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Frequencies of Bacteriophage-Resistant and Slow Acid-Producing Variants of *Streptococcus cremoris*

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The frequencies of bacteriophage-resistant and slow acid-producing variants in 10 purified strains of *Streptococcus cremoris* were studied. There were considerable differences among the strains in the occurrence of both slow acid-producing and phage-resistant mutants. Nevertheless, the spontaneous rates of mutation to slow acid production were three to five orders of magnitude greater than the corresponding rates of mutation to phage resistance, suggesting that slow acid production and phage resistance are not genetically linked, although they appear in cultures concomitantly. The frequencies of slow acid-producing variants among resistant and sensitive isolates from the same parent culture were similar and appeared to be strain dependent. All phage-resistant mutants tested were found to be deficient in adsorption of the homologous bacteriophage.

Selection of bacteriophage-resistant mutants has been desired as a means of increasing the pool of phage-unrelated strains of lactic streptococci available for use in the manufacture of cultured dairy products (3, 9, 10, 18, 21, 29). Most isolated mutants lacked the rapid acid production of their parent cultures, reverted to sensitivity to the original bacteriophages, or were attacked by new bacteriophages. The instability of lactic streptococci with respect to both rate of acid production and bacteriophage specificity is well documented (2–4, 8, 24), but the possibility of a direct linkage between these two properties has not been confirmed. Lawrence (14) mentioned the difficulty of isolating phage-resistant mutants that possess the acid-producing activity of the parent and suggested that mutations altering phage receptor sites might interfere with proteinase and peptide transport systems also located at the cell surface. Lactose metabolism and proteinase activity in lactic streptococci are plasmid borne (1, 11, 19, 20), and whether these activities are genetically linked has not been resolved (12, 13, 15, 20, 23). Some proteinases coded by plasmids are associated with the cell walls of lactic streptococci (5–7, 22, 27), and if such proteinases or associated structures are involved in phage adsorption, their loss might result in changed phage sensitivity. We studied rates of accumulation of phage-resistant variants and slow acid-producing variants to provide evidence of whether these properties are genetically linked.

MATERIALS AND METHODS

Bacteria and bacteriophages. A total of 10 strains of *Streptococcus cremoris* that are used commercially were obtained from DPL Culture Service, San Francisco, Calif., purified by single-colony isolations, and propagated routinely at 22°C in sterile 10% reconstituted skim milk (RSM). Bacteriophages corresponding to the strains were isolated from cheese factory wheys designated as positive by DPL. Activity against the appropriate culture was first confirmed in litmus milk at 22°C. Subsequently, dilutions of the lysate were streaked on M 17 agar plates (26) spread with 0.1 ml of the corresponding culture, and the plates were incubated at 30°C. Plaques were selected and purified twice by restreaking on M 17 agar, and phage stocks were prepared by the method of Terzaghi and Sandine (26). Titers of the phage stocks, subsequently stored at 4°C, ranged from 10^6 to 10^9 PFU/ml.

Rates of acid production. The rates of acid production by bacterial isolates were determined in RSM incubated at 22°C. Freshly set cultures were inoculated (1%) into tubes of RSM (9 ml), and those that resulted in coagulation of the RSM in less than 18 h were designated as fast acid producers. Those that required 18 to 28 h were designated as slow acid producers. Any tubes in which RSM was not coagulated at 28 h were discarded.

Tests for phage sensitivity and adsorption. Cultures grown from selected colonies were tested for phage sensitivity in tubes of litmus milk that were inoculated with 1% freshly coagulated culture with and without 1% of the corresponding phage stock and that were incubated at 22°C. The absence of coagulation in the tube containing phage stock, with corresponding coagulation in the control tube, was taken to indicate that the isolate was sensitive to the phage. Simultaneous

coagulation of the litmus milk in both tubes was interpreted as indicating that the isolate was resistant to the phage.

Adsorption of bacteriophages by cultures grown from selected colonies was tested in M 17 broth by a method similar to that of Limsowtin and Terzaghi (16). Cultures were grown in the broth for 18 h at 22°C, and fresh broth was added to give a turbidity reading of 60 to 80 (equivalent to an absorbance at 580 nm of about 0.8) on a Klelt-Summerson colorimeter (filter no. 54). The corresponding phage stocks were diluted to contain about 5×10^4 PFU/ml and 0.02 M $CaCl_2$. Diluted phage stock (1 ml) was added to 1 ml of the broth culture to give a multiplicity of infection of 0.001. Exactly 15 min at 30°C was allowed for adsorption, the mixture was diluted with 98 ml of chilled 10% M 17 broth containing 0.02 M $CaCl_2$ and shaken, and 10 ml was passed through a syringe-mounted 25-mm membrane filter (0.2 μm) to remove adsorbed bacteriophage particles. The filtrate (0.2 ml) then was plated in duplicate on M 17 agar plates spread with the appropriate indicator bacterial strain, and the plates were incubated overnight at 30°C. Preliminary experiments revealed that with this method 90% or more of the bacteriophage particles are adsorbed by sensitive hosts.

RESULTS

Phage-resistant and slow acid-producing mutants of strain L. Strain L, the cells of which occur principally as diplococci, was repurified by single-colony isolation, and the resulting culture, grown overnight at 22°C, was used for inoculating two 1-liter quantities of RSM, one of which also received 20 ml of phage stock. Portions from each flask were immediately diluted and plated on M 17 agar. Plate counts were subsequently determined at 4-h intervals during incubation of the flasks at 22°C until milk coagulation. The plates were incubated at 25°C for 48 h and, subsequent to counting, various numbers of colonies were selected and inoculated into tubes of RSM (9 ml) as follows: 100 from the uninfected zero-time culture, 100 from the infected zero-time culture, 90 from the infected coagulated culture, and 72 from the infected coagulated culture after eight daily transfers (ca. 60 cell divisions). The tubes of inoculated RSM were incubated at 22°C until coagulation and then tested for activity, sensitivity to bacteriophage L, and adsorption of the bacteriophage.

Results indicated that the original culture contained about one phage-resistant mutant per 10^5 sensitive cells (Fig. 1) and that about 1% of the sensitive bacteria were slow acid producers (99 fast; 1 slow). The phage-resistant mutants grew at approximately the same rate as the sensitive bacteria (about 1.25 h per cell division) (Fig. 1). Most of the isolates from the infected zero-time culture were phage-resistant mutants (7 sensitive; 92 resistant). None of the 7 sensitive isolates and only 2 of the 92 resistant isolates were

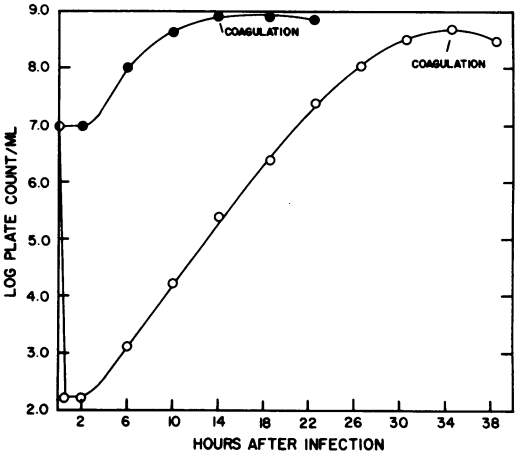

FIG. 1. Plate counts of *S. cremoris* strain L grown at 22°C in RSM with (○) and without (●) added bacteriophage.

slow acid producers. All of the 90 isolates from the infected coagulated culture and all of the 72 isolates from the infected coagulated culture after eight daily transfers were phage resistant. Only two were slow acid producers. The fast and slow acid-producing sensitive isolates adsorbed bacteriophage L (adsorption percentages, ≥90%), but neither the fast nor the slow acid-producing resistant isolates adsorbed the phage (adsorption percentages, <30%).

Phage resistance and slow acid production in other strains. The possible correlation between phage resistance and slow acid production in the 10 *S. cremoris* strains that differed in phage sensitivity was examined with a more rapid technique. The strains were grown in RSM, blended at high speed for 1 min to promote homogeneity of chain length, and plated on M 17 agar with and without the addition of their respective phage stocks (0.1 ml). The plates were incubated at 30°C for 48 h, colonies were counted, and plate infection survivor frequencies were determined. Subsequent to counting, 12 colonies from each plate were inoculated into tubes of RSM. After coagulation, each isolate from an infected plate was purified twice by colony isolation to free it from the bacteriophage and retested to ensure its resistance. Both sensitive and resistant isolates were tested for rate of acid production. The 10 original *S. cremoris* cultures were transferred daily in RSM at 22°C to allow approximately 150 generations and again tested for phage-resistant and slow acid-producing mutants.

The occurrence of ghost colonies on infected plates of some strains made enumeration of resistant colonies difficult. However, random

picking and study of thin, transparent colonies indicated that they were phage-resistant variants. Despite difficulties in enumerating the survivors, the daily propagations resulted in increases in the frequencies of phage-resistant mutants for six of nine strains (Table 1). Simultaneously, the frequencies of slow acid production for the phage-resistant mutants were quite similar to those for the corresponding phage-sensitive isolates and appeared to be strain dependent. With strains 12 and 72 as possible exceptions, equilibriums between fast and slow variants apparently had been reached, and the proportions of slow variants did not change appreciably during propagation of the original cultures for the approximately 150 generations.

Minimal spontaneous mutation rates. Freshly set cultures of the above strains were blended at high speed for 1 min and diluted serially (five tubes per dilution) in RSM, and the tubes were incubated at 22°C until those tubes that contained organisms showed coagulation. Based on the assumption that positive cultures at the limiting dilution had originated from single CFU, the approximate number of cell divisions required for each of the cultures to coagulate RSM was calculated. The most dilute positive tube for each strain then was propagated daily in RSM to permit approximately 145 generations from the assumed isogenic (single-cell, fast and phage-sensitive) state. Subsequently, the plate infection experiment was performed to determine the frequencies of phage-resistant variants, and 24 clones were picked from each selected plate to determine the frequencies of slow acid production. The mutation rates per generation were calculated by dividing the frequencies of phage resistance and slow acid production by the approximate number of generations from the isogenic state. The calculated rates are reported as minimal mutation rates, because reverse mutations were not determined. We assumed that increases in the frequencies of phage-resistant and slow acid-producing variants would depend on the occurrence of new mutations at constant rates throughout the measured period and that the slow and fast variants in each culture would grow symbiotically and at the same rate until they were separated by plating.

The results presented in Table 2 show that there were large differences among the 10 strains in rates of mutation to phage resistance and that the rates of mutation to phage resistance were considerably lower than the rates of mutation to slow acid production. There also were considerable differences among the strains in the incidence of slow acid-producing variants, but our values for the strains in which slow acid-producing variants were detected fall well within the potential equilibrium range of 1 to 95% reported in previous studies (24, 28).

DISCUSSION

Concomitant accumulation of spontaneous slow acid-producing and phage-resistant variants occurred in each of 10 *S. cremoris* strains. Cultures derived from theoretically isogenic CFU increased in heterogeneity with respect to both characteristics during routine propagations. However, we detected no direct correlation between phage resistance and slow acid

TABLE 1. Frequency of slow acid production[a] among phage-sensitive (preinfection) and phage-resistant (postinfection) clones of 10 different *S. cremoris* strains.

Strain	Zero-time cultures			Cultures after 150 generations		
	Plate infection survivor frequency	No. of slow acid-producing clones[b]		Plate infection survivor frequency	No. of slow acid-producing clones[b]	
		Pre-infection	Post-infection		Pre-infection	Post-infection
12	6.1×10^{-7}	5	7	2.7×10^{-6}	8	5[c]
72	1.5×10^{-5}	5	5	2.4×10^{-5}	8	12
L	2.7×10^{-6}	1	0	8.9×10^{-6}	0	0
70	7.3×10^{-6}	0	2	1.1×10^{-4}	0	0
66	3.5×10^{-6}	0	2[d]	1.3×10^{-5}	0	0
13	2.9×10^{-5}	1	0	2.8×10^{-5}	0	0
P	8.1×10^{-4}	0	0		0	0
V	1.6×10^{-5}	10	12	2.5×10^{-5}	9	10
29	2.6×10^{-5}	0	1	1.7×10^{-5}	0	0
16	1.8×10^{-4}	5	4	3.7×10^{-5}	5	1[e]

[a] Slow acid production was defined as the failure of a 1% inoculum to coagulate RSM in <18 h at 22°C.
[b] Unless otherwise noted, 12 colonies from each plate were tested.
[c] A total of 11 colonies were tested.
[d] A total of 10 colonies were tested.
[e] A total of 6 colonies were tested.

TABLE 2. Minimal spontaneous mutation rates to phage resistance and slow acid production in 10 different *S. cremoris* strains

Strain	Frequency of occurrence after 145 generations from the isogenic state		Minimal mutation rate per generation	
	Phage-resistant variants	Slow acid-producing variants[a]	To phage resistance	To slow acid production
12	2.9×10^{-6}	3.75×10^{-1}	2.0×10^{-8}	2.6×10^{-3}
72	7.8×10^{-6}	$<4.20 \times 10^{-2}$	5.4×10^{-8}	
L	9.3×10^{-6}	$<4.20 \times 10^{-2}$	6.4×10^{-8}	
70	1.0×10^{-5}	1.67×10^{-1}	6.9×10^{-8}	1.2×10^{-3}
66	1.1×10^{-5}	1.67×10^{-1}	7.6×10^{-8}	1.2×10^{-3}
13	1.5×10^{-5}	1.67×10^{-1}	1.00×10^{-7}	1.2×10^{-3}
P	2.1×10^{-5}	$<4.20 \times 10^{-2}$	1.45×10^{-7}	
V	2.3×10^{-5}	7.60×10^{-1}	1.59×10^{-7}	5.2×10^{-3}
29	6.2×10^{-5}	$<4.20 \times 10^{-2}$	4.28×10^{-7}	
16	3.1×10^{-4}	7.60×10^{-1}	2.13×10^{-6}	5.2×10^{-3}

[a] A total of 24 clones were examined for each strain.

production in any of the strains. Phage-resistant variants selected at random exhibited about the same proportions of slow acid-producing variants as did phage-sensitive parent cultures.

Large differences were found between the rates of accumulation of phage-resistant variants and those of slow acid-producing variants. For those six strains in which slow acid-producing variants were detected (Table 2), the rates of accumulation of slow acid-producing variants were three to five orders of magnitude greater than the rates of accumulation of phage-resistant variants. The actual differences in rates of accumulation might have been even greater, because it is possible that the slow variants grew more slowly than the fast variants before separation. It previously has been shown that the loss of plasmids coding for proteinases or lactose-utilizing enzymes may be the underlying mechanism that results in the loss of fast acid-producing ability (1, 11–13, 19, 20, 23, 25), and it consequently is possible that proteinase activity was impaired in the slow acid-producing isolates we studied. Nevertheless, the rates of accumulation of phage-resistant variants seem more typical of mutation frequencies for classical loss of function by point or frameshift mutation, and the substantial differences between rates of accumulation of slow acid-producing variants and those of phage-resistant variants are indicative of different underlying genetic mechanisms. This conclusion is supported also by our finding that many phage-resistant mutants produced acid as rapidly as corresponding phage-sensitive isolates.

The physical mechanism of phage resistance was found to involve changed adsorption properties in the variants tested. The altered receptor sites responsible for changed adsorption apparently did not affect the transport or proteinase systems required for fast acid production. Conversely, the loss of fast acid-producing ability occurred at high levels in a number of the strains studied, without any change in sensitivities to the homologous phages.

Our results show that cultures containing up to 76% slow acid-producing variants still can meet the criteria of Thomas and Lowrie (28) for "fast-coagulating" starters. This may be because wild-type cells hydrolyze sufficient protein in milk to support the needs of slow acid-producing variants for organic nitrogen and amino acids (25). Thus, a minority of fast acid-producing cells may stimulate the fast growth of a culture that is predominantly slow in nature. In studies of various commercial mixed cultures, Thomas and Lowrie (28) found that only 10 to 20% of the colonies they isolated from routinely propagated cultures coagulated milk as rapidly as did colonies from their corresponding parent cultures. This suggests that one reason for difficulties in obtaining fast acid-producing, phage-resistant variants from some fast acid-producing strains of lactic streptococci is that the strains actually are composed largely of slow acid-producing variants. Another possibility is that some resistant cultures grown from isolates may contain both resistant and sensitive cells and a bacteriophage that continuously lyses the sensitive cells as they are produced. It has been reported that some phage-resistant mutants still carry the bacteriophages to which they were originally sensitive, even after repeated single-colony isolations (17).

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