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Grouping of Lactic Streptococci by Gel Electrophoresis of Soluble Cell Extracts

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Soluble cell proteins obtained from 35 strains of lactic streptococci were examined by gel electrophoresis. A mathematical analysis of the densitometer scans of the gels enabled strains to be grouped according to their overall similarity. Strains which were known to be variants of the same parent strain fell into the same group, supporting the validity of the method. It is suggested that strains which are alike according to their gel electrophoretic patterns when grown under standard conditions have an overall phenotypic similarity and that this indicates a similarity in genotype. The relevance of this to selection of strains of lactic streptococci for cheesemaking is discussed.

In classical taxonomy, organisms are differentiated according to one or a few characteristics. At the other extreme, differentiation may be based on determinations made at the genetic level, such as by estimations of base ratios or deoxyribonucleic acid (DNA)-DNA homology. Another method of classification, numerical taxonomy (10), relies upon arithmetical manipulations to classify organisms on the basis of their overall phenotypic similarity to one another. Organisms are clustered into groups according to a large number of properties, with the assumption that the characters used are representative of the genome. Gel electrophoretic patterns of the proteins in bacterial cell extracts have been used to distinguish between genera and species and to group strains within a species according to their similarities (4, 8, 17). Grouping bacteria by gel electrophoretic protein patterns correlated well with results obtained by DNA-DNA hybridization and with numerical taxonomy. In the present study, gel electrophoresis of bacterial proteins was used to investigate the overall similarities among 35 strains of lactic streptococci. The data obtained have been analyzed to assign numerical values to the similarities obtained for gel electrophoretic patterns from different strains.

MATERIALS AND METHODS

Bacterial strains. The strains of *Streptococcus cremoris, Streptococcus lactis, Streptococcus diacetylactis,* and *Streptococcus thermophilus* used are listed in Table 1. Organisms were grown in M17 broth (18), and stock cultures were stored at −10°C in the same medium.

Growth of organisms. M17 broth (4 liters in two flasks) was inoculated (1%) with a 16-h broth culture and incubated at 22°C for 16 h, except that *S. thermophilus* strains were incubated at 37°C. The cells were harvested, washed once in 0.01 M phosphate buffer (pH 7.0) and washed twice in buffer [3 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.0], by centrifugation at 10,000 × *g* for 15 min. Strains 104 and 108 were harvested by centrifugation at 35,000 × *g* for 10 min because of poor pelleting. The washed bacteria were resuspended in 2 to 4 ml of a freshly prepared solution of deoxyribonuclease (0.01%, wt/vol; Sigma Chemical Co.) in 6.4 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.4).

Preparation of cell extracts. Preparation of cell extracts was carried out as described by Kersters and De Ley (8), with some modifications. The suspensions were disrupted in a French pressure cell (Aminco, Silver Spring, Md.) at 8.3 × 10^4 kilopascals. Intact bacteria and debris were removed by centrifugation at 15,000 × *g* for 15 min at 4°C in a Sorval RC-2 centrifuge, followed by centrifugation of the turbid supernatant at 80,000 × *g* and 4°C for 1 h (Beckman model L2-68 ultracentrifuge; type 30 rotor). The protein concentration in the supernatant was determined (12) and adjusted to 2.5 mg/ml with 6.4 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.4). After centrifugation of a 2-ml sample at 80,000 × *g* and 4°C for 4 h, the protein concentration of the supernatant again was determined and adjusted to 1 mg/ml with buffer [6.4 mM tris(hydroxymethyl)-aminomethane-hydrochloride buffer, pH 8.4) containing 5% (wt/vol) sucrose. This cell-free extract contained the soluble proteins and could be stored at −10°C for up to 6 weeks before electrophoresis.

Electrophoresis. The disc gel electrophoresis technique of Davis (3) was used, with the following modification. The sample (50 μl containing 50 μg of protein) was applied to the top surface of a large-pore spacer gel, over a small-pore gel; consequently, a sample gel was not necessary. Electrophoresis buffer was layered over the sample, and electrophoresis was carried out at room temperature with a current of 50 mA/

TABLE 1. *Origin of strains of lactic streptococci*

Strain	Species	Origin[a]	r^a
AM_1	*S. cremoris*	Collins, UCD	0.81
AM_2	*S. cremoris*	Collins, UCD	0.82
E_8	*S. cremoris*	CMS	0.91
H_2	*S. cremoris*	CMS	0.84
SK_{11}	*S. cremoris*	Phage-resistant AM_1 derivative	0.88
104	*S. cremoris*	CAG	0.97
108	*S. cremoris*	CAG	0.99
114	*S. cremoris*	GCW	0.89
130	*S. cremoris*	NIRD	0.84
134	*S. cremoris*	Phage-resistant AM_2 derivative	0.85
158	*S. cremoris*	CMS	0.89
166	*S. cremoris*	CMS	0.89
240	*S. cremoris*	CMS	0.91
266	*S. cremoris*	DRI	0.95
298	*S. cremoris*	Streptomycin-resistant E_8 derivative	0.87
368	*S. cremoris*	AM_1 derivative	0.82
386	*S. cremoris*	MTF	0.92
398	*S. cremoris*	Phage-resistant E_8 derivative	0.84
402	*S. cremoris*	Phage-resistant 158 derivative	0.90
438	*S. cremoris*	CMS	0.85
448	*S. cremoris*	DRI	0.81
480	*S. cremoris*	CMS	0.86
484	*S. cremoris*	CSIRO	0.85
H_1	*S. lactis*	CMS	0.82
ML_3	*S. lactis*	UD	0.83
ML_8	*S. lactis*	UD	0.82
SK_1	*S. lactis*	Phage-resistant ML_3 derivative	0.80
DRC_1	*S. diacetylactis*	CSIRO	0.88
DRC_2	*S. diacetylactis*	CSIRO	0.82
SD_1	*S. diacetylactis*	DRI	0.85
SD_{10}	*S. diacetylactis*	CMS	0.80
MK_{13}	*S. thermophilus*	DRI	0.81
33	*S. thermophilus*	NP	0.88
573	*S. thermophilus*	NIRD	0.83
1242	*S. thermophilus*	NIRD	0.93

[a] Abbreviations: CAG, Co-opérative Agricole de Granby, Quebec, Canada; CMS, an isolate from commercial mixed starter; CSIRO, Commonwealth Scientific and Industrial Research Organization, Food Research Division, Highett, Australia; DRI, New Zealand Dairy Research Institute collection; GCW, Gilbert Chandler Research Institute, Werribee, Australia; MTF, Microlife Technics, Sarasota, Fla.; NIRD, National Institute for Research in Dairying, Reading, England; NP, Nestlé Products, Lausanne, Switzerland; UCD, University of California, Davis; UD, United Dairies, London, England.

[b] r is the correlation coefficient of four gels of each strain with one another (see text).

gel (180 V). The run was stopped when the bromophenol dye marker was 1 cm from the bottom of the tube (1.25 h). Gels were removed from the tubes and stained overnight in 0.1% (wt/vol) amido black 10B (Merck) in 7% (vol/vol) acetic acid. Gels were destained electrophoretically in 7% acetic acid.

Gels were scanned with a Canalco model G densitometer. Optimal settings of the instrument were as follows: scale, 3; signal, 6; scan speed, 8; chart speed, 15 cm/min; signal range, 50.

Adjustment of the length of the gel scan by normalization. The distance travelled by the dye front markers and the proteins in the extracts varied slightly among gels, even in the same electrophoresis run. All gels contained a similar protein band just behind the dye front marker, and this could be used as a marker for the protein front. The addition of the internal reference proteins ovalbumin and thyroglobulin to the protein sample interfered with the bands of bacterial proteins on the gels. However, the inclusion of these standard proteins on separate gels in the same electrophoretic run as the samples showed that the beginning of the gel and the protein front were consistently in the same position relative to the standard proteins. Therefore, densitometer scans of different lengths could be compared by assigning positions 0

and 100 to the points corresponding to the most mobile protein band and the beginning of the small-pore gel, respectively. The scan between these points was then divided into 100 equal parts. The extinction was expressed as height in millimeters at positions 1 to 100, and the figures obtained were used for a mathematical comparison of the gel electrophoretic patterns.

The process of allowing for different scan lengths in this way is referred to as normalization, following the nomenclature used by Kersters and De Ley (8).

Compensation of normalized densitometer scans. Even after scans had been normalized, there were sometimes small differences in the position of corresponding protein bands in gels of the same extract. This was corrected by a process which has been called compensation (8). For each set of four electrophoretic patterns from one strain, four or five main bands were selected. The positions of the bands were determined, and the average position for each band was calculated. In each scan, each selected band was moved to its average position. The numbers of the positions in between bands were then adjusted at the points of lowest extinction to allow for any changes that had been made in the position numbers of the peaks. The resulting scans are referred to as compensated normalized scans.

Mathematical comparison of gel electrophoretic patterns. The data from different electrophoretic patterns was compared by using the computer program of Kersters and De Ley (8). This program compared the electrophoretic pattern from each strain with that from every other strain. Correlation coefficients (r values) between the electrophoretic patterns were used to construct a dendrogram relating the strains. Very similar patterns had correlation coefficients approaching 1.0. Because relative densities of gels rather than absolute values were used for comparison, small variations in the amount of protein applied to the gels or in the background densities of the gels did not affect the result.

Biochemical characters and phage sensitivity. All strains were examined for the principal distinguishing characters of the four species. Catalase production was determined as described by Sandine et al. (16). Strains were incubated in M17 broth at 10°C for 2 weeks and at 40 and 45°C for 48 h, and visible turbidity was recorded. The ability to initiate growth in 4 or 6.5% NaCl and at pH 9.2 was similarly recorded after incubation in modified M17 broth for 48 h at 22°C. Citrate utilization was positive if gas was detected in a Durham tube in a citrate broth (15) culture which had been incubated at 30°C for 24 h. Arginine utilization was detected by the production of ammonia on bromocresol purple agar (14) after 48 h at 22°C. The production of diacetyl plus acetoin was detected by the King test (9).

Phage stocks containing at least 10^9 plaque-forming units per ml were spotted undiluted and at 10^{-2} dilutions on lawns of bacterial strains. Phage sensitivity was confirmed by replating from zones of lysis to produce plaques.

RESULTS

Reproducibility of protein patterns. Each of the 35 strains (Table 1) was grown on two separate occasions, and the protein extracts were analyzed on duplicate gels. To minimize variability, the same growth medium, time, and temperature of incubation of cultures were used unless otherwise specified, and centrifugation and electrophoretic procedures were standardized. Gels typically contained 10 to 15 main bands, which were readily distinguished on the densitometer scans (Fig. 1), and 5 to 7 minor bands, which were more easily distinguished visually.

Data from normalized and normalized compensated gel scans were compared for six strains. In each instance compensation improved the reproducibility; e.g., r values were 0.87 for both normalized scans and 0.89 and 0.95 for normalized compensated scans for strains 114 and 266, respectively (Fig. 2).

The densitometer scans for each set of four gels per strain were normalized and compensated, and the correlation coefficients among the four gels from each strain were determined as a measure of reproducibility (Table 1). Correlation coefficients between gels within each set were between 0.80 and 0.99. Gels of protein extracts of the same strain grown on different occasions were no more different from one another than duplicate gels of the same extract in the same electrophoretic run. Differences between gels were caused by the electrophoretic techniques, and despite careful standardization of techniques, there were minor differences between gels from different electrophoretic runs. Therefore, in comparing strains, a correlation coefficient of <0.80 was required before strains could be regarded as different from one another by this technique.

Comparison of gel electrophoretic patterns from different strains. One normalized compensated densitometer scan was selected as representative of each strain, and the data from these 35 scans were compared (Fig. 3). A total of 18 of the strains fell into one of two large groups (groups 1 and 6). Group 1 contained nine strains; two pairs of these strains, *S. diacetylactis* DRC_1 and DRC_2 and *S. cremoris* 240 and 480, had correlation coefficients above 0.80. The second group contained three of the four *S. lactis* strains investigated; strain ML_3 and its phage-resistant derivative SK_1 had a correlation coefficient of 0.82. The third group consisted of two strains, *S. cremoris* 158 and its phage-resistant derivative 402, the correlation coefficient between them being 0.92. Group 4 contained only *S. cremoris* 108, which was not closely related to any other strain. Group 5 contained two *S. thermophilus* strains, 1243 and 573, and *S. cremoris* 104. Group 6 contained one *S. diacetylactis* strain and eight *S. cremoris* strains, including AM_1, its protein-

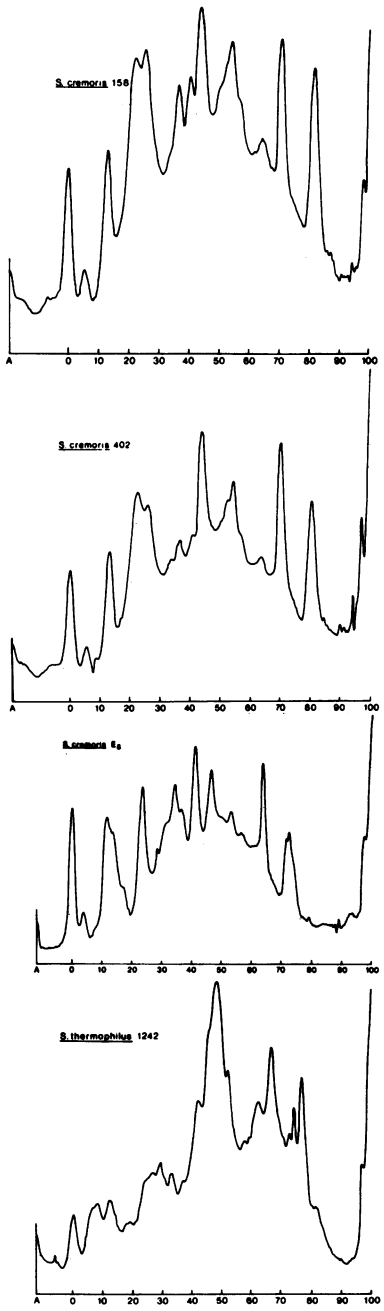

FIG. 1. *Densitometer scans of gels from extracts from two similar strains (S. cremoris 158 and 402) and two dissimilar strains (S. cremoris E_8 and S. thermophilus 1242).*

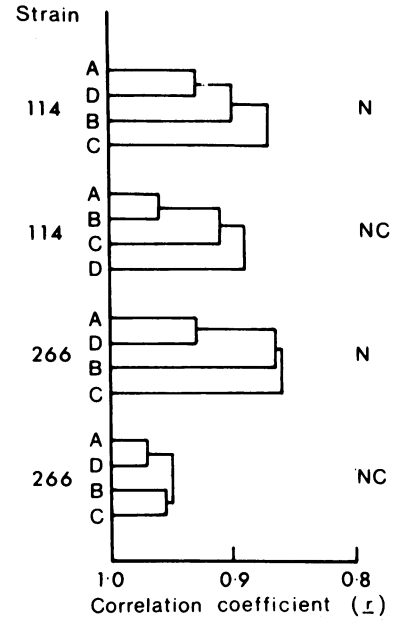

FIG. 2. *Numerical analysis of normalized (N) and normalized compensated (NC) scans of S. cremoris 114 and 266. A and B are duplicate gels from one extract; C and D are duplicate gels from another extract of the same strain.*

ase-negative derivative strain 368, and its phage-resistant derivative SK_{11}. It also included strain AM_2, which is similar to strain AM_1 in cheese-making qualities and phage sensitivity, and a phage-resistant AM_2 derivative, strain 134. *S. cremoris* H_2 was alone in group 7. Group 8 consisted of *S. cremoris* E_8 and its two derivatives, strains 298 and 398, the correlation coefficient between strains E_8 and 398 being 0.86. Group 9 contained *S. cremoris* 114 and 266, and group 10 contained two *S. thermophilus* strains, MK_{13} and 33.

Comparison of *S. lactis* ML_3 electrophoretic patterns after growth on different sugars. Lactic streptococci grown on glucose were deficient in at least six enzymes involved in carbohydrate metabolism, as compared with cells grown on galactose or lactose (2). Therefore, an experiment was designed to determine whether changes in intracellular proteins resulting from growth on different sugars were reflected in the gel electrophoretic patterns. *S. lactis* ML_3 was grown in M17 broth containing 0.5% lactose, glucose, or galactose, and gel electrophoretic patterns were obtained for extracts of the three cultures. The patterns obtained were identical visually (Fig. 4, gels B, C, and D) and analysis of the densitometer tracing showed a correlation coefficient of 0.86 between the gels (Fig. 5). Gels obtained from *S. thermophilus* MK_{13} in the same electrophoretic run, for comparison, were noticeably different (Fig. 4, gel A), having a correlation coefficient of 0.57 with *S. lactis* ML_3.

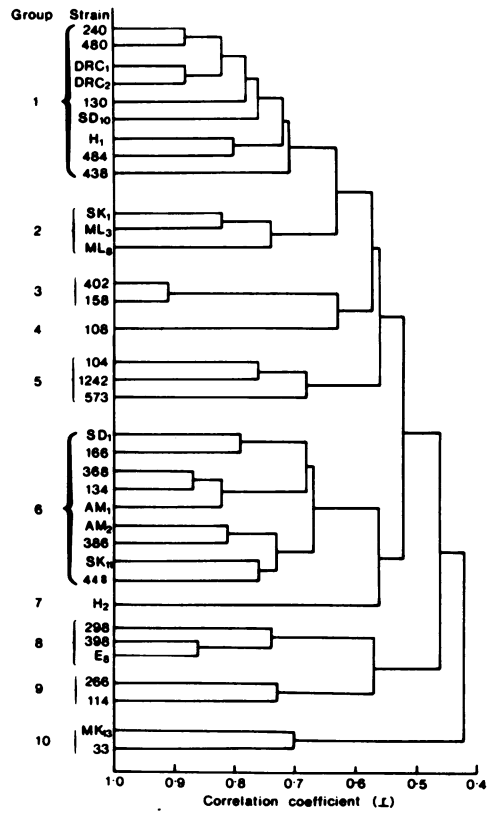

FIG. 3. *Dendrogram showing correlation coefficients between lactic streptococcal strains as determined by analysis of gel electrophoretic patterns.*

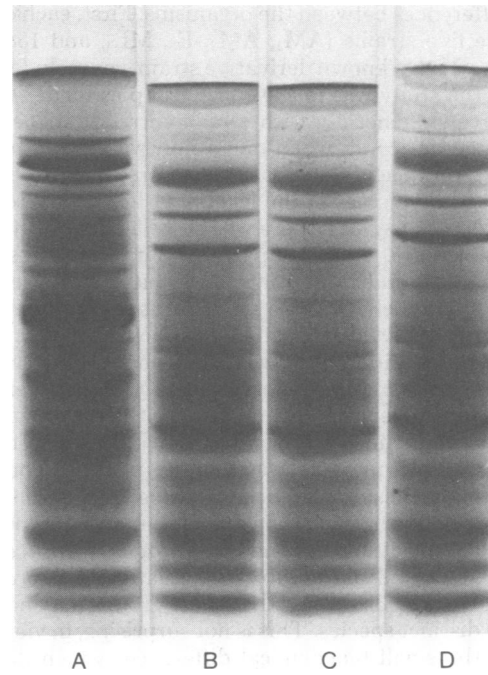

FIG. 4. *Gel electrophoretic patterns of soluble cell extracts from S. thermophilus MK13 (A) and S. lactis ML3 grown on lactose (B), galactose (C), and glucose (D).*

Morphological and biochemical characteristics. All strains were morphologically typical streptococci, occurring in chains of 2 to 10 cocci in milk, and were catalase negative. Of the 23 *S. cremoris* strains, 12 grew in M17 broth containing 4% NaCl (Table 2), although the ability to grow in 4% NaCl is reported to be diagnostic for *S. lactis*. Only three of four *S. lactis* strains initiated growth at pH 9.2 in M17 broth, although ability to initiate growth at this pH is reported to be diagnostic for *S. lactis*. *S. thermophilus* gave weak positive reactions in the King test, and two *S. thermophilus* strains utilized citrate, which would place them with *S. diacetylactis* except for temperature requirements. It is possible that characters which have been reported as diagnostic for species of lactic streptococci may depend on both the strains and the media employed.

Phage sensitivity. The sensitivity of 25 of the strains to 50 phages is reported in Table 3. The remaining strains were not attacked by any of the phages in the collection of the New Zealand Dairy Research Institute. Two phages which had been propagated on each of the 25 strains were selected and spotted onto lawns of the bacterial strains being tested. The results indicate that strains within one group were frequently attacked by the same phage, but that the same phages were also able to attack bacterial strains from different groups. Thus, one phage attacked strains AM_1, AM_2, and SK_{11}, which are all in group 6 (Fig. 1), but the same phage also attacked strains H_1 (group 1) and 114 (group 9).

DISCUSSION

In the present study, gel electrophoretic patterns of proteins in bacterial cell extracts were used to group strains of lactic streptococci according to their overall similarity. The gels contained 20 to 25 bands, in agreement with data obtained for other genera by Kersters and De Ley (8) and Fox and McClain (4). Most bands must contain several proteins, some of which would be present in such small amounts that they would not be detected by this technique. However, Swings and De Ley (17) found that grouping bacteria by this technique gave good correlation with results from DNA-DNA hybridization and numerical taxonomy.

Data presented here indicate the extent to which differences between strains of lactic streptococci revealed by this technique reflected real

differences between the organisms. First, each of the five strains (AM_1, AM_2, E_8, ML_3, and 158) for which a known derivative strain was included in the study was in the same group as its derivative(s), generally with a high correlation coefficient. Second, when one strain was grown on different sugars, so that there were likely to be marked changes in the level of at least six cytoplasmic enzymes, there was no reflection of this change in the protein patterns obtained. These findings indicate that differences between strains which involve a small number of proteins were not revealed by gel electrophoretic patterns and that differences which are found between strains, therefore, reflect relatively large differences between the phenotypes of the organisms involved. Since the soluble cell proteins were obtained from cells grown under identical conditions, these differences may be expected to represent corresponding differences in genotype.

The positions assigned to *S. lactis, S. diacetylactis,* and *S. thermophilus* relative to *S. cremoris* (Fig. 3) suggest that these groups may not be distinct species. This is not surprising, in view of the small biochemical differences which define these species and the similarities between the species which have been found by other workers. Knittel (Ph.D. thesis, University of Wisconsin, Madison, 1975) found the mean per-

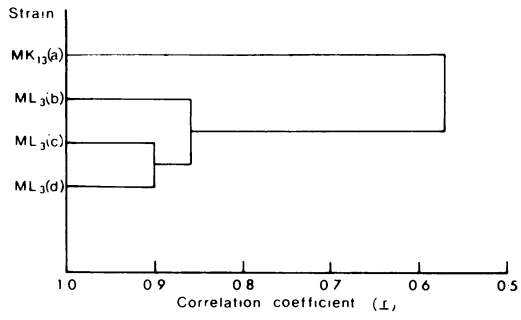

FIG. 5. *Comparison of gel electrophoretic patterns of soluble cell extracts from S. thermophilus MK_{13} (a) and S. lactis ML_3 grown on lactose (b), galactose (c), and glucose (d).*

cent guanine plus cytosine contents were similar for *S. lactis, S. cremoris,* and *S. diacetylactis.* An investigation into the immunological relationships among fructose diphosphate aldolases produced by streptococci (11) showed that five groups could be differentiated, with *S. lactis, S. cremoris, S. diacetylactis, S. thermophilus,* and *S. mitis* forming one group.

Cultures of lactic streptococci are normally considered to be different strains if they have come from different sources or if they have been isolated at different times. The interchange of cultures which takes place throughout the world's dairy industries and the isolation of strains from dairy products or commercial mixed starters make it likely, however, that many cultures which are regarded as being different strains are in fact isolates of the same strain. In selecting starter strains for use in cheese factories, it is necessary to ensure that the strains differ from one another in phage sensitivity, for instance, by an appropriate procedure, such as that of Heap and Lawrence (5). In their review, Jones and Sneath (7) reported that numerous investigations have shown that the ability to propagate phages seldom crosses wide taxonomic barriers and that the closer organisms are taxonomically to one another, the more phages they propagate in common. This suggests that organisms which have an overall similarity of genotype are most likely to share the genes necessary for the propagation of a particular phage. In the present study it was found that strains which fell into one group according to protein profile often shared sensitivity to a common phage. However, grouping of lactic streptococcal strains by their overall similarity did not always correspond to differentiation by phage sensitivity; many phages were able to attack bacterial strains from several groups. Phages were able, therefore, to attack strains which differed considerably in overall similarity, but presumably shared the necessary characters for propagation of particular phages.

Obviously, differentiation of starters by a technique such as that used in this study is not

TABLE 2. *Biochemical reactions of 35 strains of lactic streptococci*

Organism	No. of strains	No. of positive strains							
		Growth at:[a]					Arginine utilization	King test[b]	Citrate utilization
		10°C	40°C	45°C	pH 9.2	4% NaCl			
S. cremoris	23	23	0	0	0	12	0	0	0
S. lactis	4	4	4	0	3	4	4	0	0
S. diacetylactis	4	2	2	0	1	4	4	4	4
S. thermophilus	4	0	4	4	1	0	0	4	2

[a] Incubation was for 48 h, except at 10°C at which temperature incubation was for 14 days.
[b] Test for diacetyl plus acetoin.

TABLE 3. *Phage sensitivity of 25 lactic streptococcal strains*

Test bacterial strain	Bacterial strain on which phages were propagated[a]																								
	AM_1	AM_2	E_8	H_2	SK_{11}	104	108	114	130	158	166	240	266	298	368	386	398	402	448	480	484	H_1	ML_3	ML_8	SK_1
AM_1	+	+		+	+				+						+	+				+	+				
AM_2	+	+		+	+				+							+									
E_8			+																						
H_2				+					+									+	+		+	+			
SK_{11}					+													+					+		+
104				+		+	+									+			+		+	+			
108						+	+														+				
114								+													+				
130		+							+							+					+				
158										+			+					+						+	
166											+										+				
240	+											+													
266										+			+								+			+	
298														+											
368															+			+							
386																+		+						+	
398																	+								
402																		+						+	
448				+														+	+		+	+			
4480																		+		+	+				
484																					+				
H_1				+												+		+	+		+	+			
ML_3																		+			+		+		+
ML_8																		+						+	
SK_1																		+					+		+

[a] + indicates sensitivity to phage.

a substitute for testing for phage sensitivity, which remains the first step in selecting starters. However, this method of differentiation may be useful as an additional step in selecting strains to combine in a multiple starter. A phage-resistant mutant of a bacterial strain frequently lacks the ability to adsorb the phage to which it has acquired resistance, but host range mutants of phage which can adsorb to the resistant bacterial strains can be obtained readily (1, 13). It would not be normal practice, therefore, to include a strain and its phage-resistant derivative when assembling a multiple starter. The methods used to isolate starter cultures, however, make it possible for two strains to be related in this way without this fact being known. The data presented here show that such strains are likely to have a high overall similarity, as determined by gel electrophoretic pattern of their protein extracts. Strains 114 and 266 have been grouped together in this study, and it has been shown (6) that a host range mutant phage which propagated on strain 266 was readily isolated from a phage which grew on strain 114. Strain SK_1 was isolated as a phage-resistant variant of strain ML_3, but a phage isolated on strain SK_1 will attack strain ML_3. Similarly, strain 402 was derived as a phage-resistant variant of strain 158, but a phage which can attack strain 402 can also grow on strain 158.

Gel electrophoretic patterns of soluble cell extracts can therefore be used to determine which strains of lactic streptococci are most different from one another in overall genotype, as determined by their relative position in the resulting dendrogram. It is reasonable to assume that such strains will be least likely to have in common the requisite information for propagation of a particular phage and that these data might therefore be utilized in the selection of starter strains.

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