GIDA (2010) 35 (5): 317-323 *Research* /Araştırma

MORPHOLOGICAL CHARACTERIZATION OF *STREPTOCOCCUS THERMOPHILUS* AND *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* VIRULENT PHAGES

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Received / Geliş tarihi: 05.12.2009
Received in revised form / Düzeltilerek geliş tarihi: 11.02.2010
Accepted / Kabul tarihi: 14.02.2010

Abstract

In this study, 25 phages of *S. thermophilus* and 25 phages of *L. bulgaricus* were inspected and identified morphologically by electron microscopy. In *S. thermophilus* phages the diameters of hexagonal heads were found to range between 47-74 nm, while non-contractile tails were 182 to 290 nm long and 7-14 nm wide. In these phages tail plaque, collar and fiber like structures were not found. Identified *S. thermophilus* phages were placed in the *Siphoviridae* family according to Ackermann's and/or Group B in Bradley's classification. Also for *L. bulgaricus* phages isometric hexagonal capsid and non-contractile tail structures were also determined. The capsids measured between 47-73 nm in diameter, and the tails were between 117-162 nm long and 7 to 13 nm wide. These phages were also placed in *Siphoviridae* family in Ackermann's classification and/or group B in Bradley's. Importantly, it was also determined that in some of these phages collar and tail plaque structures were present. It is thought that minor modifications in the preparation of the *S. thermophilus* and *L. bulgaricus* phages, and the type of electron microscope used were significant factors that affected the visibility of the tail structures.

Keywords: *S. thermophilus*, *L. bulgaricus*, phage, morphological characterization, electron microscope

STREPTOCOCCUS THERMOPHİLUS VE *LACTOBACİLLUS DELBRUECKİİ* SUBSP. *BULGARİCUS* VİRÜLENT FAJLARININ MORFOLOJİK KARAKTERİZASYONU

Özet

Bu çalışmada 25 adet *S. thermophilus* ve 25 adet *L. bulgaricus* fajının elektron mikroskobik incelemesi yapılarak morfolojik karakterizasyonu gerçekleştirilmiştir. *S. thermophilus* fajlarında izometrik, hegzagonal baş çapının 53-74 nm, kontraktil olmayan kuyruk uzunluğunun 182-290 nm ve kuyruk genişliğinin de 7-14 nm arasında değiştiği görülmüştür. Bu fajlarda yaka, kuyruk plağı ve fibril benzeri yapıya rastlanmamıştır. İncelenen tüm fajlar, elde edilen verilere dayanılarak diğer *S. thermophilus* fajları gibi Ackermann sınıflaması *Siphoviridae* familyasına ve/veya Bradley sınıflaması B grubuna dâhil edilmiştir. *S. thermophilus* fajlarında olduğu gibi *Lb. bulgaricus* fajlarında da izometrik, hegzagonal kapsit ve kontraktil olmayan kuyruk yapısı belirlenmiştir. Kapsit çapları 47-73 nm arasında değişirken, kontraktil olmayan kuyruk uzunlukları 117-162 nm ve kuyruk enleri 7-13 nm arasında bulunmuştur. Ackermann sınıflaması *Siphoviridae* familyasına ve/veya Bradley sınıflaması B grubuna dâhil edilen bu fajlarda yaka, kuyruk tablası ve fibril yapısının varlığı dikkat çekmiştir. *S. thermophilus* ve *L. bulgaricus* faj örneklerinin hazırlanmasındaki farklılıkların ve kullanılan elektron mikroskop tiplerinin kuyruk yapılarının görünebilirliğini etkilediği düşünülmüştür.

Anahtar kelimeler: *S. thermophilus*, *L. bulgaricus*, faj, morfolojik karakterizasyon, elektron mikroskop

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INTRODUCTION

Knowledge of thermophilic phages is quite restricted when compared to that of lactococcal phages (1), owing to the high incidence of phage infections in the cheese production industry (2). Thus lactococcal phages have been under investigation since first being determined by Whitehead and Cox in 1935 (3). However the first phage specific to *Streptococcus thermophilus* (*S. thermophilus*) was determined in 1952 (4) and a *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) phage was isolated first by Reddy and Reinbold in 1974 with further investigations being done in 1982 (5). In addition, in recent years researches have focused more on thermophilic phages because of the frequent infections in cheese and yogurt plants which they cause (6).

To this day, morphological studies have shown *S. thermophilus* and *L. bulgaricus* phages to have a single morphotype. All have a hexagonal capsid and a non-contractile tail, and all of them are placed in the *Siphoviridae* family according to Ackermann's classification and/or in group B in Bradley's classification (2, 5-9). A *L. bulgaricus* phage that shows a different morphotype from other thermophilic phages is the only phage having a contractile tail and is placed in the *Myoviridae* family according to Ackermann's classification (5).

During the last 6 years, a thermophilic phage problem has periodically occurred in modern yogurt factories in Turkey (10). In order to solve the phage problem, it is important that, in addition to conventional prevention measures, factories should rotate between commercial strains the sensitivity of which has been tested against native phage collections (11, 12). It is also important that factories should use natural (indigenous) phage resistant strains because it has been shown that natural strains are much more resistant to phages than commercial strains (6). For this purpose 23 *S. thermophilus* and 25 *L. bulgaricus* phages were provided from our phage collection. This research focuses on the morphological characterization of these 48 phages using a transmission electron microscope (TEM). Owing to the limited data on the isolation of *S. thermophilus* phages from raw milk samples (12) the incidence of such phages in raw milk was also investigated. Two *S. thermophilus* phages isolated from raw milk samples were characterized morphologically and identified taxonomically along with other 48 phages.

MATERIALS AND METHODS

Phage and strain cultures

Twenty three *S. thermophilus* phages and twenty five *L. bulgaricus* phages were provided from our collection. These phages had been isolated and purified from bulk, yogurt, whey and ayran-Turkish buttermilk collected from dairy plants in the Ankara region. Bacterial strains (*S. thermophilus* B3, 709, 231 and *L. bulgaricus* Y4, V1, V2, 231) from which the phages were isolated were mixed starter cultures used industrially. The two *S. thermophilus* phages (Φ1B3-A, Φ2B3-A) from raw milk samples from the Afyon region were also isolated using the B3 industrial strain as a host organism. The isolation of all phages was carried out single plaque isolation technique as described previous study (13). To study the growth of *S. thermophilus* strains and their phages, modified M17 Broth (thMl7 Broth), modified M17 Agar (th M17 Agar) and modified M17 Soft Agar (th M17 Soft Agar) (0.45% agar) were used (14). *L. bulgaricus* strains and their phages were grown in MRS Broth (Merck, Darmstadt, Germany), MRS Agar (1.5% agar) and MRS Soft Agar (0.6% agar). In order to achieve a better adsorption of *L. bulgaricus* phages, $CaCl_2$ (10 mmol/l) was added to all three types of culture medium (15). All cultures and phages were incubated for 18 h at 43°C.

Electron microscopy of phages

S. thermophilus and *L. bulgaricus* phages were concentrated through the centrifugation of phage lysates containing 10^7-10^8 phage particles (pfu/ml) for 2 h at 20000 rpm at 4°C. Some of *S. thermophilus* phage pellets were dissolved in 20 µl 0.3 M ammonium acetate and an equal volume of dye solution (10 µl amonium molibdate and 10 µl sodium phosphotungstate, 2 % - 3 %, w/v, pH 5.0 ± 0.02, Sigma Chem. Co., USA) was added (16). The phage-dye mixture was then dropped on to 400 mesh grids covered with carbon formvar (3.05 mm, Agar Scientific Ltd. UK). After waiting for 15 minutes, the excess dye was removed. Electron micrographs were taken with TEM, models JEOL JEM S 100 and JEOL JEM 100-C under 80 kV power. The pellets of the other *S. thermophilus* (Φ1B3-A, Φ2B3-A, ΦB3-X12, ΦB3-X13, ΦB3-X18, Φ231-X9, Φ231-X23) and all of the *L. bulgaricus* phages were suspended in 0.1 % (w/v) ammonium acetate first

(17), then 20 µl 3 % phosphotungstate was added and finally they were placed on the grids (18). Fifteen minutes later, the grids were washed with deionised water and dried on a filter paper (19). Micrographs of the phages were taken under 80 kV power by using a LEO 906 E electron microscope. Phage size calculations are based on the averages of 5 to 10 measurements (20).

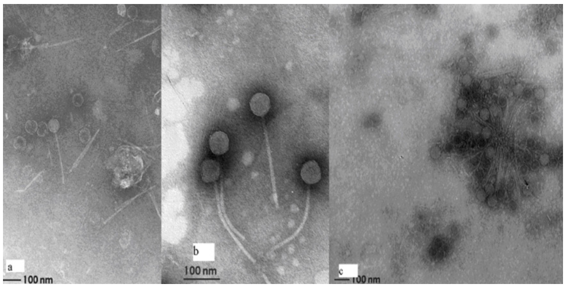

Figure 1. S. thermophilus phages. (a) Φ231-X9, 60000x; (b) ΦB3-X18, 100000x; (c) Φ709-X4, 45 000x

RESULTS

Morphology of *S. thermophilus* phages

Phages isolated by using *S. thermophilus* 709, 231 and B3 industrial hosts had isometric, hexagonal and assumptively icosahedral capsids, 47-74 nm in diameter, and non-contractile tails 182-290 nm long and 7-14 nm wide (Table 1). None of the phages displayed collar, tail plaque or fiber structures. The presence of a plaque-like structure was suspected only in phages Φ231-X9 and ΦB3-X18 (Figure 1a,b). The morphological characterization of 25 *S. thermophilus* phages showed that the phages belong to the *Siphoviridae* family in Ackermann's and/or in Group B in Bradley's classification.

Morphology of *L. bulgaricus* phages

The four industrial strains of *L. bulgaricus* were 231, Y4, V1 and V2, and were used as host organisms for these 25 phages. The capsids were 47 to 73 nm in diameter and isometric hexagonally shaped. It was determined that all of the phages carried non-contractile tails without a cover structure and the tails were 117 to 162 nm long and 7 to 13 nm wide (Table 2). While in the phages ΦHV1 (Figure 2a), ΦGV2, ΦG1V2 and ΦG4V2 the presence of the structures like collar pieces and tail plaques were determined, in the phages ΦH2Y4, ΦJY4, ΦFY4

Table 1 Morphological properties of S. thermophilus phages

Phage Code	Capsid diameter (nm)	Tail length (nm)	Tail width (nm)	Collar (nm)	Tail plaque (nm)	Fiber
Φ709-X1	64	235	12	-[1]	-	-
Φ709-X2	61	245	12	-	-	-
Φ709-X3	63	190	8	-	-	-
Φ709-X4	74	290	10	-	-	-
Φ709-X5	67	220	12	-	-	-
Φ231-X6	62	182	12	-	-	-
Φ231-X7	69	290	12	-	-	-
Φ231-X9	60	230	12	-	-	-
Φ231-X10	68	221	12	-	-	-
ΦB3-X11	53	193	9	-	-	-
ΦB3-X12	53	214	9	-	-	-
ΦB3-X13	60	220	12	-	-	-
ΦB3-X14	55	235	13	-	-	-
ΦB3-X15	73	230	14	-	-	-
ΦB3-X16	54	234	11	-	-	-
ΦB3-X17	57	210	10	-	-	-
ΦB3-X18	54	220	10	-	-	-
ΦB3-X19	63	217	12	-	-	-
ΦB3-X20	47	224	10	-	-	-
Φ231-X21	62	272	7	-	-	-
Φ231-X22	67	230	10	-	-	-
Φ231-X23	73	220	10	-	-	-
Φ1B3-A	57	244	10	-	-	-
Φ2B3-A	57	244	10	-	-	-

[1] not exist

Table 2 Morphological properties of L. bulgaricus phages

Phage Code	Capsid diameter (nm)	Tail length (nm)	Tail width (nm)	Collar (nm)	Tail plaque (nm)	Fiber
H2Y4Φ(X1)	54	134	11	-[1]	30x15	-
MY4Φ(X2)	55	151	11	-	-	-
JY4Φ(X3)	55	138	13	-	32x15	-
FY4Φ(X4)	55	141	13	-	24x13	-
H3Y4Φ(X5)	58	131	13	-	22x13	-
H1Y4Φ(X6)	49	128	11	-	22x14	-
IY4Φ(X7)	56	156	10	-	+[2]	-
SİYY4Φ(X8)	58	142	9	-	+	-
709BY4Φ(X9)	50	138	9	-	15x9	-
SİBY4Φ(X10)	55	138	11	-	22x12	-
GY4Φ(X11)	73	127	10	-	+	-
PY4Φ(X12)	56	151	10	-	+	-
F231Φ(X17)	53	147	9	-	+	-
HV1Φ(X19)	55	143	11	30x7	19x11	-
FV1Φ(X20)	52	144	11	+	22x13	-
GV2Φ(X21)	57	137	11	34x15	28x19	-
G1V2Φ(X22)	54	136	11	38x7	24x13	-
G3V2Φ(X23)	47	133	7	-	-	-
G4V2Φ(X24)	56	143	9	30x9	24x13	-
G5V2Φ(X25)	58	162	8	-	+	-
GKV2Φ(X26)	51	133	9	-	-	-
KV2Φ(X27)	54	145	10	-	-	-
LV2Φ(X28)	53	117	10	-	34x13	-
AV2Φ(X29)	58	138	9	-	-	-
SİYV2Φ(X30)	51	149	10	-	+	-

[1] not exist [2] exist but can not be measured

(Figure 2b), ΦH3Y4, ΦH1Y4, Φ709BY4, ΦSIBY4, ΦFV1, ΦLV2 only the presence of tail plaque was observed. Also, the presence of these structures was suspected in the phages ΦIY4, ΦSIYY4, ΦGY4 (Figure 2c), ΦPY4, ΦF231, ΦG5V2, ΦSIYV2. There was no evidence of contractile tail covers in any of the phages. In the light of the characterization studies these 25 native *L. bulgaricus* phages were placed in the *Siphoviridae* family in Ackermann's classification and/or Group B in Bradley's.

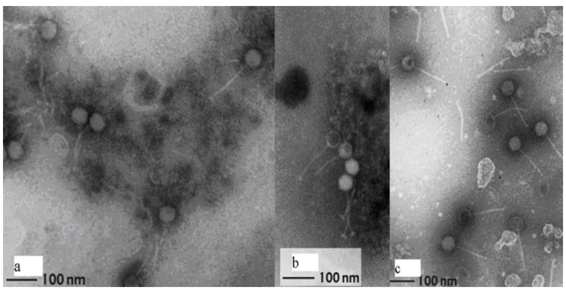

Figure 2. L. bulgaricus phages (a) ΦHV1, 60000x; (b) ΦFY4, 60000x; (c) ΦGY4, 60000x.

DISCUSSION

It was observed that all of *S. thermophilus* phages had isometric hexagonal heads. Although it is possible to determine the taxonomical characteristics of the phages through the use of electron micrographs, it is difficult to identify whether the capsid structures were icosahedral, octahedral or dodecahedral (15). In addition, regarding the characteristics mentioned by Ackermann and DuBow (17), in electron micrographs of *S. thermophilus* phages Φ709-X5, Φ231-X7, Φ231-X21, Φ231-X22, Φ231-X23, ΦB3-X11, ΦB3-X18 (Figure 1b) and ΦB3-X19, hexagonal and pentagonal profiles were observed together while, importantly, hexagonal and spherical profiles were present together in the micrographs of Φ709-X4 (Figure 1c) and ΦB3-X20. It can thus be said that the phages have an icosahedral head structure. In electron micrographs of other *S. thermophilus* phages, only hexagonal capsid profiles were determined. Furthermore, the tails penetrated the capsids, which is a sign of icosahedral head structure, in the cases of phages Φ231-X9 (Figure 1a), Φ231-X22, ΦB3-X12, ΦB3-X13 and ΦB3-X14. On the other hand, the determination of the pentagonal capsid only in tailed phages (17) increases the probability of them being placed in the *Siphoviridae* family with an isometric hexagonal profile having icosahedral capsid members. In this study, depending on the increasing number of the phages examined, the values of capsid diameter, tail length and tail width generally differed within wider limits. However, the 5 nm tail width determined by Kivi et al. (16) in one of 9 *S. thermophilus* phages and the 42 nm capsid diameter determined by Benbadis et al. (20) in one of 7 phages, differed from our phages in their extremely low values.

In *S. thermophilus* phages, the diameter of the capsids were found to be between 45 to 65 nm, the tails were 210-270 nm long and 7-13 nm wide (1, 14, 16, 19-25). Among the phages that we examined, the shortest (182 nm) and the longest tail (290 nm) was about 30 nm longer or shorter than the values found by other researchers. A phage determined by Reinbold et al. (5) also had a 290 nm long tail. In addition to that Suárez et al. (6) and Quiberoni et al. (2) finally isolated a phage having a 330 nm long tail. In another study electron microscopic analysis showed that phage 2972 had a 55-nm-diameter isometric capsid and a 260-nm-long noncontractile tail (25). Among 25 native *S. thermophilus* phages that were inspected morphologically, the presence of tail plaque was suspected in only two of the phages (Φ231-X9 and ΦB3-X18) (Figure 1a,b) but in none of the phages were fiber or collar structures determined. Moreover Krusch et al. (14), Kivi et al. (16) and Prevots et al. (21) did not determine any collar structures out of 59, 7 and 9 phages they examined respectively. Krusch et al. (14) determined tail plaque structures in all of the phages they examined. It is known that fiber structures are rarely found (14, 22), and it is speculated that this is because these fragile structures are easily detached by mechanical effects or that perhaps mutants without fibers frequently occur. (26). However, in some *S. thermophilus* phages tail plaque and fibers were shown clearly (19, 21).

In this study while collar and tail plaques were observed in many of the *L. bulgaricus* phages, these structures could not be seen in *S. thermophilus* phages although the micrographs were taken at the same magnification. The reason for this may be either the modifications in the dying procedure or the use of three different electron microscopes.

L. bulgaricus phages had also isometric hexagonal heads and noncontractile tails. In one exceptional case Reinbold et al., isolated and micrographed a *L. bulgaricus* phage having a contractile cover. This phage was categorised in *Myoviridae* in Ackermann's classification (5). The temperate phages mVl and mV4 were studied by Cluzel et al. (27), and the morphology of the ch2 virulent phage was determined by Chow et al. (28). The lb 539 temperate phage was examined by Auad et al. (15). These four phages were also placed in Group B in Bradley's classification.

The capsid diameters (50 nm) of the mV1 and mV4 phages (27) were the same as in the case of the ch2 virulent phage (28). Their tail lengths were also close to each other, being 180 nm and 170 nm respectively. However in the lb 539 temperate phage the diameter of the capsid was 47 nm and the tail was 159 nm long (15). The new virulent phage phiLdb which was isolated by Wang et al. has an icosahedral capsid of 47.7±0.9 nm in diameter and a long noncontractible tail of 129.8±2 nm and several fibres (9). 25 native *L. bulgaricus* phages examined in this study were found to have a wider range of capsid diameter (47-73 nm), tail length (117-162 nm) and width (7-13 nm).

In this study, the capsid diameters and tail width determined in *L. bulgaricus* phages were similar to the other *L. bulgaricus* phages. However when the tail lengths were compared, it was found that they had smaller tails. Only the lb 539 (15) and phiLdb (9) phages with 159 and 129 nm tail length lay in between the values that we measured, respectively. One other difference was clearly apparent in collar and tail plaque structures. According to the measurements of Chow et al. (28), both collar and tail plaque structures showed considerably higher values (Figure 2a). However it is thought that the differences in the values are not because of the structural differences of the native phages originating in Turkey, but because tail structures are not clearly identified since the micrographs in this study were taken at a lower magnification.

The frequency of thermophilic phage isolation from raw milk

In our former studies using native and industrial strains, although the lactococcal phages were isolated from raw milk at a high frequency (29, 30), a lack of success in the isolation of *S. thermophilus* and *L. bulgaricus* phages with native and industrial hosts, led us to reconsider raw milk as a highly suitable isolation source for these phages (31). Since there is only a limited literature on this subject (12) a minor trial study was conducted; first, 18 raw milk samples were compared with 3 *S. thermophilus* (B3, 709, 231) strains, 2 phages (Φ1B3-A and Φ2B3-A), which were effective only with the B3 host, were isolated from 2 raw milk samples and their morphological characterization was undertaken with the other phages in the collection. Then 9 industrial *S. thermophilus* (231, 632, 709, V1, V2, Y1, Y4, CH-1, B3) strains were used as a host. However, out of 3 raw milk samples, no phage could be isolated. Additionally, the same 3 samples were tried with a total of 6 *L. bulgaricus* strains, five of which were industrial (Y1, Y4, V1, V2, 231) and one being a reference strain (*Lactobacillus bulgaricus* ATTC 11842), from the 2 raw milk samples 7 of the phages which were effective with V1, V2, Y4 and *L. bulgaricus* ATTC 11842 strains could be isolated. In the light of the data, phages specific to *L. bulgaricus* are more frequently isolated than the phages specific to *S. thermophilus* from raw milk. However it is much more difficult and less common to isolate thermophilic phages from raw milk samples.

ACKNOWLEDGMENT

We thank The Scientific and Technological Research Council of Turkey for the financial support. (Project No: TÜBİTAK/TARP 2106).

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