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Classification of Virulent and Temperate Bacteriophages of *Listeria* spp. on the Basis of Morphology and Protein Analysis

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A set of 22 phages of *Listeria* species (listeriaphages) (21 temperate and 1 virulent) were compared on the basis of morphology and protein composition. All 22 phages had icosahedral heads and exhibited either contractile or noncontractile tails. They represented two different morphotypes. Twenty phages belonged to the *Siphoviridae* family and could be differentiated only on the basis of tail length. Accordingly, they could be assigned to previously defined listeriaphage species. Two other phages were classified as members of the *Myoviridae* family, one of which (A511) should be regarded as a new species. It was found to be substantially different from all other known listeriaphages. All phages exhibited typical protein profiles, which were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent laser densitometrical analysis of the gels. It was then possible to distinguish eight protein subgroups on the basis of unique protein patterns. This classification corresponds well to the previous groupings based on host range. Most of the phages revealed two or three major proteins ranging from 21 to 24 kDa and 30 to 36 kDa. In addition, at least 10 minor proteins could be observed for each phage. Our results indicate that the major proteins are structural proteins of the capsid and tail, and the protein band ranging from 30 to 35 kDa could clearly be assigned to the proteins of the phage capsid.

To date, more than 300 phages of *Listeria* species (listeriaphages) have been isolated. Almost all of them stem from lysogenic strains (3, 8, 13, 14, 22, 26, 28, 32), except for some phages which were found in water or sewage (11, 13, 14). All described listeriaphages are temperate, with the exception of A511, which was isolated and characterized in this laboratory (14, 17).

Most investigations regarding listeriaphages used phage typing (4, 13, 14, 20, 22, 25, 26) for elucidating the epidemiology of listeriosis, which had been the focus of interest following some recent epidemics (9, 10, 31). This method of differentiation can also be used as a means for identifying faulty or insufficiently sanitized equipment in food production (15).

Although ultrastructure is the most important criterion for phage taxonomy, only 66 listeriaphages have been investigated by electron microscopy. Two of them belong to the *Myoviridae* family (Bradley's morphotype A1; tail with contractile sheath [5, 24]), whereas the remaining isolates belong to the *Siphoviridae* family (morphotype B1; noncontractile tail [1, 24]). Those investigations resulted in a classification of the listeriaphages into five species which were differentiated by head diameter in addition to tail length and diameter (1, 3, 23, 24). The proportions of morphological groups A1 and B1 to each other reflect the commonly found distribution for all bacteriophages of gram-positive bacteria, since only very few particles of the A1 type have been reported. Regarding biochemical and genetic characteristics of listeriaphages, very few data are available. DNA-DNA hybridizations (27) indicated a good correlation between ultrastructure and DNA relatedness.

In this laboratory, 20 temperate and 1 virulent listeriaphage are routinely employed for phage typing. The aim of the present study was to investigate those phages by electron microscopy and electrophoresis of phage proteins. The major structural proteins, which contribute to the morphology, were identified. In order to correlate our classification to other previously defined listeriaphage types, morphological data were related to already investigated listeriaphages. This should provide a stable basis for future biochemical and taxonomical studies.

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MATERIALS AND METHODS

Bacteria, bacteriophages, and media used. A selected set of host strains of *Listeria monocytogenes* (Weihenstephan Listeria Collection [WSLC] 1003, 1019, and 1042) and *Listeria ivanovii* (WSLC 3009) was used. All strains were maintained on slants of tryptose agar (Merck, Darmstadt, Germany) at 4°C. A total of 21 phages, which are used for listerial phage typing in this laboratory, were examined (13). In addition, phage B604 was included in this study. Phage stocks were stored at −60°C. Prior to use, host strains were subcultured in tryptose broth (Merck) at 30°C for 16 h. All media were supplemented with Ca^{2+} (1.25 mM), and the pHs were adjusted (7.00) as previously described (14).

Propagation, purification, and concentration of phages. Large-scale bacteriophage production (250 ml of each phage; PFU > 10^9) was essentially carried out as outlined earlier (14). The concentration and purification of the phages were largely drawn from the methods of Yamamoto et al. (33). Phages were precipitated overnight at 4°C by two-step precipitation with 10% (wt/vol) and 15% (wt/vol) polyethylene glycol 6000 (Merck) in the presence of 0.6 M NaCl. The precipitate was then collected by centrifugation for 30 min at 15,300 × g_{max} at 4°C. After the supernatant was discarded, the precipitate was resuspended in 5 ml of SM buffer (0.05 M Tris-HCl [pH 7.5], 5.8 g of NaCl per liter, 2.0 g of $MgSO_4 \cdot 7H_2O$ per liter [29]) and kept at 4°C.

The concentrated phage suspension was then layered onto a preformed CsCl step gradient, consisting of six layers

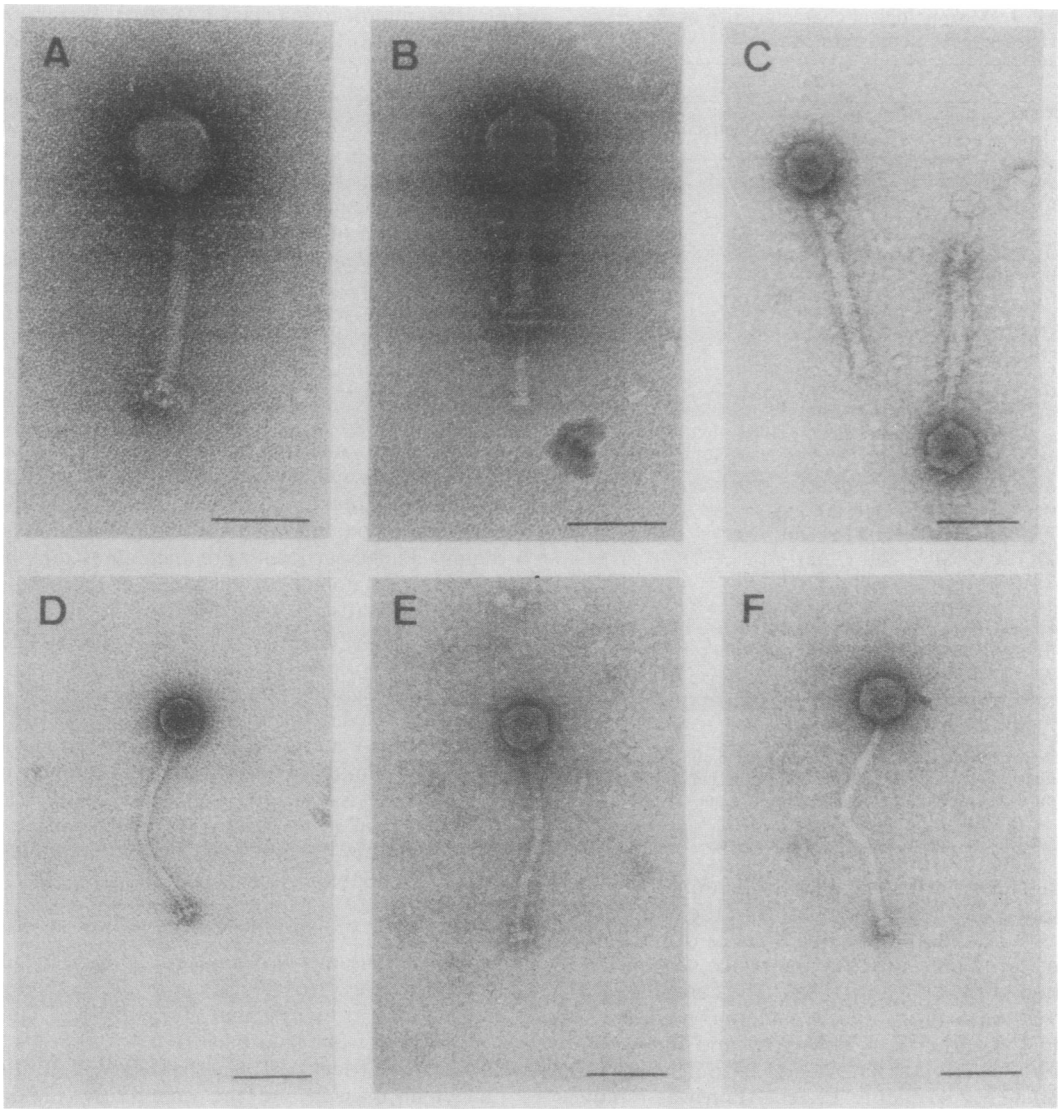

FIG. 1. Electron micrographs of different morphotypes of listeriaphages (bars = 100 nm). (A) A511 (noncontracted tail); (B) A511 (contracted tail); (C) B054 (noncontracted tail [left] and contracted tail [right]); (D) B024; (E) C707; (F) A500.

(densities were 1.70, 1.50, 1.45, 1.40, 1.30, and 1.20 g/cm^3 in SM buffer), and centrifuged to equilibrium (15 h at 93,000 × g_{max}, Rotor SW-28.1; Beckman). The bluish opalescent bands observed at densities close to 1.45 g/cm^3 were carefully withdrawn by insertion of sterile syringes (5 ml) through the tube wall just below the bands.

Once collected, the phage suspensions were dialyzed to remove all salts and low-molecular-mass debris. This was done in cleaned and autoclaved dialysis tubings (Visking 8/32, 6 mm by 15 cm; Serva, Heidelberg, Germany) against two successive changes in 2 liters of SM buffer (30 min each) and two additional changes in 5 liters of double-distilled water (15 h).

The dialyzed suspensions were then transferred into 3-ml centrifuge tubes, and the phage particles were pelleted at 216,000 × g_{max} (Beckman SW60 rotor, 90 min, 4°C). At last, the pellets were resuspended in double-distilled water (50 to 150 μl) and stored at 4°C. These suspensions served in the determination of phage morphology by electron microscopy and the analysis of phage proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Apart from the protein pattern of intact phage particles, we investigated the broken-off capsids of four listeriaphages (A118, A502, B012, and B056). These particles sedimented at a density of about 1.50 g/cm^3 and could, therefore, easily be separated. Electron microscopy and determination of proteins were carried out in the same manner as described for the intact phage particles.

Electron microscopy of bacteriophages. Although electron microscopy of phages can be performed with crude lysates or directly from plaques in order to determine the morphology, we obtained all electron micrographs from phage suspensions purified by density gradient centrifugation. The concentrated phages were diluted 1:20 with double-distilled water and served directly as probes for the negative staining procedure.

Negative staining. Carbon-coated mica plates, which had been rendered hydrophilic by glow discharge, were im-

TABLE 1. Morphological characteristics of the investigated listeriaphages

Phage	Head diameter, nm (SD)	Tail				n^a
		Diameter, nm (SD)	Length, nm (SD)	Contractile	S/F[b]	
A511	88 (2.44)	22 (2.62)[c]	201 (4.70)[c]	+	S	11
B054	64 (1.96)	16 (2.07)	244 (5.31)	+	S	7
B024	59 (4.15)	11 (1.00)	239 (7.46)	−	F	8
B055	62 (1.62)	11 (1.00)	242 (5.77)	−	F	5
C707	60 (1.60)	10 (1.41)	243 (6.28)	−	F	5
B053	59 (3.30)	10 (1.00)	244 (11.48)	−	F	5
B051	62 (3.49)	10 (1.00)	245 (10.13)	−	F	9
D441	63 (3.52)	11 (1.00)	247 (11.48)	−	F	4
B604	58 (2.26)	11 (1.00)	248 (10.55)	−	F	9
A020	63 (1.66)	11 (1.00)	248 (5.96)	−	F	4
B025	63 (2.91)	11 (1.04)	252 (7.08)	−	F	5
B545	62 (1.54)	11 (1.00)	258 (9.69)	−	F	6
B653	61 (2.95)	10 (1.00)	260 (7.56)	−	F	5
A500	62 (1.16)	12 (1.35)	274 (9.94)	−	F	4
A006	62 (1.80)	10 (1.00)	280 (1.18)	−	F	3
B101	61 (1.58)	10 (1.00)	280 (15.86)	−	F	2
B056	59 (1.62)	10 (1.00)	285 (3.12)	−	F	3
B012	61 (1.62)	11 (1.41)	286 (8.12)	−	F	3
B110	57 (2.81)	10 (1.00)	288 (13.73)	−	F	5
B035	63 (1.68)	11 (1.00)	294 (6.36)	−	F	7
A118	61 (1.55)	10 (1.22)	298 (8.10)	−	F	4
A502	62 (1.47)	11 (1.00)	302 (4.46)	−	F	6

[a] Number of measured particles.
[b] S, stiff; F, flexible.
[c] Data are for the noncontracted tail. For the contracted tail, the total length was 201 nm (standard deviation, 7.32), the length of the tail core was 85 nm (standard deviation, 7.13), and the tail diameter was 24 nm.

mersed into a clean petri dish filled with double-distilled water in a manner which allowed floating of the carbon film. With the aid of a platinum loop, small pieces of the carbon film were transferred onto drops of the phage suspension to allow adsorption of the phage particles. The carbon film was then transferred onto drops of the staining solution (2% uranyl acetate [wt/vol], pH 4.2; Merck) and immediately picked up with cleaned copper grids (3.05 mm, 400 mesh; Balzers, Wiesbaden, Germany). Excess stain was removed with filter paper, and the grids were allowed to air dry. They could then directly be used for viewing in a transmission electron microscope (Zeiss EM-10A). Pictures were taken at an acceleration voltage of 80 kV and an objective aperture of 90 μm (HS7 Cronolar film; DuPont). Phages were photographed at magnifications of ×80,000 and ×100,000. Actual magnification was determined with the aid of catalase crystals (18) and a standard cross grating (d = 0.463 μm; both from Balzers). For every phage, 4 to 11 measurements were made, and means were calculated to minimize the error in determination of bacteriophage particle dimensions.

SDS-PAGE protein profiles of phages. To determine protein compositions of the phage particles, the bacteriophage proteins were separated on an ultrathin (0.5 mm), discontinuous SDS-polyacrylamide gel (ExcelGel System, 8 to 18% T; Pharmacia, Freiburg, Germany) on a horizontal electrophoresis unit (Ultrophor; Pharmacia). The concentrated phage preparations were mixed with an equal volume (50 to 150 μl) of double-concentrated sample buffer (12) (modified according to the gel manufacturer's instructions [30]). Dithiothreitol (26 mmol/liter; Sigma) was used instead of mercaptoethanol, and its oxidation was inhibited by EDTA (3 mg/100 ml; Sigma). Phage particles were disintegrated by boiling for 8 to 10 min. The molecular mass marker mixtures SDS-6H and SDS-7 (Sigma) were used for calibration (0.5 mg of protein per ml). They were prepared in the same manner as described above.

The gel was loaded by use of an applicator strip, with 10 to 30 μl sample per well. Electrophoresis was carried out at 600 V and 50 mA at 12°C for approximately 90 min. Proteins were then simultaneously fixed and stained in Coomassie brilliant blue R-350 (Phastgel Blue System; Pharmacia) at 55°C for about 15 to 20 min. Destaining of the gel was done three times for 15 min each in 25% methanol–10% acetic acid–65% double-distilled water. Glycerol (10%, vol/vol) was added for gel impregnation to the last decolorization step.

Laser densitometrical analysis. The dried gels were analyzed with an Ultroscan XL laser densitometer, which was connected with a personal computer and Gelscan XL-2.1 software (all from Pharmacia).

Densitometrical analysis enabled us to determine the molecular masses and the quantitative amount of single bacteriophage protein bands by signal integration charging the relative peak area.

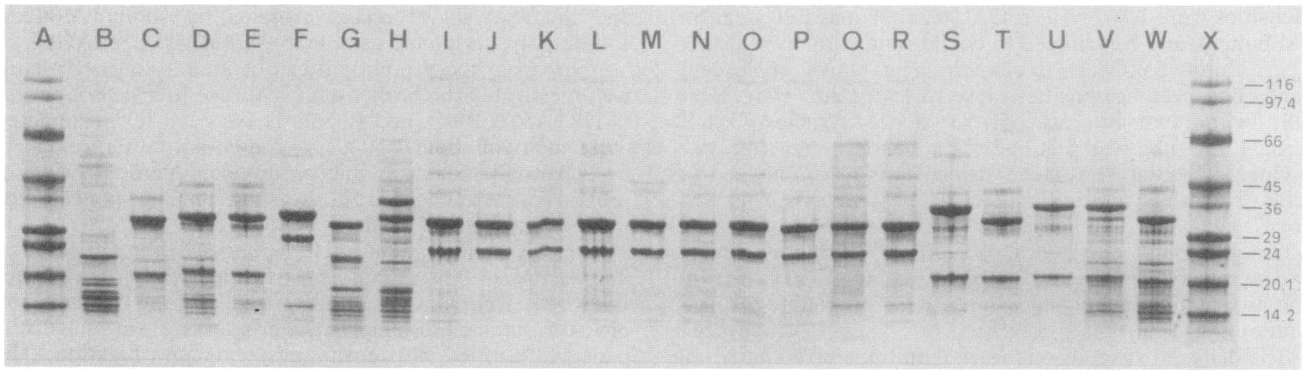

FIG. 2. Protein profiles of 22 listeriaphages. Lanes A and X contain marker proteins as follows: α-lactalbumin, 14.2 kDa; trypsin inhibitor (soybean), 20.1 kDa; trypsinogen (phenylmethylsulfonyl fluoride treated), 24 kDa; carbonic anhydrase, 29 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; albumen (egg), 45 kDa; albumin (bovine), 66 kDa; phosphorylase *b*, 97.4 kDa; and β-galactosidase, 116 kDa). Other lanes are as follows: B, A511; C, A500; D, A118; E, A502; F, A006; G, B653; H, B054; I, B051; J, B055; K, B604; L, B025; M, D441; N, B545; O, C707; P, B024; Q, B053; R, A020; S, B012; T, B035; U, B056; V, B101; W, B110.

TABLE 2. Molecular masses of the major and some minor proteins of the phages and percent relative proportions

Phage	No. of bands[a]	Molecular mass (kDa) and relative proportions[b]		Phage group[c]
		Major proteins	Minor proteins	
A511	27	15.0 (17.0), 22.0 (14.0), 13.5 (12.5)	12.8 (9.5), 16.0 (8.0), 17.5 (7.0)	I
A500	17	31.0 (54.0), 20.0 (22.5)	35.5 (6.0), 21.5 (3.0), 14.0 (2.5)	IV
A118	23	31.0 (33.0), 20.5 (17.5), 15.0 (11.0)	41.0 (4.0, 14.0 (3.5), 22.0 (3.0)	IIa
A502	20	31.0 (34.0), 20.0 (17.5), 29.5 (12.0)	41.0 (6.0), 14.5 (5.5), 57.5 (2.5)	IIa
A006	16	31.5 (53.0), 26.0 (21.0)	14.0 (6.0), 55.0 (3.0), 106.0 (2.0)	IIb
B653	25	30.0 (24.0), 22.0 (16.5), 17.5 (11.0)	13.5 (7.5), 68.0 (6.0), 15.0 (5.0)	IIIa
B054	29	31.5 (16.5), 35.0 (16.0), 17.0 (12.0)	29.5 (6.5), 13.5 (6.0), 14.5 (5.5)	IIIa
B051	13	30.0 (43.0), 23.0 (31.0)	29.5 (7.0), 56.5 (5.0), 39.0 (3.0)	IIIb
B055	15	30.0 (43.5), 23.5 (29.0)	29.0 (6.0), 16.5 (5.0), 38.5 (3.5)	IIIb
B604	15	31.0 (42.5), 23.5 (27.5)	116.0 (6.0), 18.0 (3.5), 61.0 (3.0)	IIIb
B025	15	30.5 (44.5), 23.5 (30.0)	48.5 (4.0), 15.0 (3.5), 17.0 (3.0)	IIIb
D441	16	31.0 (49.0), 24.0 (28.5)	58.0 (4.5), 45.5 (3.0), 40.7 (3.0)	IIIb
B545	17	31.0 (48.0), 24.0 (28.0)	57.5 (4.5), 17.5 (3.0), 48.5 (2.0)	IIIb
C707	13	30.5 (45.0), 23.5 (31.5)	30.0 (7.0), 63.0 (3.0), 40.0 (2.5)	IIIb
B024	14	30.5 (51.0), 23.5 (31.5)	57.0 (3.5), 16.5 (3.0), 39.0 (2.5)	IIIb
B053	25	31.0 (27.0), 24.0 (25.0)	16.0 (7.0), 30.0 (6.0), 18.5 (5.5)	IIIb
A020	19	31.0 (41.5), 24.0 (29.0)	30.0 (6.5), 16.0 (4.5), 64.0 (4.5)	IIIb
B012	14	34.5 (48.0), 21.0 (26.5), 31.0 (10.0)	29.5 (4.0), 58.0 (3.0), 15.0 (2.5)	IIIc
B035	13	32.5 (45.0), 21.0 (24.0)	31.5 (8.5), 43.0 (7.5), 16.0 (3.0)	IIIc
B056	17	36.0 (42.5), 21.5 (25.0), 32.0 (12.0)	77.5 (4.5), 16.0 (4.5), 34.0 (3.0)	IIIc
B101	17	36.0 (24.0), 15.5 (13.5), 21.5 (13.0)	34.0 (8.0), 20.5 (7.5), 16.5 (7.0)	IIId
B110	22	33.0 (25.0), 21.0 (13.5), 15.5 (10.0)	16.5 (8.5), 31.0 (6.0), 23.0 (4.5)	IIId

[a] Total number of protein bands which could be resolved by laser densitometry.
[b] Percent relative proportions are in parentheses. For major proteins, protein share ≥10% of the total protein. For minor proteins, protein share <10% of the total protein.
[c] Grouping of phages according to their protein relatedness and host ranges (14).

RESULTS

Electron microscopy. For clear morphological distinction of listeriaphages, the following criteria were taken into consideration: head diameter, tail length and diameter, and flexibility and contractility of the tail.

All 22 phages investigated had icosahedral heads and exhibited contractile or noncontractile and flexible or stiff tails. These phages basically represented two different morphotypes, which can be seen in Fig. 1. Their primary dimensions are listed in Table 1. However, the new species A511, as described here, shows a deviating morphology compared with that of phage B054 (i.e., phage 4286 [24]), since the type of sheath contraction is considerably different (Fig. 1).

Twenty of the 22 phages belong to the *Siphoviridae* family (morphotype B1 [2]). Their relatively long tails (lengths, 239 to 302 nm; widths, 10 to 12 nm) showed noncontractile sheaths and were somewhat flexible. The phages were morphologically very similar to each other and therefore could be distinguished only on the basis of their different tail lengths. Most phages possessed structural elements at the distal end of their tails, sometimes forming a six-pointed baseplate. However, the fine structure could not be clearly resolved.

Two other phages had contractile, nonflexible tails. Hence, they were classified into the *Myoviridae* family (morphotype A1) as described earlier (24).

Phage A511 was substantially different from B054 with respect to tail structure and particle size. The sheath contraction seemed to occur upwardly, from the receptor region toward the head region. Because of this, part of the tail core (about 85 nm) was exposed, which resembles 40% of the total length of the tail.

Protein profiles. Preparations of all phages showed two or three intensive bands (major proteins), corresponding to the structural proteins of the head and the tail, and at least 10 minor proteins. Protein profiles of the 22 phage particles are presented in Fig. 2. By using laser densitometrical analysis, it was possible to discriminate between 13 and 29 protein bands, depending on the phage examined. Table 2 summarizes the analytical data with respect to the quantitative evaluation. Major and minor proteins were differentiated according to the relative proportions of the single proteins to the total amount of individual phage proteins. Proteins with a share of 10% or more were designated major proteins; those with less than 10% were labeled minor proteins. Proteins with an amount of less than 1% were not taken into consideration for the analysis of protein composition. Most of the phages showed major proteins with molecular masses

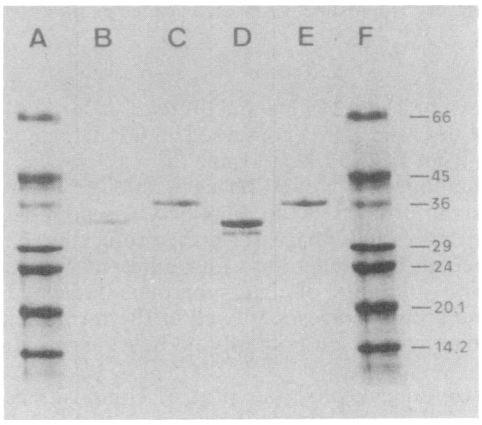

FIG. 3. Capsid proteins of four listeriaphages. Lanes A and F, marker proteins (see legend to Fig. 2). Other lanes are as follows: B, A502; C, B012; D, A118; E, B056.

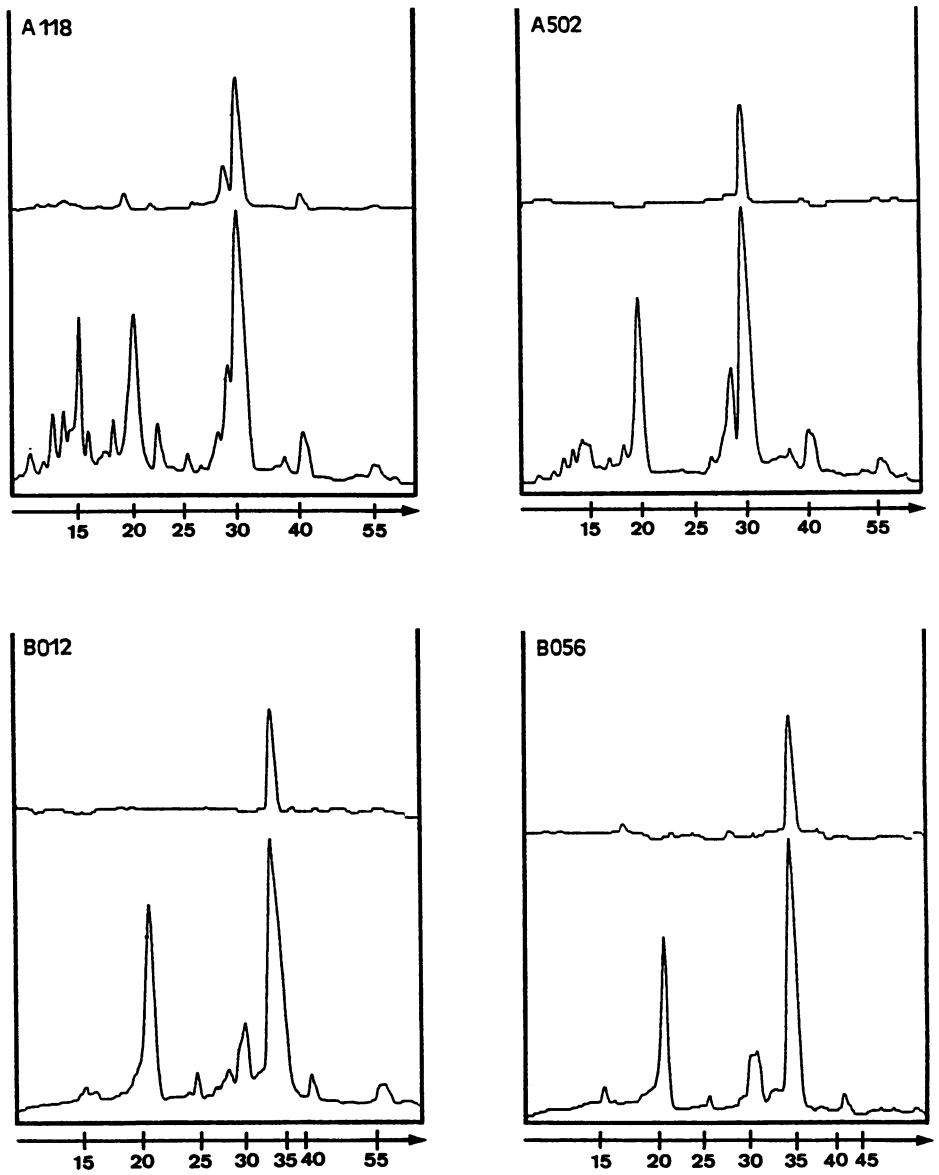

FIG. 4. Comparison of the laser densitograms of total protein profiles (bottom spectra) and capsid proteins (top spectra) of four listeriaphages. The respective molecular masses (in kilodaltons) are indicated below each graph.

ranging from 21 to 24 kDa and 30 to 36 kDa. Some phages (A511, B054, and B653) possessed additional major proteins ranging from 14 to 17 kDa (Table 2).

As mentioned above, all phages exhibited relatively specific protein profiles. Accordingly, it was possible to characterize individual phages. Furthermore, the 22 listeriaphages could be divided into four different groups (protein clusters), with a total of eight subgroups (indicated in Table 2). These groups correspond well to the former differentiation, which was based primarily on host range patterns (14, 15).

We also investigated the protein compositions of broken-off capsids of four listeriaphages (A118, A502, B012, and B056). The molecular masses of the protein bands ranged from 31 to 35 kDa (Fig. 3). Laser densitometry of these gels revealed that those protein bands are in perfect agreement with the profiles drawn from intact phages (Fig. 4). It can be concluded that the upper major protein band (30- to 36-kDa range) includes the structural proteins of the phage capsid, at least with regard to the four phages investigated in this experiment.

DISCUSSION

Sixty-six listeriaphages have already been morphologically characterized (1, 7, 23, 24). Electron micrographs showed that most of them have isometric heads and noncontractile tails (B1 morphotype). These phages were of very similar shapes and sizes and could not be clearly differentiated on the basis of their fine structures. From our study, 18 new phages have been added. Seventeen of them can be assigned to hitherto existing species, although some minor

deviations (in tail length) were observed. Rocourt et al. (24) classified phage 4286 under the *Myoviridae* (phage species 4211). B054 appeared, as expected, to be identical to 4286.

The *Siphoviridae* are, at present, differentiated into five species on the basis of tail length. No members of species 2389 (1, 3) or species H387 (23) were observed among our phages. Nine of the new listeriaphages investigated here (B024, B055, C707, D441, B604, A020, B025, B545, and B653), with tail lengths from 239 to 260 nm (Table 1), corresponded to phages already described (7, 24); they resembled phage species 2685 (tail lengths, 250 to 255 nm). The fourth phage species described previously (2671; tail lengths of about 290 nm [1]) corresponded to our phages A500, A006, B101, B012, B110, B035, A118, and A502 (tail lengths, 274 to 302 nm).

This differentiation should not be established definitively, since we observed high variability within single phage species. Accordingly, it seems somewhat inappropriate to consider this characteristic for taxonomic purposes.

The phages B051 (=4211), B053 (=4277), B054 (=4286), and B056 (=5337) have been characterized recently (24). However, concerning the identity of the temperate phage B051, some discrepancies were obvious. At first, this phage seemed to be identical to phage 4211, since the lysogenic carriers were identical (SLCC 4211 = WSLC 2051 [13]). However, the phages were totally different in morphology. In our study, B051 could undoubtedly be regarded as a member of the *Siphoviridae* family, whereas 4211 was reported to belong to the *Myoviridae* (24). These differing results can be explained only by a loss or exchange of prophages or an accidental exchange of bacterial strains.

Phage A511 appeared to be completely different from all other known listeriaphages. Because of its unique morphology (morphotype A1; Fig. 1), this phage should be regarded as the type phage of a new species designated A511. The syringelike type of sheath contraction suggests a penetration of the host cell by the tail core, followed by injection of the genetic material. In contrast, such a mechanism cannot be assumed for the other members of the *Myoviridae* (i.e., species 4211). In addition, A511 featured a substantially larger capsid and a thicker but shorter tail than phage species 4211.

By SDS-PAGE, considerably different phage protein patterns could be observed, thus allowing the distinction of eight subgroups. Also, protein profiles confirmed that most members of the previously established lytic groups (14) were, with respect to their proteins, relatively similar to each other (in some cases nearly identical). As expected, the different phage groups appeared not as clearly associated, reflecting their different lytic specificity.

Comparison of the laser densitograms of capsid fractions with those of complete phage particles rendered it possible to assign certain proteins to the structural proteins of the phage capsid (Fig. 4). Therefore, the corresponding other major protein bands are likely to originate from the structural components of the phage tail. The minor proteins observed for each phage might be subordinate components of the head or tail (e.g., determinating the specificity of the phage receptors).

A clear relation of the proportions of capsid and tail proteins to the morphology (i.e., tail length) of the *Siphoviridae* could be observed. In other words, tail length directly corresponded to the percent share of the lower major protein band (21 to 24 kDa).

The diverging protein profiles of the two *Myoviridae* included in this study (A511 and B054) can be correlated to their different tail structures.

Investigations concerning protein profiles have been conducted for a variety of phages, e.g., phages from enterobacteria and lactococci (6, 12, 19, 21). For lactococcal phages, a close connection was observed between data from those studies and taxonomical approaches based on DNA-DNA relatedness.

In general, a good correlation was observed between the classification based on protein profiles and that based on host ranges and phage-mediated resistance clusters (14, 16). Hence, overall taxonomic differentiation could be further improved. We feel that protein profile data should be used more widely in the taxonomy of more than listeriaphages, because the entire profile can be regarded as a more-or-less individual attribute of each phage. Protein analysis possesses a greater taxonomical potential than any system relying solely on morphology.

Supplementary genetic analysis of the investigated phages is presently being conducted. It may be of interest to see whether our phages can be subordinated into the DNA homology groups already established (27).

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