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## Classification of Virulent Lactococcal Bacteriophages Based on Protein Composition and Restriction Endonuclease Analysis

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**Abstract:** Twenty-four virulent bacteriophages isolated from raw milk and whey samples were characterised by structural protein composition and bacteriophage DNA restriction endonuclease fragments. Twenty-four lactococcal bacteriophages were possessed among 24-31 total structural proteins with molecular masses of 6.5-208.9 kDa and 2-8 major structural proteins with molecular masses of 6.5-38 kDa. Lactococcal bacteriophages were classified as 5 different groups by total structural proteins and 4 different groups by major structural proteins. The genome sizes of bacteriophages were estimated between 19.6 and 39 kb. Twenty-four lactococcal bacteriophages were classified into 8 groups based on the same restriction endonuclease recognition and restriction sites on the lactococcal bacteriophage genomes.

**Key Words:** *Lactococcus lactis*, bacteriophage, protein profile, restriction analysis

### Virulent Laktokok Bakteriyofajlarının Protein Kompozisyonu ve Restriksiyon Endonukleaz Analizleri Esas Alınarak Sınıflandırılması

**Özet:** Çiğ süt ve peyniraltı sularından izole edilen yirmidört bakteriyofaj, yapısal protein kompozisyonu ve faj DNA restriksiyon endonukleaz fragmentleri kullanılarak karakterize edildi. 24 laktokok bakteriyofajında 6,5-208,9 kDa moleküler büyüklükte 24-31 adet arasında değişen sayıda toplam yapısal protein ve 6,5-38 kDa moleküler büyüklükte 2-8 adet arasında değişen sayıda major yapısal protein saptandı. Laktokok bakteriyofajları toplam yapısal protein içeriklerine göre 5 farklı grup, major yapısal protein içeriklerine göre ise 4 farklı grup altında sınıflandırıldı. 24 laktokok bakteriyofajının toplam genom büyüklükleri 19,6-39 kb arasında tanımlandı. Laktokok bakteriyofaj genomlarında restriksiyon endonukleaz enzim tanıma ve kesim bölgelerinin benzerliğine göre 8 farklı faj grubu oluşturuldu.

**Anahtar Sözcükler:** *Lactococcus lactis*, bakteriyofaj, protein profili, restriksiyon analizi

### Introduction

*Lactococcus lactis* species are susceptible to bacteriophage infections. These infections are the major cause of significant product losses in the dairy industry worldwide. Because of this, studies have focused on the bacteriophage, bacteriophage-host relationships and their genetic nature. A number of studies have been undertaken to define reliable bacteriophage classification criteria and to form phage collections (1-3). Initially, lactococcal bacteriophages were classified by host range, serology and morphology (4-6), but with the development of molecular technology they have also been classified on a DNA and structural protein basis (5,7,8). Analyses of lactococcal bacteriophages at the genetic and

molecular levels have only recently been undertaken, but despite this there have been significant advantages in the understanding of their origins, evolution, relationships, and genome structure (1,9,10). In contrast to the genomic studies, the proteins of the lactococcal bacteriophages have received little attention except for the preparation of structural protein profiles of bacteriophages c6A and MU1 (5,9-12).

In this study, we classified 24 bacteriophages isolated from raw milk and whey samples of traditional cheeses produced in Turkey based on the structural protein composition, genome size and restriction endonuclease digest profiles.

## Materials and Methods

### Bacterial Strains, Bacteriophages and Growth Media

The 98 bacterial strains and 24 virulent bacteriophages used in this study were obtained from the Culture Service of Ankara University. *Lactococcus lactis* strains were propagated at 30 °C in M17 broth or on M17 agar plates supplemented with 0.5% glucose per litre medium (13). M17 broth was also used for bacteriophage propagation. Bacteriophages and culture stocks were kept at -18 °C in 40% glycerol.

### Bacteriophage Assays

All bacteriophages isolated from a single plaque and host range studies were conducted by spot-test as described earlier (14).

### SDS-Polyacrylamide Gel Electrophoresis

SDS-Page analyses of bacteriophage structural proteins were done as described by Laemmli (15). Bacteriophage particles were precipitated by ultracentrifugation (60 min, 50000 rev min<sup>-1</sup>, + 4 °C). The samples were suspended with equal volume of sample buffer. They were boiled for 15 min and then electrophoresed on a polyacrylamide gel at 35 mA. Protein molecular weight marker was used for calibration as references (SDS molecular weight marker, Cat. No.161-0317, Bio-Rad, USA).

### Bacteriophage DNA Isolation

Bacteriophage deoxyribonucleic acid was isolated from high titre bacteriophage preparations. Bacteriophage particles were pelleted as described above. They were suspended in TE buffer (pH 7.5) and treated with 10% SDS and proteinase K (25 mg/ml). After incubation at 37 °C for 10 min, they were extracted by chloroform-isoamyl alcohol and concentrated by ethanol precipitation as described by Maniatis et al. (16).

### Restriction Enzyme Analysis of Bacteriophage DNA Samples

CsCl-ethidium bromide density gradient centrifugation was used for purifying bacteriophage DNA samples as described by Klaenhammer et al. (17). Purified DNA samples were digested with *EcoRI* and *BamHI* (Sigma Chem. Co., USA). Enzyme preparations were added in bacteriophage DNA samples suspended in TE buffer (pH 7.5). Restriction fragments were heat-

treated (37 °C for 60 min) (16). They were separated on 0.7% agarose by gel electrophoresis in TAE buffer (pH 8), followed by staining in ethidium bromide and visualised by UV as described by Meyers et al. (18).

## Results

Twenty-four bacteriophages were isolated from raw milk and whey samples of traditional cheeses produced in Turkey against 10 strains of *L. lactis* (Table 1). Host range of all 24 bacteriophages was determined on 98 different *L. lactis* strains. Only two bacteriophages ( $\Phi$ plc 61-56 and  $\Phi$ plc 61-58) showed identical host range (data not shown). Bacteriophage structural protein profiles were examined by SDS-polyacrylamide gel electrophoresis (Figure 1). Twenty-four lactococcal bacteriophages were possessed among 24-31 total structural proteins with molecular masses of 6.5-208.9

Table 1. Bacteriophages and *Lactococcus lactis* host strains used in this study.

Phages	Homologous Host Strains
$\Phi$ plc 61-56	<i>L. lactis</i> subsp. <i>cremoris</i> PLC 61
$\Phi$ plc 61-58	<i>L. lactis</i> subsp. <i>cremoris</i> PLC 61
$\Phi$ pll 35-6	<i>L. lactis</i> subsp. <i>lactis</i> PLL 35
$\Phi$ pll 35-8	<i>L. lactis</i> subsp. <i>lactis</i> PLL 35
$\Phi$ pll 36-10	<i>L. lactis</i> subsp. <i>lactis</i> PLL 36
$\Phi$ pll 36-14	<i>L. lactis</i> subsp. <i>lactis</i> PLL 36
$\Phi$ pll 36-15	<i>L. lactis</i> subsp. <i>lactis</i> PLL 36
$\Phi$ pll 47-21	<i>L. lactis</i> subsp. <i>lactis</i> PLL 47
$\Phi$ pll 98-22	<i>L. lactis</i> subsp. <i>lactis</i> PLL 98
$\Phi$ pll 98-23	<i>L. lactis</i> subsp. <i>lactis</i> PLL 98
$\Phi$ pll 98-25	<i>L. lactis</i> subsp. <i>lactis</i> PLL 98
$\Phi$ pll 98-26	<i>L. lactis</i> subsp. <i>lactis</i> PLL 98
$\Phi$ pll 98-28	<i>L. lactis</i> subsp. <i>lactis</i> PLL 98
$\Phi$ pll 98-32	<i>L. lactis</i> subsp. <i>lactis</i> PLL 98
$\Phi$ pll 6-2	<i>L. lactis</i> subsp. <i>lactis</i> PLL 6
$\Phi$ pll 10-5	<i>L. lactis</i> subsp. <i>lactis</i> PLL 10
$\Phi$ pld 64-33	<i>L. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> PLD 64
$\Phi$ pld 66-36	<i>L. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> PLD 66
$\Phi$ pld 67-38	<i>L. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> PLD 67
$\Phi$ pld 67-39	<i>L. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> PLD 67
$\Phi$ pld 67-41	<i>L. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> PLD 67
$\Phi$ pld 67-42	<i>L. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> PLD 67
$\Phi$ pld 67-43	<i>L. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> PLD 67
$\Phi$ pld 67-44	<i>L. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> PLD 67

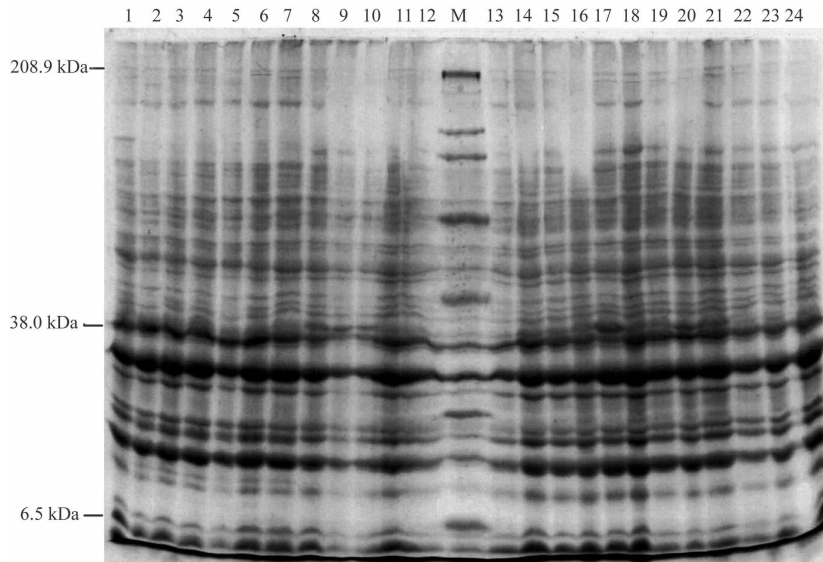


Figure 1. SDS - Polyacrylamide gel electrophoresis of structural proteins of lactococcal bacteriophages.

kDa : Kilodalton , Lane M: Protein molecular weight marker

Lanes, 1:  $\Phi$ pll 98-22; 2:  $\Phi$ pll 98-23; 3:  $\Phi$ pll 98-25; 4:  $\Phi$ pll 98-26; 5:  $\Phi$ pll 98-28; 6:  $\Phi$ pll 98-32; 7:  $\Phi$ pll 35-6; 8:  $\Phi$ pll 35-8; 9:  $\Phi$ pll 36-10; 10:  $\Phi$ pll 36-14; 11:  $\Phi$ pll 36-15; 12:  $\Phi$ pll 6-2; M: (200.0, 116.2, 97.4, 66.2, 45.0, 31.0, 21.5, 14.4, 6.5 kDa); 13:  $\Phi$ pld 67-38; 14:  $\Phi$ pld 67-39; 15:  $\Phi$ pld 67-41; 16:  $\Phi$ pld 67-42; 17:  $\Phi$ pld 67-43; 18:  $\Phi$ pld 67-44; 19:  $\Phi$ pld 64-33; 20:  $\Phi$ pld 66-36; 21:  $\Phi$ pll 10-5; 22:  $\Phi$ plc 61-56; 23:  $\Phi$ plc 61-58; 24:  $\Phi$ pll 47-21

kDa. These bacteriophages were classified as five different groups by total structural proteins. Eight bacteriophages were assigned to Group I containing 31 structural proteins, 11 bacteriophages to Group II containing 30 structural proteins, 2 bacteriophages to Group III containing 29 structural proteins, 2 bacteriophages to Group IV containing 27 structural proteins and 1 bacteriophage to Group V containing 24 structural proteins (Figure 1 and Table 2). Group II bacteriophages with the absence of 120.3 kDa protein band, Group III bacteriophages with the absence of 166.0 and 120.3 kDa protein bands, Group IV bacteriophages with the absence of 208.9, 190.5, 166.0 and 120.3 kDa protein bands, Group V bacteriophages with the absence of 208.9, 190.5, 166.0, 125.9, 120.3, 112.2 and 104.7 kDa protein bands differed from Group I bacteriophages (Figure 1). All 24 bacteriophages were possessed among 2-8 major structural proteins with molecular masses of 6.5-38 kDa. They were classified as four different groups by major structural proteins. One bacteriophage was assigned to Group I containing 8 major structural proteins (38.0, 33.1, 25.1, 24.0, 20.0,

17.8, 14.1 and 6.5 kDa), 20 bacteriophages to Group II containing 7 major structural proteins (33.1, 25.1, 24.0, 20.0, 17.8, 14.1 and 6.5 kDa), 1 bacteriophage to Group III containing 5 major structural proteins (33.1, 25.1, 17.8, 14.1 and 6.5 kDa) and 2 bacteriophages to Group IV containing 2 major structural proteins (25.1 and 14.1 kDa) (Figure 1 and Table 2). According to total structural protein groups, all Group I, Group III, Group V and Group II bacteriophages (except for  $\Phi$ pld 67-38 and  $\Phi$ pld 67-43) contained exactly the same major structural protein bands.

DNAs from each 24 bacteriophages were digested with *EcoRI* and *BamHI*. Total genome sizes of 24 lactococcal bacteriophages were 19.6 to 39 kb based on the sum of the sizes of the restriction fragment sizes (Figure 2 and Table 3). Eight different bacteriophage groups were constructed according to the same restriction endonuclease recognition and restriction sites on the lactococcal bacteriophage genomes. Two bacteriophages (genome sizes 27.2 kb) were assigned to Group I, 5 bacteriophages (19.6 kb) to Group II, 2

Table 2. Bacteriophage groups according to total and major structural protein composition.

Bacteriophage Groups Based on Total Structural Protein Composition	Bacteriophages	Bacteriophage Groups Based on Major Structural Protein Composition	Bacteriophages
Group I (31 structural proteins)	ΦpII 98-22 ΦpII 98-23 ΦpII 98-25 ΦpII 98-26 ΦpII 98-28 ΦpII 98-32 ΦpII 35-6 ΦpII 35-8	Group I (8 major structural proteins)	ΦpId 67-43
Group II (30 structural proteins)	ΦpII 36-15 ΦpII 6-2 ΦpId 67-38 ΦpId 67-39 ΦpId 67-41 ΦpId 67-43 ΦpId 67-44 ΦpII 10-5 ΦpIc 61-56 ΦpIc 61-58 ΦpII 47-21	Group II (7 major structural proteins)	ΦpII 98-22 ΦpII 98-23 ΦpII 98-25 ΦpII 98-26 ΦpII 98-28 ΦpII 98-32 ΦpII 35-6 ΦpII 35-8 ΦpII 36-15 ΦpII 6-2 ΦpId 67-39 ΦpId 67-41 ΦpId 67-44 ΦpII 10-5 ΦpIc 61-56 ΦpIc 61-58 ΦpII 47-21 ΦpId 64-33 ΦpId 66-36 ΦpId 67-42
Group III (29 structural proteins)	ΦpId 64-33 ΦpId 66-36	Group III (5 major structural proteins)	ΦpId 67-38
Group IV (27 structural proteins)	ΦpII 36-10 ΦpII 36-14	Group IV (2 major structural proteins)	ΦpII 36-10 ΦpII 36-14
Group V (24 structural proteins)	ΦpId 67-42		

bacteriophages (22.2 kb) to Group III, 4 bacteriophages (23.8 kb) to Group IV, 4 bacteriophages (24.4 kb) to Group V, 2 bacteriophages (21.2 kb) to Group VI, 3 bacteriophages (36 kb) to Group VII and 2 bacteriophages (39 kb) to Group VIII (Figure 2 and Table 3).

Exactly the same host ranges, total structural protein profiles and restriction patterns of the ΦpIc 61-56 and ΦpIc 61-58 (Figures 1 and 2) indicated that these bacteriophages were the same species.

**Discussion**

Our results and the data presented in the literature (2,5,9,10,18) showed those lactococcal bacteriophages have a similar or an identical major and minor structural protein composition. We only have found slight differences between the minor and major structural protein profiles of some tested bacteriophages. There was no correlation between the bacteriophage groups based on total and major structural protein profiles of lactococcal bacteriophages. More detailed studies of further virulent bacteriophages will be needed to clarify the significance of these results.

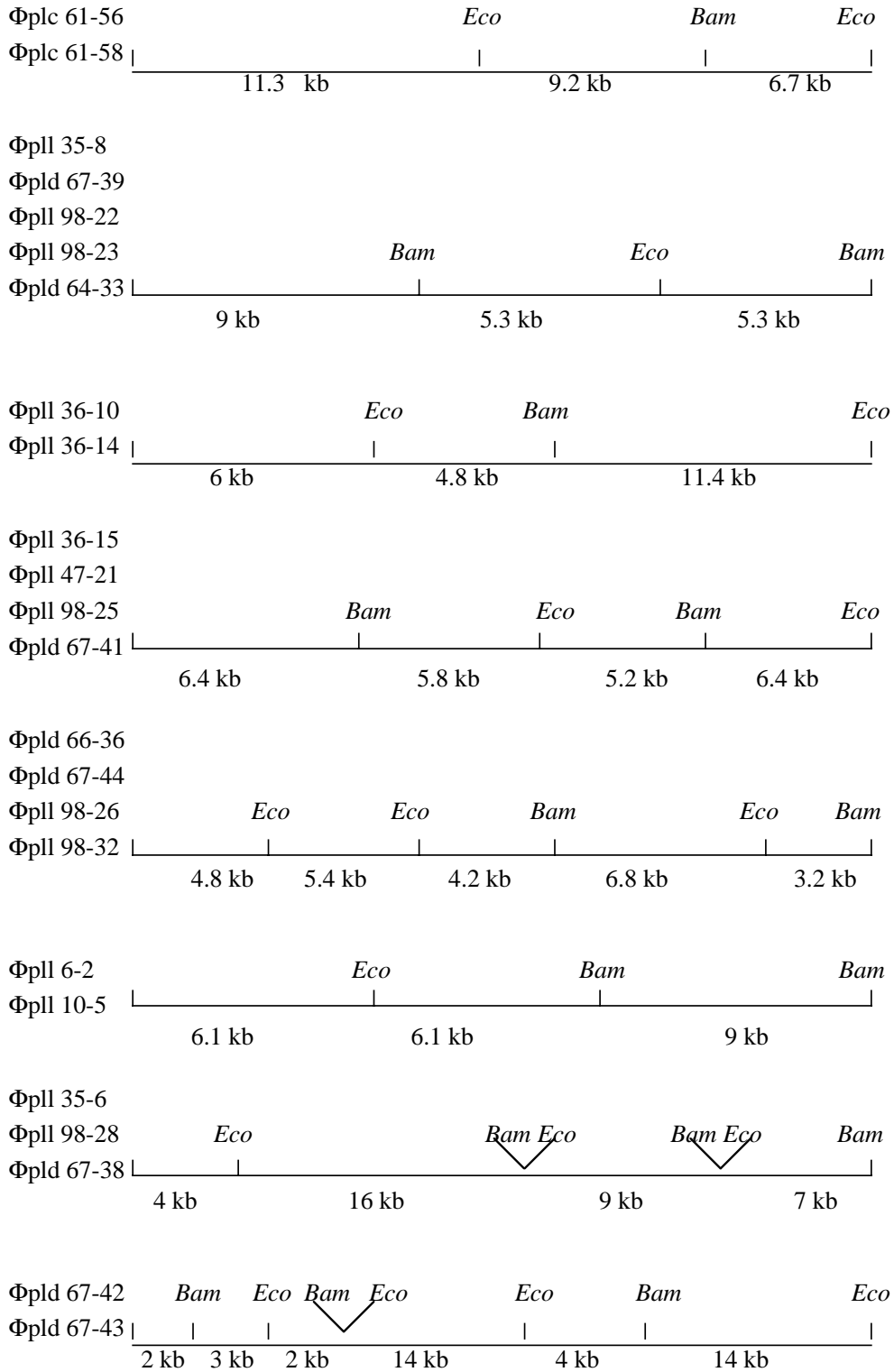


Figure 2. Endonuclease cleavage site maps of bacteriophage DNAs. Cleavage sites are marked by *Eco* (*Eco*RI) and *Bam* (*Bam*HI).  
 kb: Kilobase

Table 3. Bacteriophage groups according to the same restriction endonuclease recognition and restriction sites on the lactococcal bacteriophage genomes.

Group	Bacteriophages	Genome Size (kb)
I	Φpic 61-56 Φpic 61-58	27.2
II	ΦpII 35-8 ΦpId 67-39 ΦpII 98-22 ΦpII 98-23 ΦpId 64-33	19.6
III	ΦpII 36-10 ΦpII 36-14	22.2
IV	ΦpII 36-15 ΦpII 47-21 ΦpII 98-25 ΦpId 67-41	23.8
V	ΦpId 66-36 ΦpId 67-44 ΦpII 98-26 ΦpII 98-32	24.4
VI	ΦpII 6-2 ΦpII 10-5	21.2
VII	ΦpII 35-6 ΦpII 98-28 ΦpId 67-38	36.0
VIII	ΦpId 67-42 ΦpId 67-43	39.0

The estimated genome sizes of the 24 virulent lactococcal bacteriophages in this study were very similar to other lactococcal virulent bacteriophages described previously (4,6,18-20). The restriction mapping studies highlighted a marked paucity of restriction sites on the genomes of lactococcal bacteriophages originating from Turkish dairy samples. In certain *Lactococcus* bacteriophages the paucity or lack of recognition sites for specific restriction endonucleases has been linked to presence of the corresponding enzymes in the bacterial host (4,12,20-23).

When both classification diagrams having many members were compared, only two bacteriophages (ΦpII 36-10 and ΦpII 36-14) were determined in the same groups. Based on previous reports (3,18,21,24,25) and the results described above, there was no good correlation between grouping bacteriophages based on structural protein composition and genomic DNA restriction endonuclease patterns. Results of this study also indicate that bacteriophage DNA restriction endonuclease fragment length polymorphism was more reliable than bacteriophage structural protein profiling for the determination of similarities and differences of lactococcal bacteriophages.

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