed for two days before a new strain was substituted. Therefore, two commercial mixed cultures were used at the same time, one of which was always a D strain, and one was either H, F, or M. On the basis of plant experience, this system should result in practically no opportunity for phage build-up from day to day. If a phage resistant source of cultures could not be located, this same system might be used by rotating all cultures on a two day basis.

After the five cans of bulk starter were incubated for 14 to 16 hours at 70 to 72°F., the cultures then were mixed using equal parts of each culture just before adding

TABLE 20

Starter rotational system consisting of 16 mother cultures

Cultures	Day of the month															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
H1, F1, M1, D1	x	x							x	x						
H2, F2, M2, D2			x	x							x	x				
H3, F3, M3, D3					x	x							x	x		
H4, F4, M4, D4							x	x							x	x

to the vats. This procedure again has helped to eliminate the possibility of all but one or two strains of lactic acid producing organisms from being stopped by phage. The insurance of such a program has proven to be very successful. If only one culture per day is used and it is lysed by bacteriophage, the possibility of recovery in the vat in time to save the product is very remote. Secondary growth of a culture after lysis by phage is too slow for cheesemaking, while a second strain in a culture will often carry the full load, even though acid development is partially retarded.

From the evidence presented, it appears that a rotational system, based on phage tests with indicator strains, has considerable possibility of practical application. In large operations this might be the most accurate and sensitive of the procedures presented. However, due to equipment limitations, it is largely impractical for the smaller plant. Rotation of starter cultures on a regular basis, without phage tests, has been shown to be very desirable. This system also is very practical for all dairy plants, regardless of size, and is one means by which smaller plants can cope with the bacteriophage problem.

TABLE 23

Starter rotational system consisting of 9 mother cultures

Cultures	Day of the month																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
D1, H1	x	x																
D2, F1			x	x														
D3, M1					x	x												
D1, H2							x	x										
D2, F2									x	x								
D3, M2											x	x						
D1, M1													x	x				
D2, H1															x	x		
D3, F1																	x	x
D1, M2																		
D2, H2																		
D3, F2																		

SUMMARY AND CONCLUSIONS

A survey was conducted to determine the causative agent for inactivity of lactic starter cultures employed in the dairy industry. The primary agent was found to be bacteriophage, the incidence of which was greater than anticipated. Eighty-seven whey samples from 32 dairy plants were examined and of these, 40 samples, representing 27 plants, contained inhibitory substances definitely established as bacteriophage. Two indicator strains of S. cremoris, 144F and W, were inhibited 25 and 31 times, respectively, during the survey. Of the 18 cultures employed, only two were not inhibited during the entire survey. These two were cultures K2 (S. lactis) and R6 (S. cremoris).

As expected, S. lactis strains were attacked less frequently than S. cremoris. Certain purified races of bacteriophage isolated from filtrates were capable of attacking both S. lactis and S. cremoris.

Methods of acquiring bacteriophage-resistant strains of lactic streptococci were presented. More definite phage-resistance was obtained with culture LH3 by the plating technique, as compared to the serial transfer technique. One hundred percent phage-resistance was obtained by both procedures with culture R6. However, these data also indicate the superiority of the plating and isolation technique.

Data suggest that phage was removed sooner after

exposure, by the plating technique, and retained the longest in the serial transfer technique. The elimination of phage in the plating technique may be due to pure mathematical chance.

Results indicate that selected resistant strains may have significantly different rates of acid production than sensitive parent strains. Of exceptional interest are results which show resistant strains of 144F to be more active than sensitive strains in activity tests at 37.5°C., whereas the reverse was true at 21°C. Activity of resistant mutant strain IS was significantly lower than the sensitive parent at both temperatures. Activity tests at 37.5°C. indicate R6 sensitive and R6 resistant strains to be about the same, while 21°C. activity tests for the same strains show a great difference, the sensitive strain being far more active than the resistant mutant. There were no great differences in activity of phage-sensitive and phage-resistant cultures 11E and W based on activity tests at 21 and 37.5°C.

There was very little apparent difference between the serially exposed (step 1) and the plated isolates (step 2) of all five cultures. Culture R6 showed the greatest difference at 21°C. In this instance, R6 of step 1 produced 0.09 of one percent less acid than in step 2.

With careful repeated plating and isolation technique, it should be theoretically possible to obtain a completely phage-resistant, active culture.

Experimental studies were carried on with the cooperation of seven dairy plants in the state of Oregon. The types of products in which starter cultures were used included cottage cheese, cultured buttermilk, cheddar cheese, and blue vein cheese. A total of 51 cultures, either phage-sensitive or phage-resistant strains, were carried during this phase of the study.

A total of 82 whey samples were analyzed for inhibitory agents against starter cultures. Fifty-two of these samples were found to contain heat labile inhibitory substances against one or more of 51 cultures sent out to commercial dairy plants. It was concluded, on the basis of previous observations, that most of the 52 samples owed their inhibitory effect to bacteriophage.

From observations and data presented, it was concluded that it is actually possible by phage tests to predict the liability to phage attack of a culture before its employment in a dairy plant. In all instances where a build-up of phage was shown by the indicator strains, subsequent inactivity of the culture resulted. In plant 2, using cultures 26 and 301, the vat cultures were completely inactive within two days after effects of build-up of phage were shown by the indicator strains.

Observations indicated that when working with phage-resistant lactic streptococci, subsequent inactivity of mutant resistant bacterial strains may occur due to mutation

of the original phage, rather than to reversion to a sensitive state by the organism.

From actual data, and plant experience over a period of about three years, it was determined that rotational systems for both phage-sensitive and phage-resistant cultures have practical application. It appears that a rotational system, based on phage tests with indicator strains, has practical application. In large operations this might be the most accurate and sensitive of the procedures presented. However, due to the equipment limitations, it is largely impractical for the smaller plant.

Rotation of starter cultures on a regular basis, without phage tests, has been shown to be very desirable and also very practical for all dairy plants, regardless of size, and is of special application to smaller plants.

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