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Electron Microscopic Heteroduplex Study and Restriction Endonuclease Cleavage Analysis of the DNA Genomes of Three Lactic Streptococcal Bacteriophages

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Three lactic streptococcal bacteriophages were compared with one another by electron microscopic analysis of heteroduplex DNA molecules. The phages were almost identical in morphology and had been isolated over a period of 10 years on different strains of *Streptococcus cremoris* from cheese plants situated in different parts of New Zealand. There was a high degree of homology between the DNAs, in agreement with Southern blot hybridization data reported earlier. There were, however, distinct regions of nonhomology, mostly between 0.45 and 1.71 kilobases in length, suggestive of the occurrence of block recombination events. A deletion of 2.23 kilobases in the two more recently isolated phages, or an insertion in the first isolate, was found. All three phage DNAs showed differences in restriction endonuclease cleavage sites. Alignment of the restriction endonuclease maps with the heteroduplex maps showed that differences in cleavage sites occurred most frequently in regions of nonhomology. However, differences in cleavage sites in regions of apparent homology were also detected, indicating that point mutations may have occurred in addition to block recombination events.

Lactic streptococcal bacteriophages are of significance to the dairy industry because of their ability to propagate on strains of lactic streptococci which are used as starters in cheese manufacture. Phages isolated from cheese plants have been differentiated into groups according to morphology (6, 9), serology (6), and host range and host resistance (3). A recent study (7) of the DNA-DNA homology between phages of different morphological types showed that phages of similar morphology were related to one another as determined by Southern blot hybridizations. This technique is not, however, sufficiently quantitative to determine the degree of DNA-DNA homology between the phages. This study further examines the relationship among the three phages by restriction endonuclease mapping and by electron microscopic analysis of heteroduplex molecules of the phage DNAs. The heteroduplex and cleavage maps obtained are discussed with regard to possible evolution of these lytic phages.

MATERIALS AND METHODS

Phages. Phages were isolated from cheese wheys on *Streptococcus cremoris* starter strains (Table 1). Phages are designated by their number in the New Zealand Dairy Research Institute collection. Phage preparation, concentration, and purification were carried out as described earlier (7). Phages were stored at 4°C in CsCl and dialyzed against buffer (10 mM Tris hydrochloride [pH 7.4], 50 mM $MgCl_2$) before DNA extraction.

DNA preparation. Purified phages (250 μl, 10^{12} PFU/ml) were extracted twice with equal volumes of Tris-saturated phenol (50 mM Tris hydrochloride, pH 8), and DNA was precipitated with ethanol. The DNA preparation was dried and suspended in 10 μl of buffer (100 mM Tris hydrochloride, 10 mM EDTA, pH 8.5).

Electron microscopy of phages. Phages (10 to 10^{12} PFU/ml in CsCl) were absorbed to carbon and collodion-coated copper grids, washed twice successively in buffer (10 mM Tris hydrochloride [pH 7.0], 10 mM NaN_3, 1 mM $MgCl_2$, and stained with 2% uranyl acetate (M. Wurtz, personal communication). Grids were photographed in a Zeiss 109 microscope at ×49,000 magnification. Phage T4 tails were used as a size standard.

Restriction endonuclease cleavage analysis. Digestion with restriction endonucleases *Eco*RI, *Hae*III, *Hin*dIII, and *Hpa*II was carried out as recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Fragments were separated on 0.7% agarose vertical gels, stained with ethidium bromide, and visualized and photographed under UV light.

Size determination of phage DNA by electron microscopy. Length measurements were made of nondenatured phage DNA spread in a mixture containing 30% formamide, 100 mM Tris hydrochloride (pH 8.5), 10 mM EDTA, and 0.01% cytochrome *c* spreading onto a hypophase consisting of 10% formamide, 10 mM Tris hydrochloride (pH 8.5), and 1 mM EDTA. Circular double-stranded phage PM2 DNA (10.0 kilobases [kb] [13]) was included as an internal size standard.

TABLE 1. Phages isolated from cheese plants

Phage	Propagating strain[a]	Isolation		Host range[a,b]		
		Date	Cheese plant	AM1	158	2002
853	AM1	1973	Whaeroa	+	−	−
936	158	1975	Kaikoura	+	+	−
1374	2002	1982	Kiwi	+	+	+

[a] All *S. cremoris* strains.
[b] +, Phage growth; −, no reaction. Tested with a phage preparation of 5 × 10^9 PFU/ml.

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Photographs were taken at a magnification of ×7,000 in a Zeiss 109 electron microscope.

Heteroduplex analysis. Heteroduplex molecules were prepared by alkali denaturation of double-stranded DNA from each phage (5), followed by renaturation of each pair of phage DNAs in 50% formamide. The preparations were spread under isodenaturing conditions (4) from a solution containing 50% formamide, 100 mM Tris hydrochloride (pH 8.5), 10 mM EDTA, and 0.01% cytochrome *c* onto a hypophase consisting of 20% formamide in 10 mM Tris hydrochloride (pH 8.5)–1 mM EDTA. Double-stranded phage PM2 DNA and circular single-stranded phage fd DNA (6.408 kb [1]) were included as internal size standards. Specimens were photographed under a Zeiss 109 electron microscope at a magnification of ×7,000, and the molecules were measured as described by Meyer et al. (11).

RESULTS

Phage morphologies, host range, and genome sizes. The three phages (Table 1) were isolated on different *S. cremoris* strains (propagating strains in Table 1) from wheys in cheese plants in different locations, over a period of 10 years. Host-range data (Table 1) showed cross-reactions in that while phage 853 grew only on strain AM_1, phage 936 grew on strains AM_1 and 158, and phage 1374 grew on AM_1, 158, and 2002. Morphologies of the phages are depicted in Fig. 1 and summarized in Table 2. All have small isometric heads and long noncontractile tails containing 30 to 35 striations. The electron micrographs presented here show that in addition to a collar, phage 853 has two whiskers and a small base plate. Phages 936 and 1374 have larger, flowerlike base plates, with six and eight appendages, respectively. There were no significant differences in head or tail measurements for the three phages (Table 2).

The genome sizes of the phage DNAs (Table 2) were 30.7 kb (853), 26.5 kb (936), and 28.5 kb (1374), and these differences were statistically significant.

Heteroduplex molecules. Representative heteroduplex DNA molecules of phages 853 and 936 (a), 853 and 1374 (b), and 936 and 1374 (c) are shown in Fig. 2. A schematic representation of the three pairs of molecules is given in Fig. 3. Each measurement is the average of at least 15 determinations.

DNAs from phages 936 and 1374 showed identical regions of homology and nonhomology with phage 853 DNA, to the point indicated (C, Fig. 3), including a deletion of 2.23 kb (D) at a distance of 5.75 kb from one end (end B in Fig. 2 and 3). As would be expected, phage 936 and 1374 DNAs were homologous to one another for the corresponding lengths of DNA molecules. The percentage of homologous DNA (double-stranded DNA relative to the genomic DNA) in each heteroduplex molecule is shown in Table 3. DNAs from phages 936 and 1374 were 82 and 81% homologous, respectively, with 853 DNA and 87 to 94% homologous with one another. Genome size data suggested that the 2.23-kb deletion was in 936 and 1374 DNA, or alternatively a 2.3-kb insertion was in phage 853 DNA. The single-stranded DNA parts of each loop were assigned to each phage as appeared likely according to genome sizes and to which size segments were common to each pair. The genome sizes then determined by the sum of the segments in the heteroduplex molecules (Table 3) showed good agreement with those determined on double-stranded DNA (Table 2).

Restriction mapping. Preliminary experiments with a number of restriction endonucleases indicated that *Eco*RI, *Hae*III, *Hin*dIII, and *Hpa*II would be suitable for restriction mapping. An unexpected finding was the small number of cleavage sites for *Hae*III and *Hpa*II. Earlier work (7) showed that DNAs from these phages contained about 40% G+C, so that a low G+C content would not explain this finding. However, it is possible that they contain a large

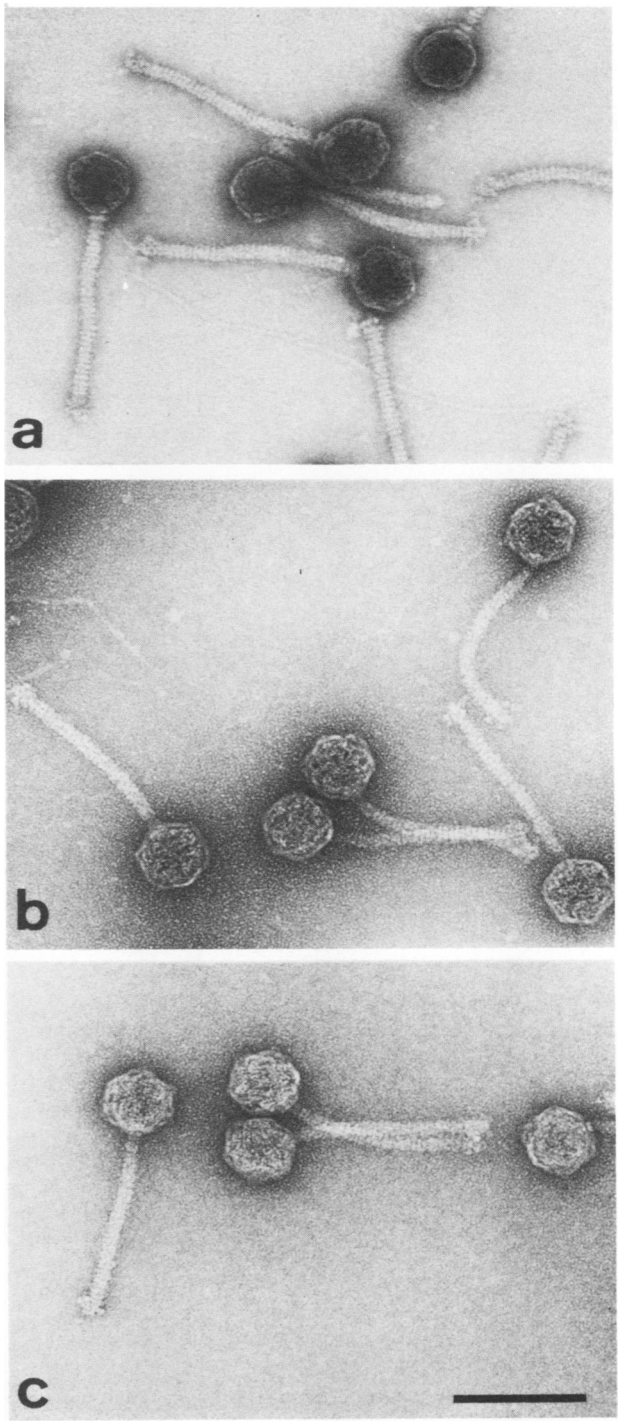

FIG. 1. Electron micrographs of phages. (a) 853; (b) 936; (c) 1374. Collars with whiskers attached can be seen on phage 853. Bar indicates 100 nm.

TABLE 2. Morphological characteristics of phages and genome sizes of phage DNAs[a]

Phage	Head (nm)	Tail (nm)[b]		Collar[c]	Other Appendages	Genome size (kb)
		Length	Diam			
853	49.2 ± 1.8	157 ± 3.2	12.3 ± 1.1	+	Whiskers	30.7 ± 0.5
936	48.9 ± 2.2	153 ± 7.7	12.5 ± 0.9	−	−	26.5 ± 0.7
1374	49.0 ± 1.4	140 ± 6.0	12.2 ± 1.1	−	−	28.5 ± 0.6

[a] Measurements made on 20 phages or molecules. Means and standard deviations are given.
[b] Tails showed 30 to 35 striations.
[c] + or − indicates present or absent, respectively.

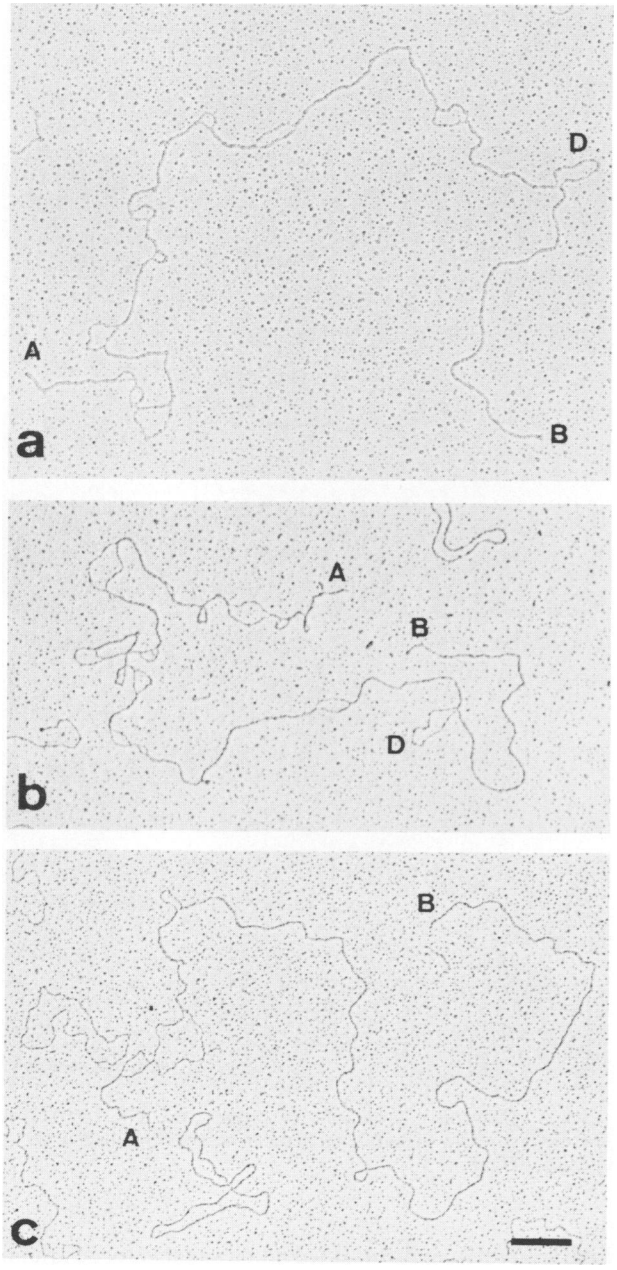

FIG. 2. Heteroduplex molecules of DNA from phages 853 and 936 (a), 853 and 1374 (b), and 936 and 1374 (c), showing ends A and B and a 2.23-kb deletion (D). The circular molecules represent single-stranded fd and double-stranded PM2 DNA, respectively. Bar indicates 1 kb.

number of methylated cytosines, which would be inhibitory to these endonucleases (10).

Each phage DNA was digested with *Eco*RI, *Hae*II, *Hin*dIII, and *Hpa*II, and the DNA fragment sizes were determined (Table 4). Double digests were carried out with *Eco*RI and *Hae*III, *Eco*RI and *Hin*dIII, and *Eco*RI and *Hpa*II, and a triple digest was carried out with *Eco*RI, *Hae*III, and *Hpa*II. The values of molecular weights of DNA obtained by restriction mapping (Table 4) were generally lower than those obtained by electron microscopy. Therefore, in aligning the restriction maps with the heteroduplex molecules, all lengths were normalized to those obtained by electron microscopy of double-stranded DNA. The restriction maps obtained were aligned with the heteroduplex maps by measuring the following heteroduplex molecules: undigested phage 853 DNA with the *Hae*III digest of phage 936 DNA; undigested 853 DNA with the *Eco*RI digest of phage 1374 DNA; undigested 936 DNA with the *Eco*RI digest of 853 DNA. In each of the partial heteroduplex molecules which contained the deletion (end B), the length of double-stranded DNA was measured.

The double-stranded DNA segment measured at end B of the molecule when phage 936 DNA was hybridized with the *Eco*RI digest of phage 853 DNA was 14.2 kb. The addition of the single-stranded 2.23-kb loop to this value gave a total of 16.4 kb for the *Eco*RI fragment of phage 853 DNA, indicating that the *Eco*RI fragment of 16.3 kb from the restriction endonuclease map of 853 DNA was at end B of the molecule (Fig. 3). Hybridization of phage 853 DNA with the *Eco*RI digest of phage 1374 DNA showed a double-stranded segment of 2.1 kb at end B of the molecule, or a segment of 8.4 kb (measured from the end of the molecule) which contained the deletion. This indicated that *Eco*RI fragments of 2.5 and 5.7 kb were at end B (Fig. 3) of the phage 1374 DNA molecule. Hybridization of DNA from phage 853 with the *Hae*III digest of phage 936 DNA similarly indicated that the 11.9-kb *Hae*III fragment was at end B of the phage 936 DNA molecule.

Comparison of the restriction endonuclease cleavage maps (Fig. 3) showed marked similarities between DNAs from phages 936 and 1374. Fourteen cleavage sites in these DNAs were in similar positions and were in regions of homology. The remaining six sites were divided between regions of homology (four) and regions of nonhomology (two). Comparison of cleavage sites in phage 936 and 853 DNAs showed that only four sites were in the same position in these DNAs, both in regions of homology. Of the remaining 19 sites in these DNAs, 8 were in regions of apparent homology, suggesting that point mutations or small deletions or insertions have occurred.

DISCUSSION

Previous studies (7) of small, isometric-headed lactic streptococcal phages showed that these phages, which con-

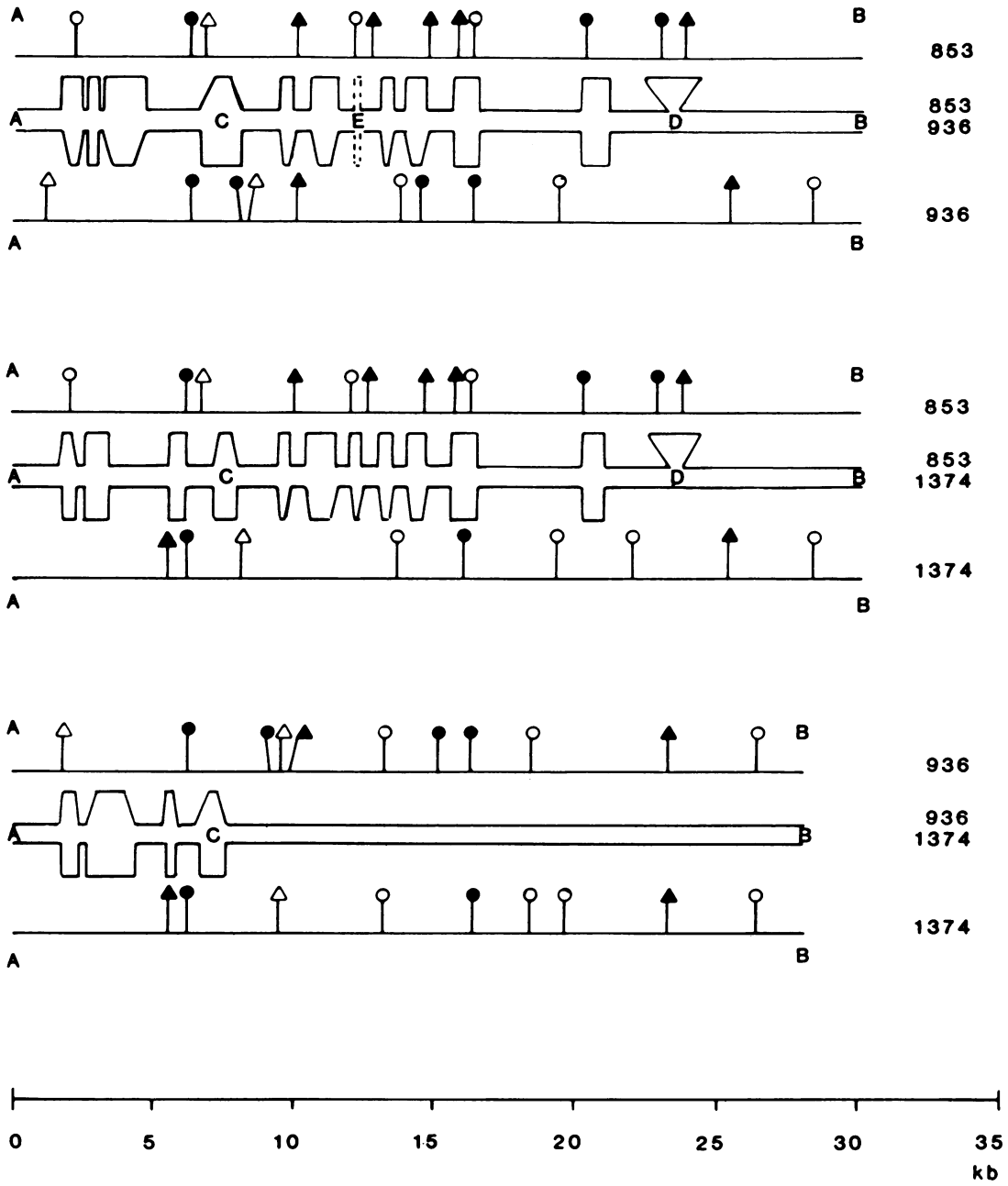

FIG. 3. Diagrammatic representation of heteroduplex molecules depicted in Fig. 2 (center), and restriction maps (upper and lower) of the phage DNAs. Symbols are as in Fig. 2. C refers to regions of nonhomology (see text). Restriction endonuclease cleavage sites *Eco*RI (○), *Hae*III (●), *Hin*dIII (△), and *Hpa*II (▲) are shown.

stitute one morphological group, also formed one group as determined by DNA-DNA homology. In this study a detailed analysis of three of these phages provided quantitative data which allow speculation on the possible evolution of these phages.

The three phages studied were isolated from different cheese plants over a period of 10 years. DNA homology of 81 to 82% between DNAs from phages 936 and 1374 with DNA from phage 853 suggested that these two phages could have evolved from phage 853. The even greater degree (87 to 94%) of DNA homology between phages 936 and 1374 suggested that they evolved from phage 853 through a common ancestor. The small segments of nonhomology between DNAs from phages 853 and 936, detected in regions of the genomes where there was homology between 853 and 1374, indicated that it is unlikely that phage 1374 evolved from phage 853 through phage 936, but it is possible that phages 936 and 1374 evolved from phage 853 or some related phage through a common ancestor.

The morphological and host-range data are in agreement with this hypothesis. All three phages show a general similarity in morphology. However, the electron micrographs presented here show details in morphology that have not previously been reported for these phages. Phage 853 had

TABLE 3. Percent homologous DNA and genome sizes of phage DNAs determined from heteroduplex molecules

Hybridized phages	Genome size[a]	% Double-stranded DNA
853	32.3	67
936	26.6	82
853	31.9	72
1374	28.4	81
936	27.3	94
1374	29.1	87

[a] Genome size of phage DNA was determined by summation of single- and double-stranded DNA of heteroduplex molecules.

whiskers and a collar, none of which were evident in phage 936 or 1374. The phage 853 base plate appeared to be different from the base plates in phages 936 and 1374. These were similar to one another in structure, but differed in the number of appendages. Phage 853 was isolated on *S. cremoris* AM1. Phage 936, if it evolved from phage 853, retained the ability to attack *S. cremoris* AM1 and acquired the ability to attack *S. cremoris* 158. Phage 1374 could propagate on both *S. cremoris* AM1 and 158 and on an additional strain, *S. cremoris* 2002. These data support the suggestion of the evolution of these lytic phages as outlined.

Campbell and Botstein (2), in discussing the lamboid phages, proposed that evolution of these phages has taken place by recombination of modules of DNA. The data presented here for these three lactic streptococcal phages suggested that such recombination occurred in the evolution of these phages, since most of the regions of nonhomology were between 0.45 and 1.7 kb in length, i.e., about gene size. Similar observations were recently reported for morphologically distinct *Bacillus licheniformis* phages (12).

The source of such recombinant DNA remains a matter for speculation. Recombination may occur between DNA of lytic phages, between phage and bacterial DNA (chromosomal or extrachromosomal DNA), or between DNA from lytic and temperate or defective prophage DNA. Earlier work with lactic streptococcal phages (8) showed a lack of DNA homology between lytic phage DNA and either temperate phage DNA or bacterial DNA. These experiments were carried out at a high level of stringency to determine whether temperate phages in lactic streptococci were likely to be a direct source of lytic phages. The question which must be asked now is whether bacterial or temperate phage DNA may contain small fragments of DNA, on the order of one-half to a few kilobases, which may be a source of recombinant DNA and so contribute to the evolution of new lytic phages. Alternatively, the source of recombinant DNA may be found in other lytic phages. From a comparison of the restriction endonuclease and heteroduplex maps, it was evident that approximately half of the differences in restriction endonuclease cutting sites were found in regions of homology as seen in the heteroduplex map. This was consistent with the occurrence of point mutations in addition to block recombination of modules of DNA. Small deletions or insertions, as well as point mutations, would not be detected by heteroduplex analysis and also may have occurred. Davis and Hyman (4) obtained evidence that point mutations were significant in the evolution of coliphages T7 and T3. The data presented here indicate that both recombination and point mutations play a part in the evolution of lactic streptococcal phages. These findings also suggest that an important source of lytic phages in cheese plants may be their evolution from other lytic lactic streptococcal phages.

TABLE 4. Molecular sizes (kb) of fragments produced from phage DNA by cleavage with restriction endonucleases[a]

Phage	*Eco*RI		*Hae*III		*Hin*dIII		*Hpa*II	
	A	N	A	N	A	N	A	N
853	15.5	16.3	11.7	12.1	23.3	24.1	10.8	10.3
	8.7	9.2	8.7	9.0	6.4	6.6	8.8	8.4
	3.0	3.1	6.3	6.5			6.5	6.2
	2.0	2.1	3.0	3.1			2.6	2.5
							2.4	2.3
							1.0	1.0
936	11.2	11.6	11.6	11.9	18.4	19.2	19.2	13.4
	7.5	7.8	5.9	6.0	5.9	6.1	8.7	8.5
	5.1	5.3	4.8	4.9	1.1	1.2	4.7	4.6
	1.8	1.8	2.1	2.2				
			1.5	1.5				
1374	12.7	13.2	13.2	13.9	17.8	18.7	18.2	17.5
	5.5	5.7	8.2	8.7	9.3	9.8	6.6	6.4
	5.2	5.3	5.6	5.9			4.8	4.6
	2.4	2.5						
	1.7	1.8						

[a] A indicates absolute values obtained. N indicates values normalized to molecular weight of DNA obtained by electron microscopy. Standards used were *Eco*RI fragments of phage P1, molecular sizes (kb) as follows: 14.6, 9.4, 9.2, 6.4, 5.8, 5.7, 5.4, 4.0, 3.7, 3.0, 2.8, 1.7, 1.5, 1.3, 1.1, 0.9, 0.6.

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