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DNA-DNA hybridizations among lactic streptococcal temperate and virulent phages belonging to distinct lytic groups

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SUMMARY

Ten lactic streptococcal temperate phages and eight lactic streptococcal virulent phages classified on the basis of host range were differentiated by DNA-DNA hybridization. Virulent phages were classified in two distinct homology groups and temperate phages in a single one. In both temperate and virulent phages, no correlation was found between DNA homology groups and lytic groups. For most of the virulent phages, no DNA-DNA hybridization occurred with the temperate phages; however, partial sequence homology was found with DNAs from two virulent phages and four temperate phages.

INTRODUCTION

Virulent phages of lactic acid bacteria, in particular of mesophilic lactic streptococci, are responsible for acidification problems during industrial fermentations. Sources of these phages are still unknown, but the existence of temperate phages in most of the lactic streptococci starters studied [6,10,12,13] suggested that virulent phages isolated in cheese factories are issued from lysogenic starter bacteria. Little is known about the genome of temperate phages of group N lactic streptococci [4,7].

In this study, DNA-DNA hybridization and eventually restriction analysis were used to (i) determine the relationships existing between temperate phages belonging to two different lytic groups [13], (ii) differentiate eight virulent phages from three lytic groups [3], and (iii) determine relationships between these temperate and virulent phages.

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MATERIALS AND METHODS

Bacterial strains, temperate and virulent phages

All bacterial strains were obtained from the CNRZ-INA collection (Centre National de Recherches Zootechniques, Institut National de la Recherche Agronomique, 78350 Jouy en Josas, France). Virulent phages (Table 1) were from M.C. Chopin, and temperate phages are listed in Table 2.

Table 1

Virulent phages and bacterial strains

Lytic group	Virulent phage		Propagating strain[a]	
	No.	CNRZ[b] No.	No.	CNRZ No.
g1	φV9	78	C9	A85
g1	φV10	129	C10	A318
g2	φV13	22	L13	Z268
g2	φV14	160	L14	A311
g2	φV15	6	L15	Z272
g3	φV12	40	C12	A15
g3	φV16	67	L16	A100
g3	φV17	170	D17	573

[a] Organisms: C = *S. lactis* ssp. *cremoris*; L = *S. lactis* ssp. *lactis*; D = *S. lactis* ssp. *diacetylactis*.
[b] CNRZ: see Materials and Methods.

Temperate bacteriophage preparation and concentration

Induction of lysogenic strains by mitomycin C (MC) was carried out as previously described [13]. Lysates (1 liter) were precipitated with 10% (w/v) polyethylene glycol MW 6000 (PEG 6000) and 500 mM NaCl [19] at 4°C. Phages were centrifuged at 16 000 × *g* for 2 h and resuspended in phage buffer (10 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 10 mM NaCl).

Propagation of the lytic and temperate phages

Phages from MC-induced lysates or lytic phages were propagated in suitable strains by the double agar layer method [1]; the upper layer was collected with M17 broth [17] and centrifuged twice at 4000 × *g*. Phages in the supernatants were dialysed against phage buffer before DNA extraction.

Preparation of DNA

Phage suspension was filtered through a nitrocellulose membrane filter (0.45 μm) and treated with 100 μg/ml of ribonuclease A (Serva) and 10 μg/ml of deoxyribonuclease I (Boehringer) at 37°C for 1 h. For phage DNA preparation, the sample was heated at 37°C for 1 h with 0.5% SDS (sodium dodecyl sulfate), 20 mM Na_2-EDTA and 0.5 mg/ml of pronase (Serva). The sample was extracted twice with an equal volume of Tris-saturated phenol, pH 7.5, and once with chloroform/isoamyl alcohol 24:1 (v/v) [11]. DNA solution was then centrifuged to equilibrium in CsCl/ethidium bromide gradient (ρ = 1.5 g/ml to the mid-point of the gradient) at 290 000 × *g* for 22 h and dialysed against DNA

Table 2

Temperate phages and bacterial strains

Phage	Lytic group	Lysogenic strain		Propagating strain	
		Strain No.	CNRZ[b] No.	Strain No.	CNRZ No.
φT20	g2	L20[a]	Z151	L13	Z268
φT23	g2	L23	Z304	L13	Z268
φT24	g2	L24	A61	L13	Z268
φT25	g2	L25	A311	L13	Z268
φT16	g3	L16	A100	L32	A45
φT34	g3	C34	A15	L32	A45
φT44	g3	D44	Z124	–	–
φT45	g3	L45	A63	–	–
φT53	g3	L53	Z146	L32	A45
φT70	g3	L70	Z254	L32	A45

[a] Organisms: C = *S. lactis* ssp. *cremoris*; L = *S. lactis* ssp. *lactis*; D = *S. lactis* ssp. *diacetylactis*.
[b] CNRZ: see Materials and Methods.

buffer (10 mM Tris-HCl, pH 8.0, and 1 mM Na_2-EDTA).

Agarose gel electrophoresis

Purified phage DNAs were digested with restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer's specifications and electrophoresed in a 0.8% agarose (Sigma, type II) horizontal slab gel at 120 V for 3 h in TE buffer (40 mM Tris acetate, pH 7.9, and 2 mM Na_2-EDTA). *Hin*dIII digests of lambda DNA were used as size markers [14]. Restriction endonuclease digests were immobilized on nitrocellulose filters (BA 85; Schleicher and Schuell, GmbH) by the Southern-blot technique [16].

Preparation of [^{32}P]DNA

Purified DNA (not less than 250 ng) was incubated at 14°C for 90 min with 12 μCi of ^{32}P-labeled dCTP in the presence of DNA polymerase (Amersham). The reaction was stopped by the addition of 10 mM Na_2-EDTA; unbound labeled nucleotides were separated from the labeled DNA by chromatography on Sephadex G-50 (Pharmacia). Immediately before use, the labeled DNA was denaturated by boiling for 5 min and quickly cooled in ice.

DNA-DNA hybridization on filters

The filters were prehybridized in Denhardt solution [11] for 2 h at 60°C in the presence of calf thymus DNA (Sigma). Hybridization was carried out for 18 h at 60°C.

The nitrocellulose filters were then washed five times in a solution containing 450 mM NaCl, 45 mM tri-sodium citrate, 0.1% SDS and 1 mM Na_2-EDTA and exposed on X-ray film (Kodak X OMAT AR).

RESULTS

Preparation and characterization of DNAs from temperate phages

Ten lysogenic strains from two different lytic groups were induced by mitomycin C to produce temperate phages [13]. With the exception of φT44 and φT45, induced lysates were used to infect appropriate indicator strains to obtain sufficient amounts of phage DNA preparation. DNAs were purified and extracted as described in Materials and Methods. Their configuration was tested by two consecutive steps of agarose gel electrophoresis as previously described [5]. The phage DNAs isolated in our studies were found to be linear double-stranded molecules.

DNA-DNA hybridization and restriction analysis of temperate phage DNAs

The ten whole temperate phage DNAs were transferred from agarose gel to a nitrocellulose filter and probed with ^{32}P-labeled DNA from φT23

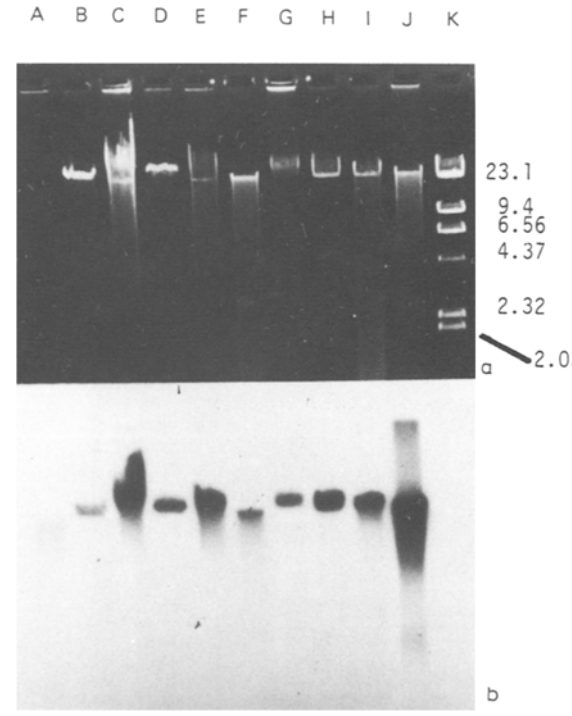

Fig. 1. (a) Agarose gel electrophoresis of the ten temperate phage whole DNAs. (b) Autoradiogram obtained after hybridization of ^{32}P-labeled DNA from phage φT70 with DNAs from: (A) φT16; (B) φT20; (C) φT23; (D) φT24; (E) φT25; (F) φT34; (G) φT44; (H) φT45; (I) φT53; (J) φT70; (K) lambda phage DNA digested with *Hin*dIII. Fragment sizes are given in kilobases. φT16 DNA could not be seen on this gel.

Table 3

Number of restriction sites obtained after hydrolysis of temperate phage DNAs by various restriction endonucleases

DNA from phage:	Cleaved by[a]:					
	*Bst*EII	*Eco*RI	*Hin*dIII	*Mbo*I	*Pst*I	*Pvu*II
φT23(MC)	2	12	12	12	7	10
φT25(MC)	0	5	4	6	6	3
φT34(MC)	4	6	10	14	6	7
φT53(MC)	5	6	8	10	7	6
φT70(MC)	1	8	15	5	0	6

[a] No site was found with *Xho*I and *Bam*HI.

(group g2) or φT70 (group g3). The φT70 DNA probe strongly hybridized with all the DNAs (Fig. 1). The φT23 DNA probe also strongly hybridized with all the DNAs except in the cases of φT16, φT24, φT44 and φT45 where reaction was weaker (data not given). These results allowed the classification of the temperate phages from two different lytic groups in a unique homology group.

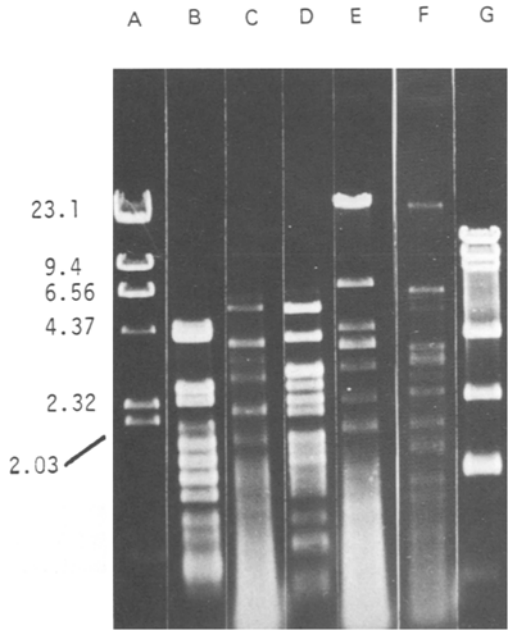

Fig. 2. Agarose gel electrophoresis of temperate phage DNAs digested with *Mbo*I. (A) lambda phage DNA digested with *Hin*dIII (in kb); (B) φT23 (MC); (C) φT25 (MC); (D) φT34 (MC); (E) φT44 (MC); (F) φT53 (MC); (G) φT70 (MC).

A more extensive study was made on phages φT23, φT25, φT34, φT44, φT45, φT53 and φT70 DNAs; these phages were concentrated directly from MC lysates to avoid eventual DNA modification by the propagating strain, and DNAs were extracted as already described.

Each bacteriophage DNA was treated with a variety of restriction endonucleases: *Ava*I, *Bam*HI, *Bst*EII, *Eco*RI, *Hin*dIII, *Mbo*I, *Pst*I, *Pvu*II and *Xho*I. φT44 and φT45 DNAs were not cut or were only partially digested.

Digestion patterns of the five temperate phage DNAs could be compared for only six of nine restriction endonucleases (Table 3). Individual DNAs exhibited characteristic restriction patterns (Fig. 2); the genome size (sum of the molecular sizes of restriction fragments) was 32 kb, 32.4 kb, 33.5 kb, 29.3 kb, 33.2 kb for φT23, φT25, φT34, φT53, φT70 DNAs, respectively.

To determine whether propagation of the induced phage through indicator strains altered restriction endonuclease patterns, φT23 and φT34 were grown in *Streptococcus lactis* L13 and L32, respectively, and φT70 was grown in *S. lactis* L32. The phages φT23 (L13), φT34 (L32) and φT70 (L32) were purified and DNAs were extracted. MC-induced lysates of φT23, φT34 and φT70, respectively named φT23 (MC), φT34 (MC) and φT70 (MC),

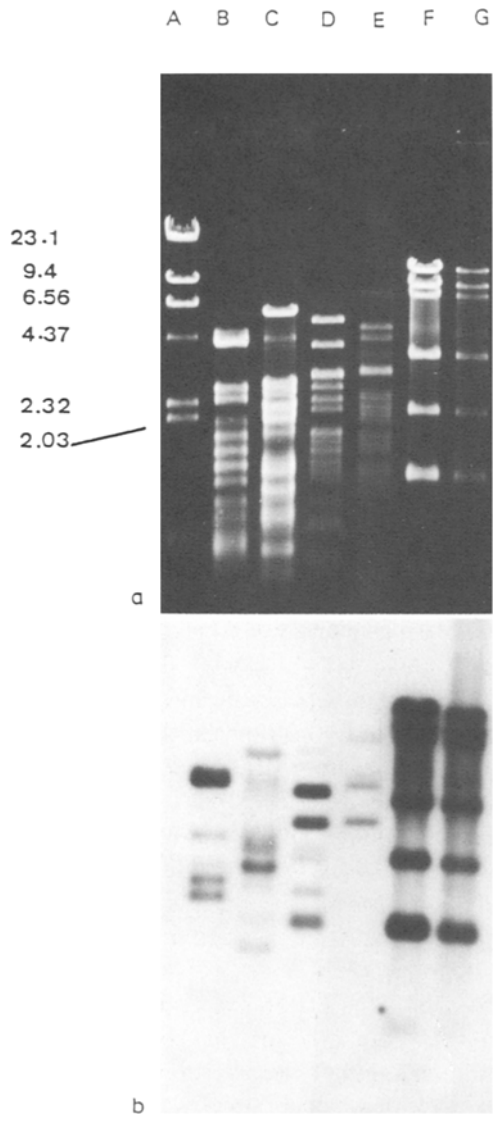

Fig. 3. (a) Agarose gel electrophoresis of *Mbo*I digests of DNAs from phages φT23, φT34 and φT70. (b) Autoradiogram prepared after hybridization of ^{32}P-labeled DNA from phage φT70 (MC). (A) lambda phage DNA digested with *Hin*dIII (fragment sizes in kb); (B) φT23 (MC); (C) φT23 (L13); (D) φT34 (MC); (E) φT34 (L32); (F) φT70 (MC); (G) φT70 (L32).

were also concentrated, purified and DNAs were extracted.

The digests of DNAs from these induced lysates and from propagated phages were compared. With the five restriction endonucleases tested, *Eco*RI, *Hin*dIII, *Mbo*I, *Pst*I and *Pvu*II, the digestion patterns of both φT70 DNAs were identical (Fig. 3A). The propagation of φT23 and φT34 in L13 and L32, respectively, resulted in modification of their DNAs and in alteration of the restriction endonuclease patterns with five restriction endonucleases (Fig. 3A).

When φT70 (MC) DNA was used as a probe on *Mbo*I digests of φT23 and φT34, not all the bands were labeled (Fig. 3B): only five of fifteen fragments of φT34 (MC) DNA and five of thirteen fragments of φT23 (MC) DNA hybridized.

When φT23 (MC) DNA was used as a probe on the same *Mbo*I digests, only four of fifteen fragments of φT34 (MC) DNA and four of six fragments of φT70 (MC) DNA hybridized (data not given).

Only partial homology was found among phages φT23, φT34 and φT70: the degree of hybridization was estimated to be 40% between φT70 and φT23 and 34.5% between φT70 and φT34.

DNA-DNA hybridization in virulent phages

DNAs from eight virulent phages belonging to three different host range groups (Table 1) were tested for DNA homology by the Southern-blot technique [16]. DNAs from phages φV10, φV13, φV14, φV15, φV16 and φV17 were labeled with ^{32}P and used as probes.

The results of hybridization (Table 4) allowed the classification of the virulent phages in two independent homology groups differing from the lytic groups: (i) φV10, φV12, φV13, φV16, and (ii) φV9, φV14, φV15, φV17.

DNA-DNA hybridization between temperate phages and virulent phages from different lytic groups

DNAs from φV10, φV13, φV14, φV15, φV16 and φV17 were labeled, and each DNA was used as a probe and hybridized with ten temperate phage DNAs (Table 2). No hybridization occurred with probes φV10, φV13, φV15 and φV17.

The φV16 DNA hybridized with three whole temperate phage DNAs: φT23, φT34 and φT70. Using this probe on *Pvu*II fragments, hybridization

Table 4

DNA-DNA hybridizations between virulent phages

+ = strong hybridization; – = no hybridization; ND = not determined.

Lytic group	Source of unlabeled DNA	Source of [^{32}P]DNA probe					
		φV10 g1	φV13 g2	φV16 g3	φV14 g2	φV15 g2	φV17 g3
g1	φV10	+	ND	+	–	–	–
g2	φV13	+	+	+	–	–	–
g3	φV12	+	+	+	–	–	–
g3	φV16	+	+	+	–	–	–
g1	φV9	–	–	–	+	+	+
g2	φV14	–	–	–	+	+	+
g2	φV15	–	–	–	+	+	+
g3	φV17	–	–	–	+	+	+

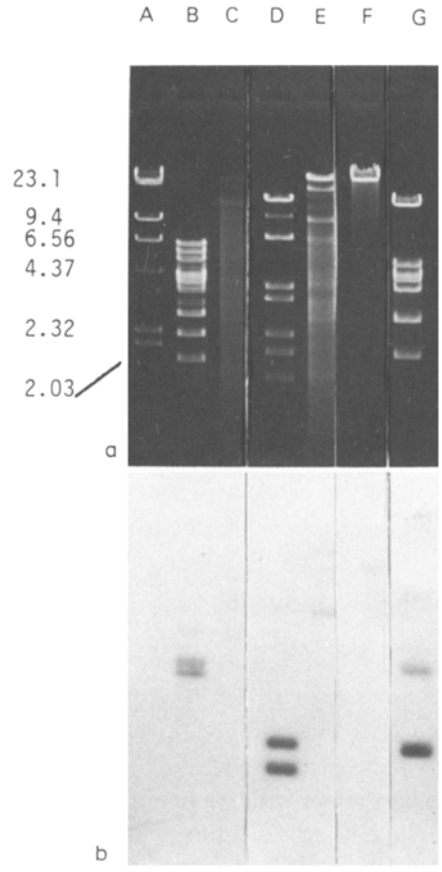

Fig. 4. (a) Agarose gel electrophoresis of *Pvu*II digests of temperate phage DNAs. (b) Autoradiogram prepared after hybridization of ^{32}P-labeled DNA from virulent phage φV16 with temperate phage DNA fragments. (A) lambda phage DNA digested with *Hin*dIII (in kb); (B) φT23 (MC); (C) φT25 (MC); (D) φT34 (MC); (E) φT44 (MC); (F) φT45 (MC); (G) φT70 (MC).

occurred with two fragments of φT23 DNA (3.8 kb and 3.4 kb), two fragments of φT34 DNA (1.76 kb and 1.34 kb), and also two fragments of φT70 DNA (3.9 kb and 1.66 kb) (Fig. 4).

The degree of hybridization with φV16 was estimated to be 22.5% for φT23 (g2), 9.2% for φT34 (g3) and 16.7% for φT70 (g3), respectively.

φV14 DNA probe showed a weak homology with φT23, φT25 and φT70 whole DNAs or DNA digests (data not given).

DISCUSSION

The relationships among eighteen temperate and virulent phages of group N streptococci, belonging to three different lytic groups, were investigated with DNA-DNA hybridization studies. The eight virulent phages were classified in two distinct homology groups, and no DNA homology was detected between phages of these two different groups. Furthermore, no correlation was found between host range and degree of DNA-DNA hybridization.

The ten temperate phages from two lytic groups were found to constitute a unique homology group.

These results suggested that the temperate and virulent phages classified into lytic groups do not represent distinct genetic entities.

Although they displayed DNA-DNA homology, restriction endonuclease patterns of temperate induced phage DNAs were always unique. Moreover,

as reported earlier by Daly and Fitzgerald [4], in two of three cases, phage DNA restriction patterns were completely modified after growth in the indicator strain. These results suggested the presence of different restriction-modification systems in these bacterial strains.

The absence of hybridization by Southern-blot technique between whole DNAs from virulent and temperate phages suggests an insignificant level of homology between the two types of phages.

However, partial homology was detected between two virulent phages and four temperate phages; for instance, small fragments of DNA of φV16 hybridized with temperate phage DNAs: these homologous fragments could result from recombination between DNA of the virulent phage and DNA from the temperate phage, as suggested by Jarvis and Meyer [8] to explain the occurrence of regions of non-homology between three lytic phages. Our results agreed with those of Teuber and Lembke [18], Jarvis [7], and Budde-Niekel et al. [2] but no evidence could be found that the virulent phages investigated originated directly from lysogenic starter strains. It is therefore improbable that all the temperate phages of mesophilic lactic streptococci could be the sources of the virulent phages appearing in cheese factories [9]. In contrast, in *Lactobacillus casei* S-1, Shimizu-Kadota et al. [15] demonstrated a complete homology between one virulent phage and the temperate phage of this strain.

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