Commun. Fac. Sci. Univ. Ank. Series C
V. 20. pp. 1 - 14 (2003)

CHARACTERIZATION OF VIRULENT BACTERIOPHAGES OF *STREPTOCOCCUS SALIVARUS* SUBSP. *THERMOPHILUS* AND *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS*

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(Received Feb. 24, 2003; Accepted June 10, 2003)

ABSTRACT

The genomes of 23 *Streptococcus salivarus* subsp. *thermophilus* bacteriophages and 24 *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteriophages were characterized by restriction endonuclease patterns and host range specificities. Bacteriophage genome sizes ranged from 21.0-72.2 kb. Four closely related bacteriophage group were designed according to the similarity in *Eco*RI fragments obtained from each bacteriophage DNA. Host range determination tests of bacteriophages indicated that *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteriophages had highly species specific host-range character.

KEY WORDS: *Lb. bulgaricus, Str. thermophilus,* bacteriophage, host range, restriction analysis

1. INTRODUCTION

Lactobacillus delbrueckii subsp. *bulgaricus* (*Lb. bulgaricus*) and *Streptococcus salivarus* subsp. *thermophilus* (*Str. thermophilus*) are two important lactic acid bacteria widely employed in the dairy industry as starter cultures in the manufacture of yoghurt and several types of cheeses (Auclair and Accolas, 1983; Fayard *et al.*, 1993). Bacteriophages to which these bacteria are sensitive can cause failure in the fermentation processess and can consequently lead to economical lossess (Coveney *et al.*, 1987; Forsman and Alatossava, 1991; Brüssow *et al.*, 1994). Although the phage problem has been recognized since 1930s (Whitehead and Cox, 1936) today there is still no expedient method to prevent the appearances of new phage infections (Forsman and Alatossava, 1991; Moineau *et al.*, 1995). We believe that taxonomic and phylogenetic research on bacteriophages will eventually help to explain the modes of genetic variation and bacteriophage-bacterium interactions, as well as the problem of the origin of bacteriophages. Early studies on bacteriophages of lactic acid bacteria mainly provided data on morphological, serological or physicological characteristics (Braun *et al.*, 1989; Bendbais *et al.*, 1990; Larbi *et al.*, 1990).

Analysis of bacteriophages, active against lactobacilli and streptococci, at genetic and molecular levels have only recently been undertaken, but despite this, there have been significant advances in the understanding of their oigins, evolution, relationships, and genome structures (Coveney *et al.*, 1987; Chow *et al.*, 1988; Sechaud *et al.*, 1988; Aud *et al.*, 2000).

The objective of this investigation was to characterize, at the genetic level, 24 virulent bacteriophages of *Lb. bulgaricus* and 23 virulent bacteriophages of *Str. thermophilus,* isolated from bulk, whey, yohurt and raw milk samples.

2. MATERIALS AND METHODS

Bacteria and bacteriophages

Strains of *Str. thermophilus* and *Lb. bulgaricus* were grown on M17 broth (Terzaghi and Sandine, 1975) at 40 C from subcultures in reconstituted milk (10 %). All bacteriophages were originally isolated from dairy industy materials, collected from cheese and yoghurt factories in Ankara. Twenty three *Str. thermophilus* bacteriophages were obtained from Kaleli and Tunail (2003). Bacteriophages were propagated in M17 broth supplemented with 10-20 mM (final concentration) calcium chloride ($CaCl_2$) at 40 C as described by Mercenier *et al.* (1987). Bacteriophage and culture stocks were stored in M17 broth containing 40 % glycerol at – 40 C.

Host range determination

Twenty five *Str. thermophilus* and 24 *Lb. bulgaricus* strains were used as indicators for the detection of bacteriophages sensitivity by an agar spotting method. Ten microliters of high-titre bacteriophage samples (> 10^7pfu/ml) were placed on each lown of indicator cells and left to stand for 30 min. Plaque formation was examined for lysis after 24-h incubation at 40 C. The results were recorded as positive (+) and negative (-) (Yoon *et al.*, 2001).

Isolation, restriction and agarose gel electrophoresis of Bacteriophage DNA

Isolation of bacteriophage DNA was carried out with concentrated bacteriophages according to the prodedure described by Maniatis *et al.* (1982).Purified bacteriophages were dialysed against buffer (5mM Tris-HCl, 10 mM $MgCl_2$) and extracted twice with tris buffer-saturated phenol. Bacteriophage DNA was precipitated with ethanol, and dissolved in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.5). Bacteriophage DNAs were digested with the restriction endonuclease *Eco*RI in a final volume of 50µl using the conditions specified by supplier (Amersham, Les Ulis, France). The fragments were subjected to electrophoresis on a 0.8 % agarose gel using TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8). Gels were stained with ethidium bromide and photographed under UV light (254 nm). Fragments of lambda DNA digests with *Hind* III were used as molecular size markers.

3. RESULTS AND DISCUSSION

Lytic activity of 24 bacteriophages, isolated from different dairy materials using by sensitive indicator dairy starter strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) (Table 1), were analysed on 24 Turkish strains of *Lb. bulgaricus*. End of double layer agar and spot tests, 24 *Lb. bulgaricus* bacteriophages were partially or completely lysed 18 strains out of 24 *Lb. bulgaricus* (Table 2). However, in the parallel research that carried out by Kaleli and Tunail (2003), all strains of *Streptococococcus salivarius* subsp. *thermophilus (Str. thermophilus)* were found to be resistant against *Lb. bulgaricus* specific bacteriophages. Because of their narrow and highly specialised host range characters, genomic analysis of *Str. thermophilus* bacteriophages were also done in this study. Codes and indicator strains of these bacteriophages, are shown in Table 3. Differentiation of bacteriophage by host susceptibility profiles is essential to the dairy industry in order to devise effective strain rotation programmes and to identify suitable bacteriophage-unrealated replacement strains (Daly *et al.*, 1995; Allison and Klaenhammer, 1998). Unfortunately, bacteriophage-host range data not adequately reflect phylogenetic relationships between various bacteriophages and are also subject to changes arising from minor DNA mutations, various genetic recombination/rearrangement events or even simply host mediated modification of bacteriophage DNA (Daly *et al.*, 1996; Akçelik *et al.*, 2000; Tükel *et al.*, 2002). Bacteriophage problems encountered in high-temperature dairy fermentations such as those used to make mozzarella cheese and yoghurt are due to mainly to *Str. thermophilus* bacteriophages (Moineau *et al.*, 1995; Mikkonen, 1996; Mikkonen *et al.*, 1996; Moineau, 1999). On the contrary of the data in these papers, we determined tahat the bacteriophages had species specificity on *Str. thermophilus* and *Lb. bulgaricus*. This character will make easy the selection of bacteriophage unrelated starter strains of *Str. thermophilus* and *Lb. bulgaricus*.

Genome sizes of the virulent bacteriophages were estimated by the cumulative sum of restriction fragment sizes after *Eco*RI digestion of genomic DNA samples (Figures: 1, 2, 3, 4 and 5). Genome sizes were ranged at 21.0 - 72.2 kb and 25.8 - 47.3 kb for *Lb. bulgaricus* and *Str. thermophilus* specific bacteriophages, respectively. The genome sizes of *Str. thermophilus* and *Lb. bulgaricus* bacteriophages usually ranged15-60 kb in lenght (Prevots *et al.*,1989; Boizet *et al.*, 1990; Hill, 1993; Garvey *et al.*, 1995; Allison and Klaenhammer, 1998, Aud *et al.*, 1999). Restriction patterns between *Str. thermophilus* and *Lb. bulgaricus* phages DNAs showed no similarty indicating that these phages derived from different parental origins. On the other hands, two group *Str. thermophilus* bacteriophages (Group 1: 709-X4, 709-X5, B3-X12, B3-X13, B3-X14, B3-X15, B3-X16, B3-X17, B3-X19, and Group 2: 231-X6, 231-X7, 231-X8, 231-X10, 231-X21 and 231-X22) (Figures 1 and 2) and two group *Lb. bulgaricus* bacteriophages (Group 1: MY4 and JY4; Group 2: GKV2, GY4 and HV1) (Figure 4 and Figure 5) showed exactly the same restriction patterns. These results pointed out that members of each groups of the bacteriophages are closely related. Furthermore, different restriction patterns of

bacteriphages indicated that these bacteriophages were derived from different parental types.

Table 1. Codes and indicator strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteriophages used in this study.

Bacteriophage	Host Bacterium	Titre (pfu/mL)
ØFY4	*Lb. bulgaricus* Y4	10^8
ØGY4	*Lb. bulgaricus* Y4	10^8
ØH1Y4	*Lb. bulgaricus* Y4	10^8
ØH2Y4	*Lb. bulgaricus* Y4	10^4
ØH3Y4	*Lb. bulgaricus* Y4	10^5
ØIY4	*Lb. bulgaricus* Y4	10^8
ØJY4	*Lb. bulgaricus* Y4	10^6
ØMY4	*Lb. bulgaricus* Y4	10^5
ØPY4	*Lb. bulgaricus* Y4	10^6
ØSIBY4	*Lb. bulgaricus* Y4	10^7
ØSIYY4	*Lb. bulgaricus* Y4	10^6
Ø709BY4	*Lb. bulgaricus* Y4	10^6
ØFV1	*Lb. bulgaricus* V1	10^6
ØHV1	*Lb. bulgaricus* V1	10^8
ØAV2	*Lb. bulgaricus* V2	10^6
ØGV2	*Lb. bulgaricus* V2	10^5
ØG1V2	*Lb. bulgaricus* V2	10^8
ØG3V2	*Lb. bulgaricus* V2	10^5
ØG4V2	*Lb. bulgaricus* V2	10^5
ØG5V2	*Lb. bulgaricus* V2	10^6
ØKV2	*Lb. bulgaricus* V2	10^7
ØGKV2	*Lb. bulgaricus* V2	10^6
ØLV2	*Lb. bulgaricus* V2	10^4
ØSIYV2	*Lb. bulgaricus* V2	10^5

Table 2. Host specificities of *Lb. bulgaricus* bacteriophages on *Lb. bulgaricus* strains originaly isolated from Turkey.

	Bacteriophages											
Bacteria l Strains	H2 Y4	M Y4	J Y4	F Y4	H3 Y4	H1 Y4	I Y4	SIY Y4	709B Y4	SIB Y4	G Y4	P Y4
406	-	-	-	-	-	-	-	-	-	-	-	-
412	-	-	-	-	-	-	-	-	-	-	-	-
2001	-	-	-	-	-	-	-	-	-	-	-	-
2004	-	-	-	-	-	-	-	-	-	-	-	-
3701	-	-	-	-	-	-	-	-	-	-	-	-
3702	-	-	-	-	10^3	10^7	10^3	-	-	-	10^7	10^4
4007	-	-	-	-	-	-	-	-	-	-	-	-
4008	10^7	10^6	10^6	10^6	10^7	10^7	10^6	10^6	10^7	10^6	10^5	10^7
4601	10^7	10^7	10^7	10^7	10^7	10^7	10^7	10^7	10^7	10^7	10^7	10^7
4603	10^7	10^6	10^7	10^7	10^7	10^6	10^7	10^7	10^5	10^6	10^6	10^7
4902	10^7	10^7	-	10^6	10^7	10^7	10^7	-	-	10^6	10^7	-
5002	10^6	10^4	-	-	10^6	10^3	10^6	-	-	-	-	10^5
5006	-	10^3	10^5	10^3	10^6	10^3	-	10^5	-	10^5	10^5	10^6
6003	10^4	10^5	10^5	-	10^5	-	10^6	10^5	10^5	10^7	10^4	10^6
6007	10^7	10^7	10^5	10^7	10^6	10^7	10^6	10^7	10^7	10^7	10^7	10^7
6301	10^6	10^6	10^6	10^7	10^7	10^7	10^6	10^7	10^6	10^6	10^7	10^7
6302	10^6	10^5	10^5	10^5	10^5	10^4	10^6	10^5	10^5	10^6	10^6	10^7
6502	-	-	-	-	-	-	-	-	-	-	-	-
6503	10^7	10^6	10^7	10^7	10^7	10^6	10^6	10^7	10^6	10^6	10^7	10^7
6604	10^7	10^6	10^6	10^6	10^7	10^6	10^6	10^7	10^7	10^6	10^7	10^6
6608	10^3	10^5	10^5	10^5	10^6	10^5	10^6	10^7	10^6	10^5	10^5	10^5
6803	10^7	10^7	10^7	10^7	10^7	10^6	10^7	10^7	10^7	10^7	10^7	10^7
7006	10^7	10^6	10^7	10^7	10^7	10^6	10^6	10^7	10^7	10^7	10^7	10^6
7004	10^7	10^7	10^7	10^7	10^7	10^6	10^7	10^7	10^7	10^7	10^7	10^7

Table 2. Host specificities of *Lb. bulgaricus* bacteriophages on *Lb. bulgaricus* strains originaly isolated from Turkey (Continue).

	Bacteriophages											
Bacteria l Strains	H V1	F V1	G V2	G1 V2	G3 V2	G4 V2	G5 V2	G K V2	K V2	L V2	A V2	SIY V2
406	-	-	-	-	-	-	-	-	-	-	-	-
412	-	-	-	-	-	-	-	-	-	-	-	-
2001	-	-	-	-	-	-	-	-	-	-	-	-
2004	-	-	-	-	-	-	-	-	-	-	-	-
3701	-	-	10^7	-	-	10^4	-	-	-	-	-	-
3702	-	10^6	10^7	10^5	10^3	-	10^5	-	-	-	-	-
4007	-	-	-	-	-	-	-	-	-	-	-	-
4008	10^7	10^6	10^6	10^7	10^7	10^6	10^7	10^6	10^7	10^7	10^6	10^5
4601	10^7	10^7	10^7	10^7	10^7	-	10^7	10^7	10^7	10^7	10^7	10^7
4603	10^7	10^7	10^7	10^7	10^5	10^6	10^7	10^7	10^7	10^6	10^7	10^7
4902	10^6	10^6	10^7	10^7	10^6	-	10^7	10^7	10^7	10^6	10^7	10^7
5002	-	-	10^3	-	10^4	-	10^6	-	10^7	-	10^5	-
5006	10^5	-	10^5	10^5	10^5	10^5	10^5	10^5	10^7	10^6	10^5	10^6
6003	10^6	10^5	10^5	10^5	10^5	10^5	-	10^7	-	10^3	10^5	10^6
6007	10^4	10^7	10^6	10^7	10^7	10^7	10^7	10^7	10^7	10^7	10^6	10^4
6301	10^6	10^6	10^6	10^6	10^6	10^7	10^7	10^6	10^6	10^6	10^6	10^7
6302	10^7	10^6	10^7	10^3	10^5	10^7	10^4	10^6	10^6	10^5	10^7	10^7
6502	-	-	-	-	-	-	-	-	-	-	-	-
6503	10^7	10^7	10^7	10^7	10^7	10^7	10^7	10^6	10^7	10^7	10^6	10^7
6604	10^6	10^7	10^6	10^6	10^7	10^6	10^6	10^7	10^7	10^6	10^7	10^7
6608	10^6	10^5	10^5	10^6	10^5	10^7	10^6	10^6	10^6	10^5	10^7	10^6
6803	10^5	10^6	10^6	10^6	10^7	10^6	10^7	10^6	10^6	10^7	10^6	10^6
7006	10^6	10^6	10^7	10^7	10^7	10^6	10^7	10^6	10^6	10^7	10^7	10^7
7004	10^7	10^7	10^6	10^6	10^7	10^6	10^7	10^7	10^7	10^7	10^5	10^6

-: No lysis
+: Lysis

Table 3. Codes and indicator strains of *Streptococcus salivarus* subsp. *thermophilus* bacteriophages used in this study.

Bacteriophage	Host Bacterium	Titre (pfu/mL)
Ø709-X1	*S. thermophilus* 709	$3x10^8$
Ø709-X2	*S. thermophilus* 709	$1x10^8$
Ø709-X3	*S. thermophilus* 709	$1x10^8$
Ø709-X4	*S. thermophilus* 709	$1x10^8$
Ø709-X5	*S. thermophilus* 709	$1x10^8$
Ø231-X6	*S. thermophilus* 231	$4x10^8$
Ø231-X7	*S. thermophilus* 231	$1x10^8$
Ø231-X8	*S. thermophilus* 231	$1x10^8$
Ø231-X9	*S. thermophilus* 231	$1x10^8$
Ø231-X10	*S. thermophilus* 231	$6x10^8$
ØB3-X11	*S. thermophilus* B3	$1x10^8$
ØB3-X12	*S. thermophilus* B3	$2.5x10^8$
ØB3-X13	*S. thermophilus* B3	$1x10^8$
ØB3-X14	*S. thermophilus* B3	$1x10^8$
ØB3-X15	*S. thermophilus* CH-1	$4x10^8$
ØB3-X16	*S. thermophilus* B3	$1x10^8$
ØB3-X17	*S. thermophilus* B3	$1x10^8$
ØB3-X18	*S. thermophilus* B3	$2x10^8$
ØB3-X19	*S. thermophilus* B3	$2x10^8$
ØB3-X20	*S. thermophilus* B3	$2x10^8$
Ø231-X21	*S. thermophilus* 231	$1x10^8$
Ø231-X22	*S. thermophilus* 231	$6x10^8$
Ø231-X23	*S. thermophilus* 231	$4x10^8$

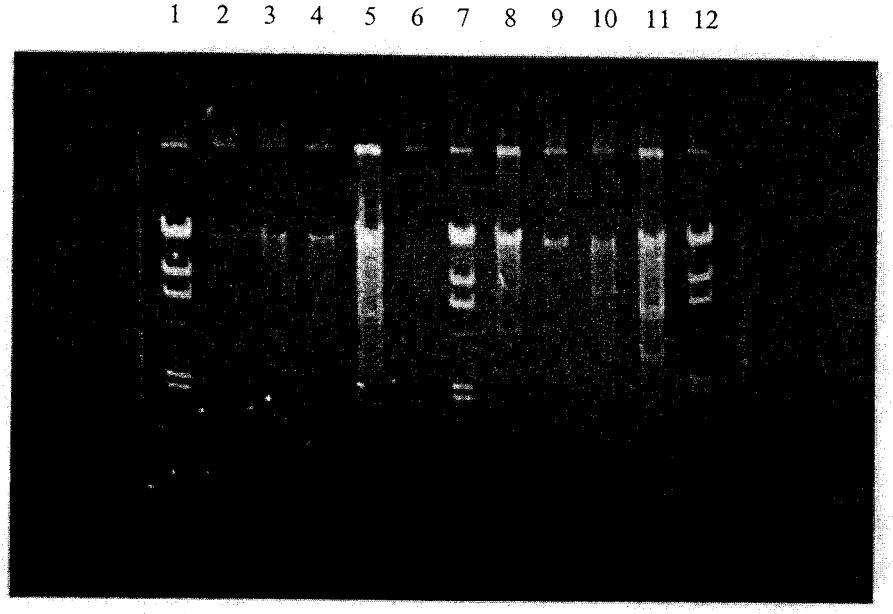

Figure 1: Restriction endonuclease patterns of *Streptococcus salivarus* subsp. *thermophilus* bacteriophage DNAs.

1 (Marker)* kb	2 (231-X6) *Eco*RI kb	3 (231-X7) *Eco*RI Kb	4 (231-X8) *Eco*RI kb	5 (231-X9) *Eco*RI Kb	6 (231-X10) *Eco*RI kb
23.1	21.2	21.2	21.2	23.1	21.2
9.4	4.6	4.6	4.6	9.4	4.6
6.5				5.7	
4.4				4.6	
2.3					
2.0					

7 (Marker)* kb	8 (231-X21) *Eco*RI kb	9 (231-X22) *Eco*RI Kb	10 (231-X23) *Eco*RI kb	11 (B3-X11) *Eco*RI Kb	12 (Marker)* kb
23.1	21.2	21.2	21.2	21.2	23.2
9.4	4.6	4.6	6.8	6.5	9.4
6.5			4.8	6.3	6.5
4.4				4.1	4.4
2.3					2.3
2.0					2.0

*λ bacteriophage DNA *Hind*III digest.

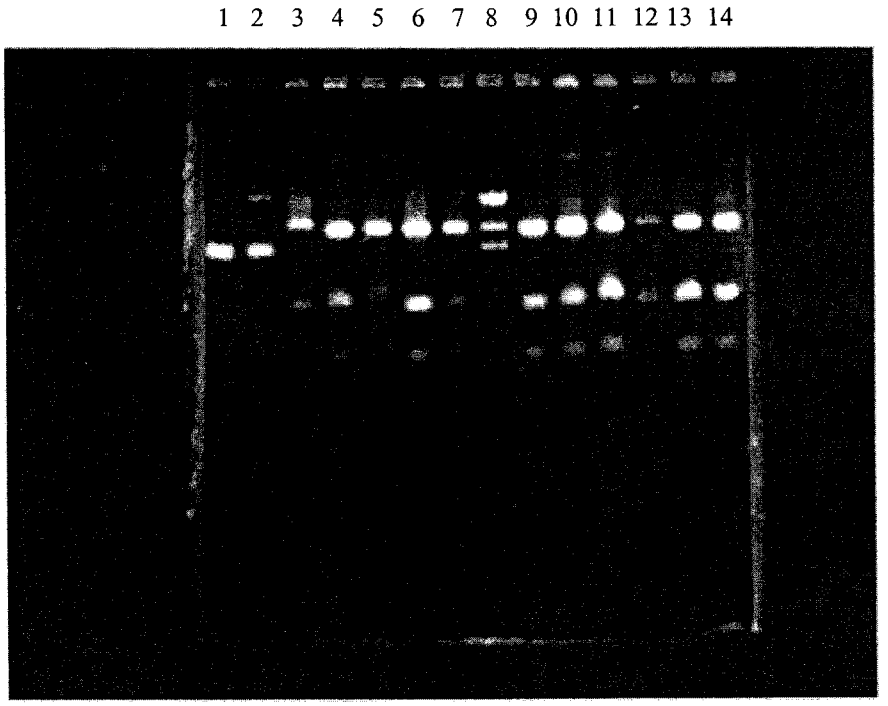

Figure 2: Restriction endonuclease patterns of *Streptococcus salivarus* subsp. *thermophilus* bacteriophage DNAs

1 (709-X1) *Eco*RI Kb	2 (709-X2) *Eco*RI Kb	3 (709-X3) *Eco*RI kb	4 (709-X4) *Eco*RI kb	5 (709-X5) *Eco*RI kb	6 (B3-X12) *Eco*RI kb	7 (B3-X13) *Eco*RI Kb
23.1	23.1	23.1	27.6	27.6	27.6	27.6
6.5	6.5	10.0	9.4	9.4	9.4	9.4
	1.8	3.5	3.5	3.5	3.5	3.5
		1.8	1.8	1.8	1.8	1.8
8 (Marker)* kb	9 (B3-X12) *Eco*RI Kb	10 (B3-X14) *Eco*RI kb	11 (B3-X15) *Eco*RI kb	12 (B3-X16) *Eco*RI kb	13 (B3-X17) *Eco*RI kb	14 (B3-X19) *Eco*RI Kb
23.1	27.6	27.6	27.6	27.6	27.6	27.6
9.4	9.4	9.4	9.4	9.4	9.4	9.4
6.5	3.5	3.5	3.5	3.5	3.5	3.5
4.4	1.8	1.8	1.8	1.8	1.8	1.8
2.3						
2.0						

*λ bacteriophage DNA *Hind*III digest.

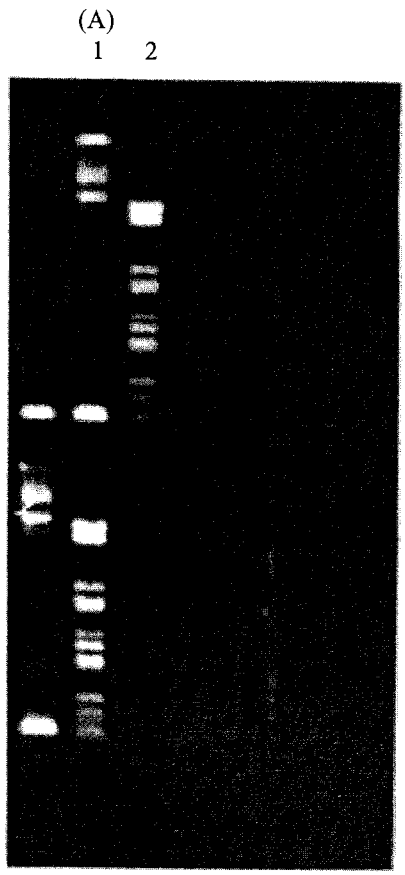

Figure 3. *Eco*RI restriction endonuclease patterns of *Streptococcus salivarus* subsp. *thermophilus* bacteriophage B3-X20 DNA.

A1-B1 (B3-X20) *Eco*RI kb	A2-B2 (Marker)* kb
24.2	21.2
21.5	5.1
1.6	5.0
	4.3
	3.5
	2.1
	1.9
	1.6

*λ bacteriophage DNA *Hind*III digest.

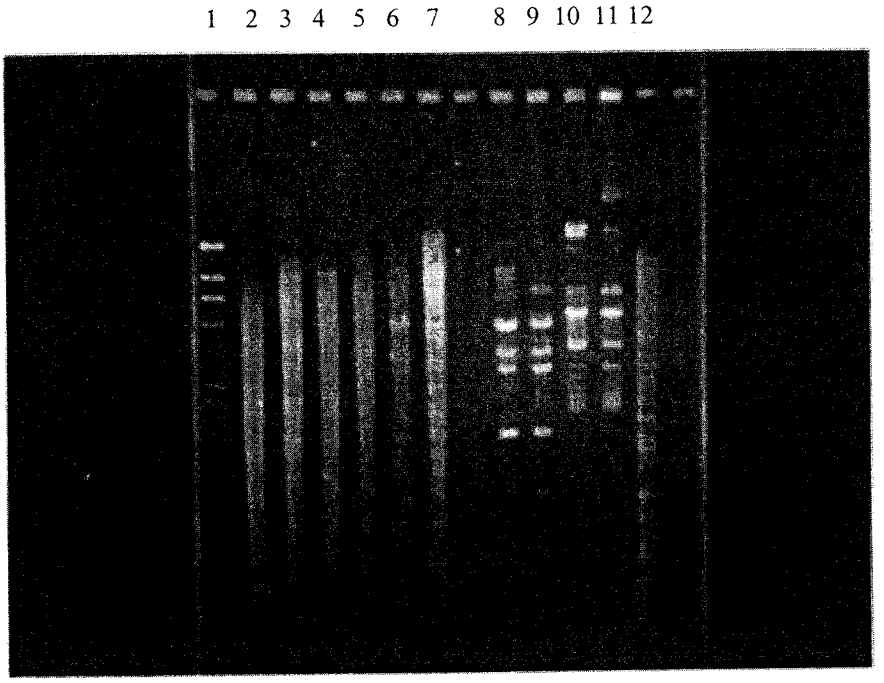

Figure 4. Restriction endonuclease patterns of *Lactobacillus delbrueckii* subsp. *bulgarius* bacteriophage DNAs.

1 (Marker)* kb	2 (PY4) *Eco*RI kb	3 (MY4) *Eco*RI Kb	4 (JY4) *Eco*RI kb	5 (IY4) *Eco*RI kb	6 (SIYY4) *Eco*RI kb
23.1	25.5	25.5	25.5	25.5	9.4
9.4	9.4	24.0	24.0	9.4	6.5
6.5		9.4	9.4	4.2	4.4
4.4					3.5
2.3					3.1
2.0					2.3
7 (709BY4) kb	8 (SIBY4) *Eco*RI kb	9 (KV2) *Eco*RI Kb	10 (LV2) *Eco*RI kb	11 (AV2) *Eco*RI kb	12 (SIYV2) kb
23.1	9.4	8.4	24.1	26.4	19.4
6.5	9.1	4.4	23.9	24.1	3.8
4.4	8.3	3.5	8.3	8.3	3.2
3.3	4.4	3.1	4.6	4.6	2.3
3.1	3.5	1.6	3.6	3.6	2.0
	3.1		3.2	3.2	
	1.6		2.0	2.0	

*λ bacteriophage DNA *Hind*III digest.

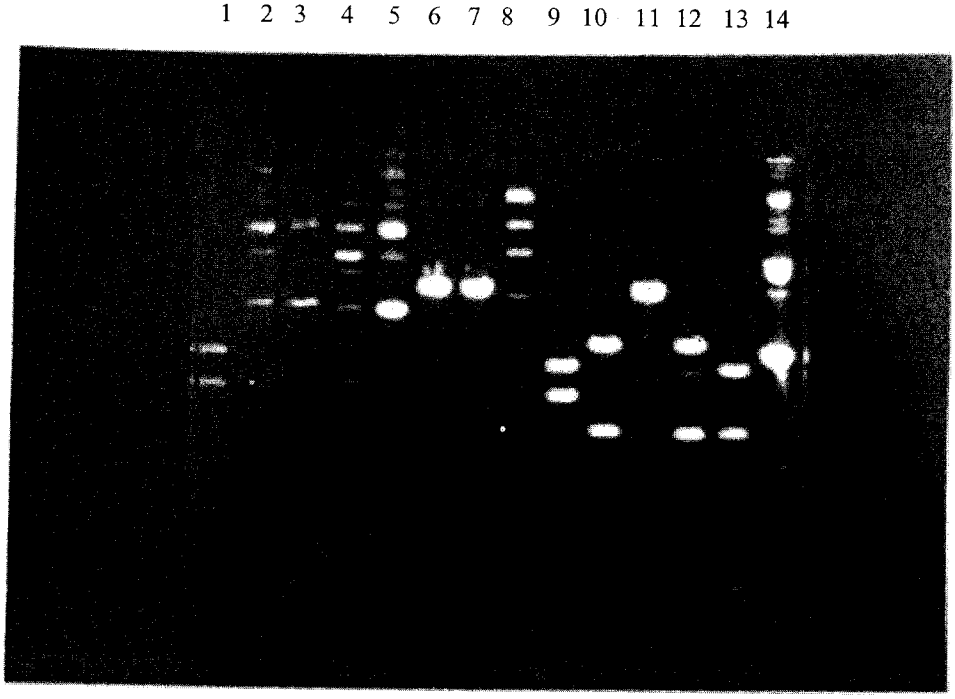

Figure 5. Restriction endonuclease patterns of *Lactobacillus dalbrueckii* subsp. *bulgaricus* bacteriophage DNAs.

1 (GV2) *Eco*RI Kb	2 (G1V2) *Eco*RI kb	3 (G3V2) *Eco*RI kb	4 (G4V2) *Eco*RI kb	5 (G5V2) *Eco*RI kb	6 (GKV2) *Eco*RI Kb	7 (GY4) *Eco*RI kb
9.4	25.3	25.3	18.8	25.3	23.1	23.1
6.5	18.8	9.4	9.4	18.8	4.6	4.6
3.5	9.4	4.2	6.5	9.4		
2.3	6.5		6.3	6.5		
	4.2		4.2	4.2		
			3.5			
			2.3			

8 (Marker)* kb	9 (FY4) *Eco*RI kb	10 (FV1) *Eco*RI kb	11 (HV1) *Eco*RI kb	12 (H1Y4) *Eco*RI kb	13 (H2Y4) *Eco*RI Kb	14 (H3Y4) *Eco*RI kb
23.1	20.6	20.6	23.1	20.6	20.6	23.1
9.4	3.3	3.7	4.6	3.7	3.5	9.6
6.5	2.1	1.8		3.5	1.8	9.4
4.4				1.8		6.5
2.3						4.6
2.0						3.6

*λ bacteriophage DNA *Hind*III digest.

ACKNOWLEDGEMENT

This research was supported by the Scientific and Technical Research Council of Turkey (TUBITAK) under project TUBITAK-TARP 2106. We thank Yasin TUNCER for technical assistance.

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