ENUMERATION OF BACTERIOPHAGE AND TYPING OF BACTERIOPHAGE AND LACTIC STREPTOCOCCI FROM LACTIC CULTURES

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INTRODUCTION

Dairy plants making cultured dairy products almost invariably experience trouble with slow acid production by lactic cultures due to bacteriophage infection. In some plants the trouble occurs only rarely, while in others slow cultures are almost a constant problem. It has been established that bacteriophage races vary in their abilities to attack different strains of lactic streptococci. Practical use is made of this information in that many plants carry several strains of cultures and use them in rotation in an attempt to avoid a build-up of a bacteriophage population sufficient to cause lysis of their cultures.

There is need for more information on the number of different strains of lactic streptococci and of races of bacteriophage. There is also need for more information on the accuracy of methods currently used for the enumeration of bacteriophage particles.

The work herein reported was undertaken in an effort to (1) compare the accuracy of the plaque count method with most probable numbers for enumerating bacteriophage, (2) determine the species of lactic streptococci predominant in lactic cultures, (3) determine the strains of lactic

streptococci by bacteriophage typing, and (4) determine the races of bacteriophage on the basis of their abilities to lyse cultures of lactic streptococci.

REVIEW OF LITERATURE

The name bacteriophage was given by d'Herelle (8) to bacterial viruses. Parmelee et al. (23) reported that the bacteriophage which attacked Streptococcus lactis was sperm shaped, 220 millimicrons in length with a head diameter of 70 millimicrons and a tail of 30 millimicrons wide and 150 millimicrons long. The term bacteriophage is commonly shortened to "phage."

Many workers have shown that bacteriophage lysis of starter cultures organisms was responsible for slowness in the manufacture of butter and cheese (3, 4, 11, 18, 19, 26, 27, 28).

Turner (26) reported that the bacteriophage particle was absorbed on the susceptible host. It penetrated into the host and multiplied. The bacteriophage particles were then liberated. He studied five bacteriophage strains. They proliferated by stepwise increase. The burst-time was 65 minutes and average burst size about 90 countable particles. The proliferation curve of each of five bacteriophages consisted of lag, logarithmic, and resting phases. Bacterial multiplication was stopped when there was more than one particle per cell. The bacteriophage continued to increase rapidly until the host cells were lysed. The

time required for mass lysis was shortest at 32°C (90°F). Secondary growth took place subsequent to mass lysis in most strains. Cultures incubated at 21°C (70°F) after a light infection showed little or no effect of bacteriophage action, while these same cultures placed at 30° - 32°C (86° - 90°F) in the cheese vat would undergo mass lysis. The optimum temperature for the multiplication of bacteria and bacteriophage was found to be 32°C (90°F).

Hunter (13) reported that in certain instances where several bacteriophage races were known to be present in the starter, one race was able to establish itself to the exclusion of the others. The presence of one race may interfere with the growth of another. When different bacterial viruses were grown on the same host, one cell liberated the virus of one type only. A bacteriophage growing in association with a partially resistant strain of Streptococcus protected the cultures from the attack by other bacteriophage races. The absorption and blocking effects were selective.

Nelson et al. (18) found that the strains of S. lactis isolated from milk or whey that had soured naturally were not inhibited by any of the available bacteria-free filtrates containing bacteriophages which were active against other organisms of this group. However, they stated that strains of bacteriophage which were active against these non-sensitive strains of S. lactis might be found if a larger number of bacteriophage strains from a greater range of sources was used.

Zehren and Whitehead (28) found that the concentration of bacteriophage in whey depended on the latent period and on the burst size, which were both characteristics of bacteriophage races.

Collins (5) stated that bacteriophage present in large numbers inhibited heterologous bacteria in the absence of host bacteria. The homolgous bacteria which was considered necessary for the occurrence of nascent phenomenon was necessary only for increasing the number of bacteriophage particles to the point where most of the heterologous bacteria would be attacked.

Kleczkowska (14) reported that bacteriophage action was indicated on solid media by formation of plaques and in broth by a clearing of turbidity. Nichols and Wolf (20) found that the choice of test medium and the establishment of a standard test for detecting bacteriophage action were difficult but observed that milk was better than broth for detecting lysis. They attempted, by means of bacteriophage affinities, to establish or identify relationships of strains from commercial starters. They stated that the bacteriophage tests had helped to unravel the relationships between many commercial starters and to confirm the identity of some. They also reported that the component strains of a commercial starter were not always lysed by the same bacteriophages. Not all strains isolated from the same starter gave identical reactions against any one bacteriophage.

The cross reactions of any one starter could not be judged by that of any one of its strains and the greater the number of strains examined the more representative those reactions would be for any particular starter. Commercial starters were found to be composed of strains or groups of strains showing distinct reactions. There also were some cross reactions between the strains of a number of starters from commercial sources.

Pette (22) indicated the weak points in the present methods of enumerating and typing the bacteriophage of lactic streptococci. He observed the possibility of using latent period or the burst size. Only slight variations were found in the latent periods of various bacteriophage races but striking differences were found in burst sizes of some races.

Foster, et al. (11) suggested the use of the limiting dilution technique for determining bacteriophage titer. They suggested using 5 tubes of medium inoculated with the host culture for each decimal dilution of bacteriophage filtrate and observing for retardation of growth after incubating 18-20 hours at 30° to 32°C (86°-90°F). The most probable number of bacteriophage particles could then be determined from a probability table.

Whitehead and Hunter (27) suggested the use of starter strains of streptococci unrelated in their bacteriophage affinities to control "slowness" in cheese making. They

found it difficult to secure more than a few such strains which were active for cheese making.

The work of Anderson and Meanwell (3) first led to the idea of rotational use of starters. They used several mixed culture starters daily and immediately discontinued the use of any starter for which a dangerous concentration of bacteriophage was detected in the factory whey. Later, they found that they could safely reintroduce into the factory, after an interval of several days, a starter which had previously been lysing.

Nichols and Hoyle (19) stated that, to control slowness in a cheese factory, information regarding bacteriophage typing, adaptation of bacteriophage and bacteriophage carrying in the selection of starter strains were significant. They identified 11 bacteriophage types of lactic streptococci. Identification of type had been based on the reaction of bacteriophage pattern and not on the reaction of a single race. The bacteriophages used in typing had been classified by means of antiphage sera. The majority of the bacteriophage races were divided into three groups. Type X was an example of a collection of strains of S. lactis where bacteriophage organism relationships were relatively simple. Bacteriophage C_8 completely lysed all strains of the type and this reaction was considered determinative. Some of the constituent strains were also lysed by two other test bacteriophages, C_{31} and t_8, but

because C_8 lysed all these and no other strains in the collection, the strains were placed together to form one bacteriophage type.

Babel (4) attempted to control slow acid production in cheese manufacture by the use of cultures that were not sensitive to the bacteriophage types. He recovered four distinct types of bacteriophages from a sample of whey taken from a vat in which a multiple strain culture was used. Ten bacteriophage types which were active against one or more of 15 cultures of S. lactis were isolated from various sources. These types were differentiated by means of their bacteriophage activity patterns. One culture was not sensitive to any bacteriophage, one was sensitive to only one type, one to two types, and one culture was sensitive to three types of bacteriophage. The remainder of the cultures were sensitive to 5 to 7 phages.

Several investigators have reported on the typing of streptococci by means of serological reactions. Evans (9) studied four seriological types of streptococcus bacteriophage and observed that the only characteristic which clearly differentiated them was behavior in the cross seriological reaction. The four races of bacteriophage in the nascent stage were examined for their abilities to attack 421 strains of hemolytic streptococci. The four bacteriophages respectively lysed 89.3%, 88.4%, 79.3% and 9.7% of the strains of hemolytic streptococci.

Collins (6) isolated 37 strains of S. cremoris from 14 cultures and 37 homologous strains of bacteriophage from wheys. On the basis of cross reactions with the S. cremoris, the bacteriophages were separated into 10 different groups.

Fisk (10) demonstrated lysogenic strains among the staphylococci by spotting the potential lysogenic cultures in high concentration on an indicator strain streaked on agar. He used numerous staphylococcal bacteriophages isolated from cultures of Staphylococcus aureus to differentiate the strains of organism, but he did not enumerate the particles or estimate quantitative effects.

Takagaki (25) studied 67 strains of coagulase positive staphylococci from bovine milk and 41 were bacteriophage typed using 13 bacteriophages of Nakagawa (17). Of these, 21 were classified as group A and 20 were group B. He used 20 bacteriophages from the National Type Culture Collection and typed 24 strains. Of these bacteriophages, 14 were of group IV and the remainder belonged to 4 different phage groups. Nakagawa (17) studied 375 strains of coagulase positive staphylococci isolated from milk and found that 46.5% were typable and belonged to types III or IV.

Malik and Singh (15) bacteriophage typed 36 coagulase positive and 17 coagulase negative staphylococci from different sources against 10 standard bacteriophages. These strains were grouped into 4 different bacteriophage types. Gulotti and Spano (12) studied 125 strains of

Staphylococcus aureus from various sources. Thirty-eight cultures isolated from foods were bacteriophage type III, one was type IV, 6 were type M, and 20 were untypable. The bacteriophage pattern of 52 staphylococcal strains from 13 food-borne outbreaks were also studied and most of them were found to be type III.

EXPERIMENTAL METHODS

A. Routine Propagation of Lactic Cultures

Litmus milk was dispensed into screw capped test tubes in approximately 10 ml quantities. The tubes were autoclaved for 10 minutes at 15 pounds pressure. The tubes were then tempered at 72°F inoculated with 0.1 ml (approximately 1%) of the lactic culture and incubated at 72°F for 16 hours. The cultures were then held at 45°F.

B. Activity Test

The activities or acid producing abilities of the cultures were determined with the Four Hour activity test (16). Briefly the test was as follows: Reconstituted non-fat dry milk was prepared by dispersing 10g of the solids in 100 ml distilled water. This was then filtered to remove any undissolved particles. Measured 9 ml quantities were dispensed in rubber stoppered test tubes (20 x 125 mm). The milk was then heated in flowing steam for 30 minutes and cooled by placing in a refrigerated room at 45°F. In conducting the test, duplicate tubes were each inoculated with 0.5 ml of cultures that had been transferred 14 to 18 hours previously. The innoculated tubes were tempered in a water bath at 100°F for 5 minutes and then placed in an

incubator at 90°F for 4 hours. After incubation, the tubes were cooled in ice water and then titrated with 0.10N NaOH, using phenolphthalein as the indicator, (1 drop of 10% solution). The results were recorded as the ml of 0.10N NaOH required to neutralize the entire contents of each tube.

C. Isolation of Pure Cultures

Pure cultures of lactic streptococci were isolated by streaking commercial lactic cultures on agar plates, incubating at 72-80°F and picking typical colonies into litmus milk. Several media were tested for their abilities to support the growth of the lactic streptococci. The medium finally selected as being most satisfactory consisted of yeast extract 0.125%, tryptone 0.25%, brain-heart infusion (dehydrated) 0.5%, tryptose 0.5%, sodium chloride 0.25%, lactose 0.5%, and agar 1.5%. One ml of 1% brom cresol purple per liter was added as an indicator of acid production to detect the acid producing lactic streptococci. One drop of a 10% suspension of calcium carbonate was also added to each plate to limit diffusion of acid produced by the colonies.

D. Differentiation of the Species of Lactic Streptococci

The tests to distinguish Streptococcus lactis from Streptococcus cremoris were based on the ability of S. lactis to grow at 40°C, in 4% sodium chloride, at pH 9.2, in 0.3% methylene blue, and its ability to produce ammonia from arginine. S. cremoris was negative to these tests.

A broth medium was prepared for use in testing tolerances for salt and high pH. The medium consisted of tryptone 0.5%, yeast extract 0.25% and dextrose 1.0%. These materials were dissolved in distilled water and 1.6 ml of a 1.0% alcoholic solution of brom cresol purple added per liter as an indicator of acid production. To portions of this basic medium, calculated amounts of sodium chloride (C.P.), were added to obtain concentrations of 3.0%, 4.0% and 5.0% of the salt. Different levels of pH were achieved by adding 0.1 ml portions of 40% NaOH to 100 ml of the broth in a beaker on a magnetic stirring device and with glass electrodes attached to a pH meter immersed therein. From the titration curve obtained, the amount of 40% NaOH required to give final pH values of 8.8, 9.0, 9.2 and 9.4 were calculated and added to portions of the broth. The various lots containing salt and at different pH levels were then tubed and sterilized.

The tubes were inoculated with 1 drop each of a pure culture of lactic streptococcus, incubated at room temperature (about 80°F) and observed for evidence of growth by turbidity and/or acid production.

In testing for tolerances for methylene blue, a 4.5% aqueous methylene blue solution was prepared and sterilized. Reconstituted milk was also prepared, divided into three portions and heated in flowing steam for 30 minutes. Measured quantities of the 4.5% methylene blue solution were added to

the heated and cooled milk to give concentration of 0.1%, 0.2% and 0.3% methylene blue, respectively. These lots of milk were then dispensed into sterile test tubes. The tubes were then inoculated with 1 drop each of a pure culture of lactic streptococcus, incubated at room temperature (about 80°F) and observed for reduction of methylene blue as evidence of growth. Arginine medium was prepared according to the formula suggested by Niven et al. (21). The medium consisted of 0.5% yeast extract, 0.5% tryptone, 0.2% potassium dihydrogen phosphate, 0.05% glucose, and 0.3% d-arginine monochloride. After adjusting the pH to 7.0, the medium was placed in test tubes and then sterilized by autoclaving at 15 lbs pressure for 10 minutes. Each culture was inoculated into a tube of the sterile medium and incubated for 2 days at 37°C. Presence of ammonia was detected by addition of 1 drop of Nessler's reagent to several drops of the incubated culture on a spot plate. S. lactis gave a positive test and S. cremoris negative.

S. diacetilactis, which is similar to S. lactis in many respects, was distinguished by its ability to produce considerable amounts of acetyl methyl carbinol and biacetyl when propagated in milk, whereas, S. lactis and S. cremoris produce none or very little of these compounds. The test used to detect the presence of these flavor compounds involved the addition of 4 drops of 1% aqueous creatine hydrate solution and 1 ml of 40% sodium hydroxide to 2 ml of culture, shaking vigorously and noting the intensity of red coloration after about 30 minutes.

E. Enumeration of Bacteriophage Particles

1. Most Probable Numbers Method. The most probable numbers of bacteriophage particles per ml were determined by a modification of the method for coliforms (2). Four decimal dilutions of a bacteriophage filtrate were added to 5 tubes each of litmus milk and the tubes then inoculated with 1 drop of a 1-2,000 dilution of a sensitive strain of lactic streptococcus. This resulted in populations of lactic streptococci of less than 3,000 per ml in the tubes as determined by plate counts and calculations. The tubes were incubated at 90°F and those that failed to coagulate were considered positive to bacteriophage. The tubes that coagulated were again transferred by inoculating one drop into about 10 ml of litmus milk and observing the time required for coagulation at 90°F as compared with a control with no bacteriophage added. A table of most probable numbers was used to determine the count (2).

2. Plaque Counts. A modification of the method of Nichols and Wolf (20) was used to determine bacteriophage particles by the plaque count method. In this method, a soft agar overlay was inoculated with a sensitive strain of S. lactis and a measured portion of diluted bacteriophage filtrate. This was poured over a layer of agar in a plate, incubated in an upright position at 90°F for 24 hours and the plaques then counted. The overlay was prepared by adding to a sterile test tube 0.1 ml sterile 4.0% $CaCl_2$, 1.0 ml

weak Plate Count Agar (1.0g per 100 ml), 1.0 ml bacteriophage filtrate dilution and 0.1 ml fresh lactic culture. The base agar or underlay was trypticase soy agar (Baltimore Biological Laboratories). A thermostatically controlled water bath set at 115°F was used for holding the tubes while preparing the overlay.

F. Bacteriophage Typing

1. Preparation of Bacteriophage Filtrates. These were prepared by growing a lactic culture in contact with bacteriophage from whey, slow butter milk or other material containing bacteriophage, then filtering through a Seitz bacterial filter to remove the organism. The lactic culture was inoculated into about 100 ml of litmus milk and allowed to incubate at 90°F until the litmus began to reduce, which was evidence of a high bacterial population. The bacteriophage inoculum was then introduced and allowed to act on the culture for at least two hours. If the culture failed to coagulate, sterile 50% citric acid was added dropwise until coagulation occurred. The culture was then filtered through filter paper and the whey filtered through a Seitz filter into sterile vials or test tubes containing about 0.25 g $CaCO_3$ to neutralize the acid.

2. Purification of Bacteriophage Races. Dilutions of bacteriophage filtrate were plated in the manner described for plaque counts. Distinct and discrete plaques were inoculated

into litmus milk tubes along with the homologous host culture. After incubation to determine that lysis had occurred, a filtrate of the pure races was prepared as above.

3. Determination of Bacteriophage Sensitivities of Lactic Cultures. The lactic cultures and bacteriophage filtrates were tested against each other to determine the strains and races, respectively. The lactic culture and bacteriophage filtrates were inoculated at the rate of 1 drop each into about 8 ml of litmus milk in a test tube and observed for time required to coagulate compared to a control culture with no bacteriophage added. A culture was not considered sensitive to a bacteriophage unless it required at least 2 hours longer to coagulate at 80-90°F than the control culture.

RESULTS AND DISCUSSION

A. Identities of Lactic Streptococci from Commercial Cultures

Fifty lactic streptococci were isolated from 50 commercial cultures and classified into three species (Table I). Twenty-nine (58%) were S. lactis, 6 (12%) were S. cremoris and 15 (30%) were S. diacetilactis. Six additional cultures were identified as Leuconostoc species but were not included because of their primary function which is flavor production rather than acid production.

Since rapid acid production is of prime importance in cheese making, the pure cultures were classified into "fast" and "slow" categories. Fast cultures were those that had titration values of 4.0 ml or more when subjected to activity tests while slow cultures had values of less than 4.0 ml. The data (Table I) show that of the 29 cultures of S. lactis, 21 (72%) were fast and 8 (28%) were slow; the 6 S. cremoris included 4 (67%) fast and 2 (33%) slow, while of the 15 S. diacetilactis, 4 (27%) were fast and 11 (73%) were slow. Of the 35 cultures of S. lactis and S. cremoris, 25 (71%) were fast.

These data show that the majority of the lactic acid producing organisms in the lactic cultures were S. lactis

TABLE I

IDENTITIES OF LACTIC STREPTOCOCCI ISOLATED FROM COMMERCIAL CULTURES

	All Cultures		Fast Cultures		Slow Cultures	
	No	%	No	%	No	%
S. lactis	29	58	21	72	8	28
S. cremoris	6	12	4	67	2	33
S. diacetilactis	15	30	4	27	11	73
Total	50	100	29	58	21	42

and that S. cremoris occurred much less frequently. It was rather surprising to note that S. diacetilactis was the dominant species in many cultures (30%). It should be noted that 71% of the S. lactis and S. cremoris cultures were classified as fast cultures while only 27% of the S. diacetilactis were in this same category. Of the 29 cultures classified as fast, 24 had titration values of 6.0 ml or more which is considered to be the lowest limit for activity for use in cheese making. Of these cultures, 16 were S. lactis, 4 were S. cremoris and 4 S. diacetilactis.

It has been established that there is a symbiotic relationship between many of the strains of S. lactis and S. cremoris and the citric acid fermenters associated with them in good lactic cultures. Since the pure cultures were all isolated from commercial cultures that were considered to be fast enough for making cheese, the relatively high incidence of slow pure cultures was probably due to the absence of the stimulatory effect of the citric acid fermenters in these pure cultures.

B. Comparison of Plaque Counts and Most Probable Numbers for Enumerating Bacteriophage Particles

Numerous trials were conducted to compare plaque counts with most probable numbers. However, many of the trials resulted in failure because plaques did not develop on the plates. Several different agar media, including plate count, blood agar base, milk protein hydrolysate, brain-heart

infusion, tryptone-glucose extract, tomato, trypticase soy and antibiotic medium and combinations of several of these were used in an attempt to discover a medium on which plaque formation would regularly occur. The medium finally selected was trypticase soy agar for the base and weak plate count agar for the overlay.

The data in Table II include the plaque counts and most probable numbers obtained in 10 trials in which countable plaques were obtained. The table also includes the results obtained in 7 additional trials in which no distinct plaques were formed. In Trials 1-10 the plaque counts averaged 57×10^6 particles per ml while the most probably numbers from the first propagation averaged 25×10^6 and from the second propagation, 185×10^6.

The most probable numbers, as determined by the first propagation, appeared to be much too low as they were lower than the plaque counts in 7 out of the 10 trials and averaged less than half the counts obtained by the plaque method. Furthermore, the probable numbers from the first propagation were much lower in each of the trials than those obtained from the second propagation. The logarithmic average for the first propagation was 32×10^6 per ml compared to 227×10^6 for the second propagation.

The counts in Trials 4 to 8 inclusive, were made at the same time, using one bacteriophage race and 5 sensitive lactic cultures. Since all the counts were made from the

TABLE II

COMPARISON OF PLAQUE COUNTS WITH MOST PROBABLE NUMBERS

Trial No.	*S. lactis* No.	Bacteriophage filtrate No.	Bacteriophage particles per ml x 10^6		
			Plaque Counts	Most Probable Nos. First Propagation	Most Probable Nos. Second Propagation
1	76	MGT361	10.0	4.5	14.0
2	8	EW861	195.0	490.0	4900.0
3	102	EM861	0.23	1.3	2.2
4	8	EW861	250.0	20.0	170.0
5	22	EW861	157.0	230.0	330.0
6	76	EW861	70.0	45.0	330.0
7	100	EW861	200.0	20.0	490.0
8	102	EW861	240.0	130.0	320.0
9	8	EW861	250.0	33.0	330.0
10	22	PR16	24.0	2.0	330.0
11	76	A1059		4.5	14.0
12	8	EM861		700.0	1,100.0
13	8	EW861		330.0	2,400.0
14	8	PR16		23.0	79.0
15	22	PR16		29.0	330.0
16	100	PR16		46.0	490.0
17	102	PR16		13.0	490.0
Log. avg. Trials 1 - 10			57.0	25.0	185.0
Log. avg. all trials				32.0	227.0

same bottle of a decimal dilution of the bacteriophage filtrate, the counts should, theoretically, all be the same. The plaque counts ranged from 70 x 10^6 to 250 x 10^6 per ml. The most probably numbers from the first propagation ranged from 20 x 10^6 to 230 x 10^6 per ml while from the second propagation they ranged from 170 x 10^6 to 490 x 10^6 per ml. There appeared to be no close agreement between the plaque counts and most probable numbers for the individual cultures because culture number 8 with the highest plaque count (250 x 10^6) showed the lowest most probable numbers on both the first and second propagations (20 and 170 x 10^6, respectively). Furthermore, culture number 76 had the lowest plaque count (70 x 10^6) and next to the highest most probable number on the second propagation (330 x 10^6). In Trials 14 to 17 inclusive the most probable numbers were determined on a bacteriophage filtrate with 4 homologous cultures. On the first propagation, the most probable numbers ranged from 13 x 10^6 to 46 x 10^6 particles per ml, while on the second propagation they ranged from 79 x 10^6 to 490 x 10^6 per ml. Here again culture No. 8 had much lower values on the second propagation than those obtained with the other cultures.

The overall results indicate that the plaque counts and most probable numbers can be highly inaccurate, depending on the host culture used and perhaps other factors. It appeared that, in general, the most reliable results were

obtained with the second propagation for most probable numbers. Here the problem of secondary growth of resistant mutants could affect the results. This could possibly explain the low numbers obtained in Trials 4 and 14 in which culture number 8 was used.

The failure to obtain countable plaques in many trials and the rather inconsistent results obtained even when good plaque formation occurred emphasizes the need for improvement in methods for counting bacteriophage particles.

C. Typing of Bacteriophage Races and Lactic Streptococci

1. Pure Strains of Lactic Streptococci vs. Pure Bacteriophage Races. Each of 50 pure cultures of lactic streptococci isolated from commercial lactic cultures were tested to determine their susceptibilities to 5 pure races of bacteriophage isolated from whey from slow vats of cheese. The tests showed that 29 of the cultures were attacked by one or more of the races of bacteriophage. The results (Table III) showed that the five races appeared to be distinctly different and that they varied considerably in their abilities to attack the culture. Of the 29 cultures the number attacked by each of the races numbered 1 to 5 was 19, 15, 3, 7 and 9, respectively.

On the basis of their susceptibilities to the 5 pure races of bacteriophage, the 29 pure cultures of lactic streptococci appeared to belong to 12 different strains (Table IV). Strain I included 5 cultures; strain II, 9; strain III,

TABLE III

TYPING OF PURE RACES OF BACTERIOPHAGE WITH PURE CULTURES OF LACTIC STREPTOCOCCI

Bacteriophage Race		Cultures lysed	
No.	Identity	No.	Identities
1	MGT361	19	8,20,36,46,49,50,63,64,67,68,69,70,78,B2,E7,B8,B13,C9,T1
2	OSU660	15	8,20,36,39,46,49,50,63,64,67,68,69,70,78,B1
3	MGT661	3	36,65,C9
4	A1059	7	36,37,49,70,78,C9,T3
5	A1161	9	28,36,49,51,85,B1,B10,C9,T5

TABLE IV

TYPING OF PURE CULTURES OF LACTIC STREPTOCOCCI WITH PURE RACES OF BACTERIOPHAGE

Strain No.	Lactic Cultures No.	Identities	Susceptibility to Bacteriophage Races: MGT 361	OSU 660	MGT 661	A 1059	A 1161
			Numbers				
			1	2	3	4	5
I	5	B2,B7,B8,B13,T1	≠				
II	9	8,20,46,50,63,64,67,68,69	≠	≠			
III	2	70,78	≠	≠		≠	
IV	1	49	≠	≠		≠	≠
V	1	C9	≠		≠	≠	≠
VI	1	36	≠	≠	≠	≠	≠
VII	1	39		≠			
VIII	1	B1		≠			≠
IX	1	65			≠		
X	1	37				≠	
XI	1	T3				≠	≠
XII	4	28,51,85,B10					≠

2; strain XII, 4; and the remaining strains 1 culture each. Five strains, numbers I, VII, IX, X and XII were attacked by one race each; three strains, numbers II, VIII and XI by two races; one strain, number III by three races; two strains, numbers IV and V by four races; and one strain, number VI was attacked by all five races.

In addition to the 12 strains shown in Table IV, there was at least one or more strains represented among the 21 cultures that were not susceptible to any of the 5 pure bacteriophage races. The use of additional races of bacteriophage might have revealed the presence of more strains among the cultures.

2. Pure Strains of Lactic Streptococci vs. Bacteriophage Filtrates. Nineteen bacteriophage filtrates were tested for their abilities to attack pure strains of lactic streptococci. These filtrates were prepared from whey, slow cultures, and cheese milk from various dairy plants experiencing trouble with slow acid production. Some of the filtrates probably contained more than one bacteriophage race. The filtrates lysed 24 out of 56 pure lactic cultures. There appeared to be 13 races of bacteriophage (Table V). Race 1 lysed only 1 culture, races 2 and 3 lysed 3 each, races 4, 5 and 6 lysed 4 each, races 7, 8 and 9 lysed 5 each, race 10 lysed 11, race 11 lysed 15, race 12 lysed 18 and race 13 lysed 21 of the 24 pure lactic cultures. Races 3 and 7 included 2 filtrates each and race 6 included 5 filtrates while the remaining 10

TABLE V

TYPING OF BACTERIOPHAGE FILTRATES WITH PURE LACTIC STREPTOCOCCI

Race No.	Filtrate numbers	Cultures lysed: Number lysed	Identities
1	8	1	50
2	3	3	50,Mt1,Mt4
3	7,9	3	Mt1,Mt4,Mt5
4	1	4	50,51,Mt1,Mt4
5	6	4	50,Mt1,Mt4,Mt5
6	10,11,12, 17,19	4	50,51,T4,B1
7	2,4	5	50,51,Mt1,Mt4,Mt5
8	5	5	50,66,Mt1,Mt4,Mt5
9	18	5	50,51,T4,B13,B1
10	13	11	36A,46,49,50,50A,51,53,59,66,B1,T4
11	14	15	36A,37,46,48,49,50,50A,51,52,53,59,85,B1,B5,T4
12	15	18	8,36,36A,39,46,48,49,50,50A,51,52,53,59,63,66,B1, B5,T4
13	16	21	8,36,36A,37,39,46,48,49,50,50A,51,52,53,59,63,66, B1,B5,B13,T4,Mt4

races included 1 filtrate each. From these results it appeared that there are at least 13 races of bacteriophage and that some of the races attack very few strains of lactic streptococci, while others attack a great many strains.

On the basis of the bacteriophage sensitivity patterns (Table VI) there appeared to be 12 strains of lactic streptococci among the 24 cultures that were lysed by one or more of the bacteriophage filtrates plus at least 1 strain among the 32 cultures that were not attacked. Strain I included 4 cultures; strain II, 3; strain III, 6; strains VII and IX, 2 each and the remaining 7 strains 1 culture each. Strain VI was lysed by 1 filtrate, strains I, IV, and VIII by 2 each, strain II by 3, strains III and XII by 4, strain X by 6, strain IX by 9, strain VII by 10, strain XI by 13 and strain V by 17 of the 19 bacteriophage filtrates.

From the above results it appeared that there were at least 13 races of bacteriophage and at least 13 strains of lactic streptococci. This emphasizes the complexity of the problem of attempting to control slowness due to bacteriophage by using only a few strains of lactic streptococci. However, that fact that 32 of the 56 pure lactic streptococcus cultures were not lysed by any of the 19 filtrates indicates that control of bacteriophage may be attained by proper selection of strains and rotation of these strains in the dairy plants.

TABLE VI

TYPING OF PURE LACTIC STREPTOCOCCI WITH BACTERIOPHAGE FILTRATES

Strain No.	No.	Identities	Susceptible to Bacteriophage Filtrate Numbers
I	4	8,36,39,63	15,16
II	3	48,52,B5	14,15,16
III	6	36A,46,49,50A,53,59	13,14,15,16
IV	1	37	14,16
V	1	50	1,2,3,4,5,6,8,10,11,12,13,14,15,16,17,18,19
VI	1	85	14
VII	2	B1,T4	10,11,12,13,14,15,16,17,18,19
VIII	1	B13	16,18
IX	2	Mt1,Mt4	1,2,3,4,5,6,7,9,16
X	1	Mt5	2,4,5,6,7,9
XI	1	51	1,2,4,10,11,12,13,14,15,16,17,18,19
XII	1	66	5,13,15,16
XIII	0		

3. Commercial Cultures vs. Bacteriophage Filtrates. Ten selected bacteriophage filtrates which were strongly lytic to many pure cultures were tested to determine their abilities to cause lysis in each of 27 commercial lactic cultures. These cultures were all considered satisfactory for use in cultured dairy products. The results (Table VII) revealed that each of the filtrates had a different pattern of cultures lysed. This indicated that the filtrates represented 10 different races of bacteriophage. It was also noted that the individual filtrates were very active in that they attacked from 4 to 23 of the 27 lactic cultures.

On the basis of their lysis by the 10 bacteriophage filtrates, the 27 commercial cultures were grouped into 15 strains (Table VIII). Strains I and II were lysed by 1 filtrate each, strain III by 2, strains IV, V, VI, VII and VIII by 4, strains IX, X and XI by 5, strain XII by 6, strain XIII by 7, strain XIV by 9 and strain XV by all 10 bacteriophage filtrates.

The fact that several of the commercial cultures probably contained more than one strain of lactic streptococcus and that some of the filtrates probably contained more than one race of bacteriophage may account for the high attack rates on the commercial cultures as compared to those for the pure cultures (Table V).

Collins (5) showed that heterologous strains would be attacked if sufficient numbers of bacteriophage particles

TABLE VII

TYPING OF BACTERIOPHAGE FILTRATES
WITH COMMERCIAL CULTURES

Filtrate No.	Cultures Lysed	
	No.	Identities
1	12	7,9,10,12,19,20,21,22,27,33,34,35
2	17	1,3,4,6,7,9,10,12,17,19,20,21,22,27, 33,34,35
3	15	1,3,4,6,7,9,12,17,19,20,27,31,33,34,35
4	12	3,4,6,9,10,12,19,20,31,33,34,35
5	23	1,3,4,6,7,9,11,12,13,14,15,16,17,19,20, 21,22,23,24,31,33,34,35
6	4	6,17,19,20
7	7	12,17,20,21,33,34,35
8	7	11,19,20,27,33,34,35
9	13	1,3,4,6,7,9,11,12,19,20,33,34,35
10	15	7,9,10,11,12,13,19,20,25,27,30,31,33,34,35

TABLE VIII

TYPING OF COMMERCIAL CULTURES
WITH BACTERIOPHAGE FILTRATES

Strain No.	Filtrates Causing Lysis No.	Identities	Cultures Lysed No.	Identities
I	1	5	5	14,15,16,23,24
II	1	10	2	25,30
III	2	5,10	1	13
IV	4	1,2,5,7	2	21,22
V	4	2,3,5,9	1	1
VI	4	1,2,4,10	1	10
VII	4	5,8,9,10	1	11
VIII	4	3,4,5,10	1	31
IX	5	2,3,4,5,9	2	3,4
X	5	2,3,5,6,7	1	17
XI	5	1,2,3,8,10	1	27
XII	6	2,3,4,5,6,9	1	6
XIII	7	1,2,3,4,5,9, 10	3	7,9,12
XIV	9	1,2,3,4,5,7, 8,9,10	3	33,34,35
XV	10	1,2,3,4,5,6, 7,8,9,10	1	20

were present and that in a mixed culture the lysis of a homologous strain could produce sufficient bacteriophage particles to cause lysis of a now sensitive strain.

However, the typing of commercial cultures with whey filtrates represents the conditions that would probably prevail in dairy plant laboratories. Filtrate number 5, which lysed 23 out of the 27 commercial cultures, is an example of what trouble could occur in a plant carrying only one or two cultures. What could be accomplished by typing with whey filtrates and selection of the most resistant cultures is illustrated by strain I (Table VIII) which included 5 cultures that were lysed by filtrate number 5 only and strain II which included 2 cultures that were lysed by filtrate number 10 only. If a plant propagated one culture each of strains I and II he would apparently be safe from trouble with any of the 10 filtrates used in this experiment. On the other hand, if he had only cultures from strains XIV and XV, which were lysed by 9 and by 10 of the filtrates, respectively, he would probably be in constant trouble.

SUMMARY AND CONCLUSIONS

Pure cultures of lactic streptococci were isolated from commercial lactic cultures and identified as to species. Comparisons were made between plaque counts and most probable numbers for enumerating bacteriophage particles in bacteria-free whey filtrates.

Pure cultures of lactic streptococci were typed into strains by their susceptibilities to lysis by pure races and by whey filtrates of bacteriophage from various sources. Commercial lactic cultures were also typed by their susceptibilities to whey filtrates containing bacteriophage from various plants.

Pure races of bacteriophage and bacteriophage in whey filtrates from various plants were typed according to their affinities for pure cultures. Some of the whey filtrates were also typed with commercial lactic cultures.

Of 50 pure cultures of lactic streptococci isolated from commercial cultures, 58% were S. lactis, 12% S. cremoris and 30% S. diacetilactis. Even though the original cultures were considered to be fast enough for making cheese, only 71% of the 35 strains of S. lactis and S. cremoris and 27% of the S. diacetilactis were fast.

In 10 trials plaque counts averaged 57 x 10^6 bacteriophage particles per ml of whey filtrates while the averages for the most probable numbers were 25 x 10^6 for the first propagation and 185 x 10^6 for the second propagation. In 4 trials on one whey filtrate in which 4 pure cultures were used, the plaque counts ranged from 70 to 250 x 10^6 particles per ml while the most probable numbers from the first propagation ranged from 20 to 230 x 10^6 and from the second propagation from 170 to 490 x 10^6 per ml. These results indicated that the current methods for determining the numbers of bacteriophage particles appear to be unreliable. It appeared that the use of two propagations for the most probably numbers gave the most consistent results.

The typing of 50 pure cultures with 5 pure races of bacteriophage resulted in distinguishing 12 strains of lactic streptococci that were sensitive to one or more of the races and at least 1 more strain that was not sensitive to any of the races. The five races showed distinctly different patterns of lysis of the cultures.

Nineteen bacteriophage filtrates containing bacteriophage from various dairy plants were separated into 13 races of bacteriophage on the basis of their abilities to lyse pure cultures. The races varied greatly in their abilities to lyse cultures. Some of the races lysed only 1 culture while 1 race lysed 21 out of 24 cultures that were

sensitive to bacteriophage. The bacteriophage patterns indicated that there were 12 strains of lactic streptococci among the 24 cultures that were lysed and at least 1 more strain among the cultures that were not lysed by the bacteriophage in the whey filtrates.

All 27 commercial lactic cultures were lysed by from 1 to 10 of the bacteriophage races in whey filtrates against which they were tested. On the basis of their patterns of lysis by the filtrates, there appeared to be 15 strains of lactic streptococci among the cultures. However, since the some of the cultures probably contained multiple strains of lactic streptococci and since some of the whey filtrates probably contained multiple races of bacteriophage, the probability of cross reactions among some strains and races may have complicated the results. The typing of commercial cultures with whey filtrate from slow vats of cheese, slow cultures, etc. offers the plants an opportunity to select cultures resistant to the phage races that cause the trouble.

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