Structural proteins and DNA characteristics of 14 *Listeria* typing bacteriophages

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The major structural proteins of 13 temperate and one virulent *Listeria* typing bacteriophages were analysed and compared using isoelectric focusing in immobilized pH gradients (IPG), ultrathin-layer two-dimensional electrophoresis, amino acid analysis and N-terminal amino acid sequences of selected proteins. Isoelectric points for major capsid and tail proteins of the 12 members of the siphoviridae family included in this study ranged from 4·70 to 5·92, whereas one of the two myoviridae investigated (B054) showed structural proteins in the 6·1 to 6·3 range. In comparison to protein profiles from one-dimensional SDS gels, the IPG technique gave better resolution and improved discrimination of phage proteins. Combination of this technique and SDS gel electrophoresis made it possible to correlate M_r and isoelectric points of major structural proteins. Tail polypeptides of all siphoviridae are smaller and, with one exception, more acidic than their corresponding capsid counterparts. We also determined the amino acid composition of capsid and tail proteins. When compared with an average protein, they were found to be fairly rich in acidic and short-chain hydrophobic amino acids, as well as in lysine. In addition, the N-terminal amino acid sequences of major capsid and tail proteins of four representative listeriaphages were compared. The base composition of listeriaphage DNAs was between 37% and 39% G+C, reflecting that of their bacterial hosts. Each phage had a distinct restriction endonuclease pattern, and genome sizes ranged from 35 to 116 kb. DNA–DNA hybridization permitted the identification of five DNA homology groups. The two myoviruses studied (A511 and B054) showed no DNA homology to other phages, confirming their unique nature. The 12 siphoviruses were classified into three DNA homology groups with little cross-homology. Furthermore, phage A006 was found to share little DNA homology with the other investigated members of species 2671. Therefore, a new species (A006) is proposed. With respect to phage classification and taxonomy, a good correlation between the various approaches was observed, mostly corresponding to particle morphology.

Introduction

Approximately 320 bacteriophages specific for *Listeria* (listeriaphages) have been isolated from lysogenic strains or the environment (Andurier *et al.*, 1977; Estela *et al.*, 1992; Loessner, 1991; Loessner & Busse, 1990; Ortel & Ackermann, 1985; Rocourt *et al.*, 1982; Sword & Pickett, 1961). Their identification and description largely relies on phage morphology and host ranges. They have been classified into two different morphotypes and six species (Ackermann & DuBow, 1987; Zink & Loessner, 1992).

Listeriaphages are important tools in epidemiological research on the pathogen *Listeria monocytogenes* (Estela *et al.*, 1992; Loessner & Busse, 1990; McLauchlin *et al.*, 1986; Rocourt *et al.*, 1985), and may be useful in genetic and biotechnology applications. However, surprisingly little information is available on their molecular characteristics (Rocourt *et al.*, 1986; Zink & Loessner, 1992).

Based on the pioneering work of Laemmli (1970), many previous investigations used SDS–PAGE for analytical and comparative studies of bacteriophage proteins (Braun *et al.*, 1989; Loessner *et al.*, 1993; Mata & Ritzenthaler, 1988; Neve *et al.*, 1989; Zink & Loessner, 1992). However, little effort has been made to apply isoelectric focusing (IEF) and two-dimensional (2-D) electrophoresis to the study of phage proteins (Gersten *et al.*, 1981; Lee & Stewart, 1985), presumably owing to the somewhat poor reproducibility of specific patterns. Methodological difficulties, such as the interference by phage DNA in the samples (O'Farrell, 1975), may have been another reason for the lack of such data in the literature.

A clearer knowledge of the biochemical and, in particular, genetic relationships among listeriaphages is needed to improve understanding of their taxonomy and phylogeny, and to aid in future work on the molecular biology and genetics of these phages. Therefore, one goal

of this study was to develop and apply methods for comparative analysis of bacteriophage proteins based on IEF in immobilized pH gradients, horizontal ultrathin-layer 2-D electrophoresis, and amino acid analysis at the picomole level. Furthermore, we aimed to characterize and compare listeriaphage genomes by base composition, restriction enzyme analyses and DNA–DNA cross-hybridization. For this purpose, 14 viruses were selected from a set of listeriaphages used for phage typing in this laboratory. They included a variety of morphological types (contractile tail or non-contractile tail) and species (different particle dimensions). Among these, the only virulent listeriaphage known, A511, is unique because of its morphology and broad host range (Loessner & Busse, 1990; Zink & Loessner, 1992).

Methods

Bacterial strains and phages. L. *monocytogenes* strains of the Weihenstephan *Listeria* Collection 1003, 1019, 1042, and *L. ivanovii* strain WSLC 3009 were used as phage hosts (Loessner & Busse, 1990). They were maintained on slants of tryptose agar at 4 °C.

The one virulent and 13 temperate listeriaphages studied were from a set of 21 typing phages used in this laboratory (Loessner, 1991). They are listed in Table 1 with species affiliation and morphotype. *Escherichia coli* bacteriophages T7 (ATCC 11303-B7) and λ (ATCC 23724-B2) were grown on *E. coli* strains B and C600, respectively.

Propagation and purification of phages. High-titre phage lysates (500 ml of each) were prepared by the soft agar double layer technique, and phage particles were concentrated and purified by differential centrifugation, polyethylene glycol precipitation, isopycnic banding in CsCl density gradients, dialysis, and centrifugation as described earlier (Zink & Loessner, 1992). Phage suspensions were then adjusted to an A_{260} of 0·2 to 0·3, and stored at 4 °C.

IEF of phage proteins. Immobilized pH gradient gels (Bjellqvist *et al.*, 1982) in a horizontal, ultrathin-layer system supplemented with small amounts of carrier ampholytes (IPG–CA; Rimpiläinen & Righetti, 1985) were used for isoelectric focusing of virion proteins. Prior to rehydration of the precast, dry polyacrylamide gels (Immobiline DryPlate pH 4 to 7; Pharmacia), small rectangular holes were punched into the gels to facilitate sample application and prevent lateral band spreading (Loessner & Scherer, 1992). Gels were rehydrated overnight in a solution containing 8 M-urea (ultrapure grade; USB), 15% (w/v) glycerol, 0·25% (w/v) Nonidet P-40 (NP40), 0·25% (w/v) CHAPS (both from Sigma), 0·5% (w/v) carrier ampholytes (Servalyte pH 4 to 7; Serva) and 10 mM-dithiothreitol (Sigma). A horizontal electrophoresis apparatus (Ultrophor; Pharmacia) was used here.

For solubilization of phage proteins, 10 µl of each of the phage suspensions was mixed with 90 µl of IPG–CA buffer containing 9·5 M-urea, 1% (w/v) NP40, 1% (w/v) CHAPS, 0·8% (w/v) carrier ampholytes (Ampholine, pH 3·5 to 9·5; Pharmacia), 10 mM-DTT and 1 unit/µl of a non-specific nuclease [Benzon nuclease (pI 6·85); Merck].

After overnight incubation at 20 °C, 15 µl of each of the phage suspensions was focused at 18 °C for 13 000 Vh, with initial settings of 150 V, 2 mA (45 min), 300 V, 2 mA (45 min) and finally 3000 V, 2 mA (approximately 4·5 h).

Following fixation of proteins in 15% trichloroacetic acid for 15 min, and removal of NP40 in 7·5% TCA with 50% 2-propanol for 10 min, protein bands were silver-stained according to Blum *et al.* (1987). Since immobilized pH gradients are linearly distributed from

the anode (pH 4) to the cathode side (pH 7) of the gels, pIs could be directly calculated by migration distance.

Two-dimensional electrophoresis. Isoelectric focusing in immobilized pH gradients followed by SDS electrophoresis largely corresponded to published protocols (Görg, 1991; Görg *et al.*, 1988). In brief, individual gel strips (4 × 110 mm) were cut from the first dimension IPG–CA gels after electrophoresis was completed. They were either stored at −20 °C until use, or directly equilibrated for the second dimension SDS gel in 5 ml of a buffer containing 50 mM-Tris–HCl pH 6·8, 6 M-urea, 30% (w/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT, and traces of bromophenol blue. After 5 min, strips were transferred for 2 min into another 5 ml of equilibration buffer that additionally contained 260 mM-iodoacetamide (Sigma). This treatment reduces vertical point streaking in the second dimension gel (Görg *et al.*, 1987).

The equilibrated gel strips were then transferred gel-side-down onto the surface of a horizontal, discontinuous ultrathin-layer pore gradient SDS gel (ExcelGel, 8 to 18% T; Pharmacia), at a distance of 5 mm parallel to the cathode buffer strip. Running conditions for this second dimension were 200 V (25 min), followed by 600 V for 60 min. Proteins were then fixed in 25% methanol, 10% acetic acid for 60 min, and subsequently silver-stained. All 2-D electrophoresis analyses were run in duplicate to assess the reproducibility of protein patterns.

Blotting on PVDF membranes. Listeriaphage proteins were separated on discontinuous, pore gradient SDS gels as previously described (Zink & Loessner, 1992). Proteins were then transferred onto a PVDF membrane (Immobilon P; Millipore) with the semi-dry, discontinuous horizontal electroblotting technique (Kyhse-Andersen, 1984) at 0·8 mA/cm² for 60 min. Membranes were stained with Coomassie blue R-350 (Pharmacia) for 10 min, destained in 90% methanol, 7% acetic acid for 20 to 30 s, air-dried, and stored at −70 °C.

Amino acid analysis. Bands of interest (i.e. major structural proteins) were excised from PVDF membranes and destained completely in 95% methanol. Proteins were vapour-phase-hydrolysed in 5·7 M-HCl at 110 °C for 21 h in a protein hydrolyser (Knauer). Amino acids were extracted with 0·1 M-HCl in 20% methanol, dried under vacuum centrifugation, and redissolved in 0·2 M-sodium carbonate buffer pH 8·6. Equal volumes of a 0·3% (w/v) solution of recrystallized DABS-Cl (4-dimethylaminoazobenzene-4'-sulphonyl chloride; Pierce) in acetonitrile:acetone (25:75, v/v) were added for precolumn derivatization (15 min, 70 °C) of amino acids (modified method of Chang *et al.*, 1981). Samples were then diluted with aceto-nitrile:ethanol:water (2:1:1, v/v) and centrifuged (15000 g, 5 min). Dabsylated amino acid derivatives were analysed using reversed-phase HPLC under the following conditions: SuperPac ODS-2 column (Pharmacia); temperature 50 °C; solvent A was 9 mM-NaH₂PO₄, 0·2% triethylamine, 4% dimethylformamide, pH 6·8; solvent B was 80% aqueous acetonitrile; stepped linear gradients from 8 to 100% solvent B were run. Eluted dabsylated amino acids were detected at 436 nm. For external standards, mixed 125 pmol amounts each of the investigated amino acids (Standard AA-18; Sigma) were injected after derivatization.

For determination of baseline amino acid content, PVDF blanks were cut from areas of the blotted membrane not exposed to protein. For controls in the determination of amino acid composition, phages T7 and λ major capsid proteins were purified and analysed in the same way.

Amino-terminal sequencing of structural proteins. The sequence of the N-terminal amino acids from capsid and tail proteins of four representative phages (A511, A502, A006, B025) was determined from excised bands of the PVDF membranes, using an Applied Biosystems 477A protein sequencer (standard program).

Extraction of phage DNA and determination of base composition. For extraction of phage DNA, the standard procedure of Sambrook *et al.*

Table 1. *Morphological characteristics of the 14 listeriaphages investigated by 2-D electrophoresis, and M_r and isoelectric points of their major capsid and tail proteins*

Morphotype*	Species†	Phage	Major capsid protein		Major tail protein	
			M_r	pI	M_r	pI
A1	A511	A511	49·5K	ND‡	65·5K	ND
	4286	B054	ND	ND	ND	ND
B1	2671	A500	30·0K	5·25	20·0K	4·85
		A502	31·0K	5·20	20·5K	4·70
		A006	31·5K	5·92	26·0K	5·10
		B012	35·0K	5·00	21·5K	4·65
		B035	32·0K	5·05	21·5K	4·72
		B101	35·0K	5·05	21·5K	4·65
		B110	32·0K	4·73	21·0K	4·85
		B056§	35·5K	5·55	21·5K	4·70
	2685	B025	30·5K	5·10	23·5K	4·75
		D441	30·5K	5·45	23·5K	4·75
		B025	30·5K	5·10	23·5K	4·75
		B053	30·5K	5·10	23·5K	4·75

* Phage morphology where A1 is a myovirus with an isometric head and a contractile tail and B1 is a siphovirus with an isometric head and a long, non-contractile tail (see Ackermann & Eisenstark, 1974).

† Species are defined according to morphology and tail length (see text).

‡ ND, Not determined (protein bands could not clearly be assigned to major capsid and tail components).

§ Not shown on IEF gel (Fig. 1).

(1989) was used. Purified DNAs were resuspended in TE buffer (10 mM-Tris–HCl, 1 mM-EDTA, pH 8·0), and stored at $-20\,^{\circ}$C. In brief, the G+C contents of listeriaphage DNAs were determined as follows. Phage DNAs were heat-denatured and digested with nuclease P1. The hydrolysate and an external standard solution (equimolar amounts of all four nucleotides) were then subjected to reversed-phase HPLC (Lichrosorb RP-18 column, Merck, mobile phase 10 mM-phosphate buffer pH 7·0). G+C percentages were calculated from relative peak areas.

Restriction enzyme analysis. Purified phage DNAs (0·5 µg each) were digested with various restriction enzymes (*Afl*II, *Apa*I, *Ase*I, *Bam*HI, *Bsp*HI, *Bsp*1407I *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Eco*T22I, *Hin*dIII, *Hpa*I, *Kpn*I, *Ksp*I, *Mun*I, *Nde*I, *Pac*I, *Psp*1406I, *Pst*I, *Pvu*II, *Sca*I, *Sal*I, *Sca*I, *Sma*I, *Sna*BI, *Spe*I, *Sph*I, *Ssp*I, *Swa*I and *Xba*I) according to the suppliers' (USB, Pharmacia, Stratagene and New England Biolabs) recommendations. DNA fragments were separated by electrophoresis (0·8% agarose gels) in TAE buffer (Sambrook *et al.*, 1989) and ethidium bromide-strained. Fragment sizes were determined from comigrating *Hin*dIII and *Bst*EII digests of λ DNA (Sigma).

Southern blotting and DNA–DNA hybridization. *Cla*I-generated DNA fragments (total weight 0·3 µg) were electrophoresed as described above, vacuum-blotted (VacuGene XL, Pharmacia) onto positively charged nylon membranes (Boehringer), covalently bound by u.v.-irradiation (312 nm, 0·7 J/cm²), and hybridized to digoxigenin–dUTP-labelled DNA (Random Primed DNA Labelling Kit; Boehringer) from six phages selected according to morphology, protein profile and restriction enzyme patterns. Hybridization was carried out for 16 h under conditions of high stringency [5 × SSC (0·75 M-NaCl, 75 mM-trisodium citrate, pH 7·0), 68 °C], as were the subsequent washes (3 × 20 min; 0·1 × SSC, 0·1 % SDS; 68 °C). Chemiluminescent detection of hybridization signals was done essentially as outlined in the manufacturer's (Boehringer) protocol, except that X-ray film exposure was shortened to between 30 and 90 s.

Results

Listeriaphage proteins resolved by isoelectric focusing

Fig. 1 illustrates the protein patterns and resolution obtained with the IPG–CA technique. Interference by liberated phage nucleic acids was avoided by both
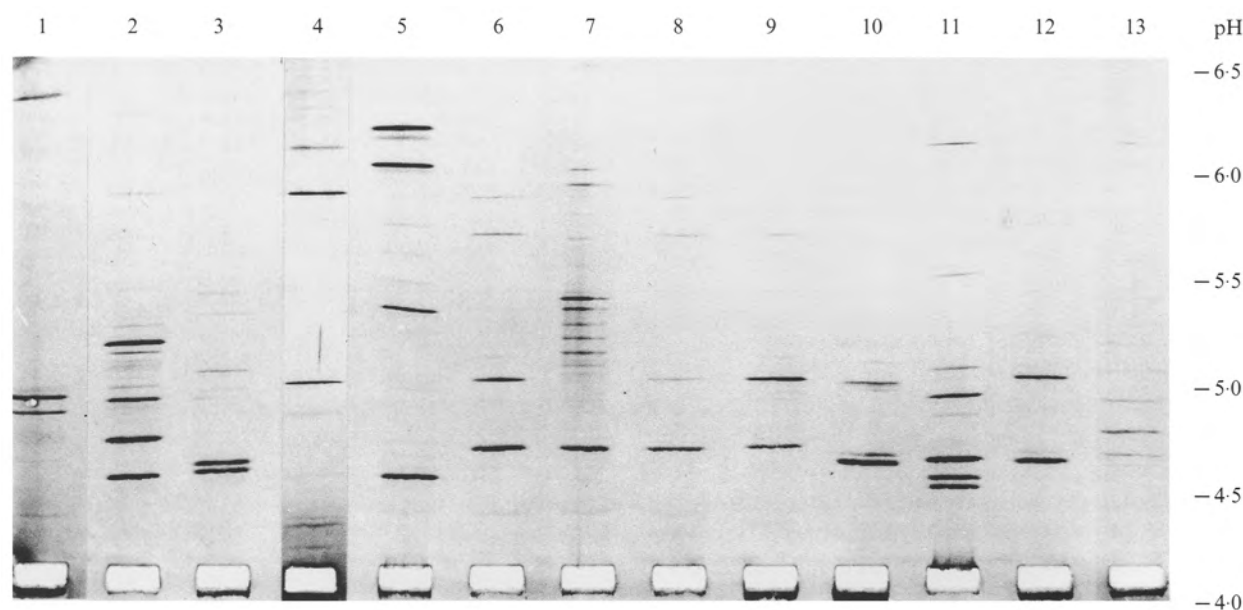

Fig. 1. IEF of structural proteins of 14 listeriaphages in immobilized pH gradient gels. Protein samples were applied in wells at the anodic gel side. Lanes are as follows: 1, A511; 2, A500; 3, A502; 4, A006; 5, B054; 6, B025; 7, D441; 8, B024; 9, B053; 10, B012; 11, B035; 12, B101; 13, B110.
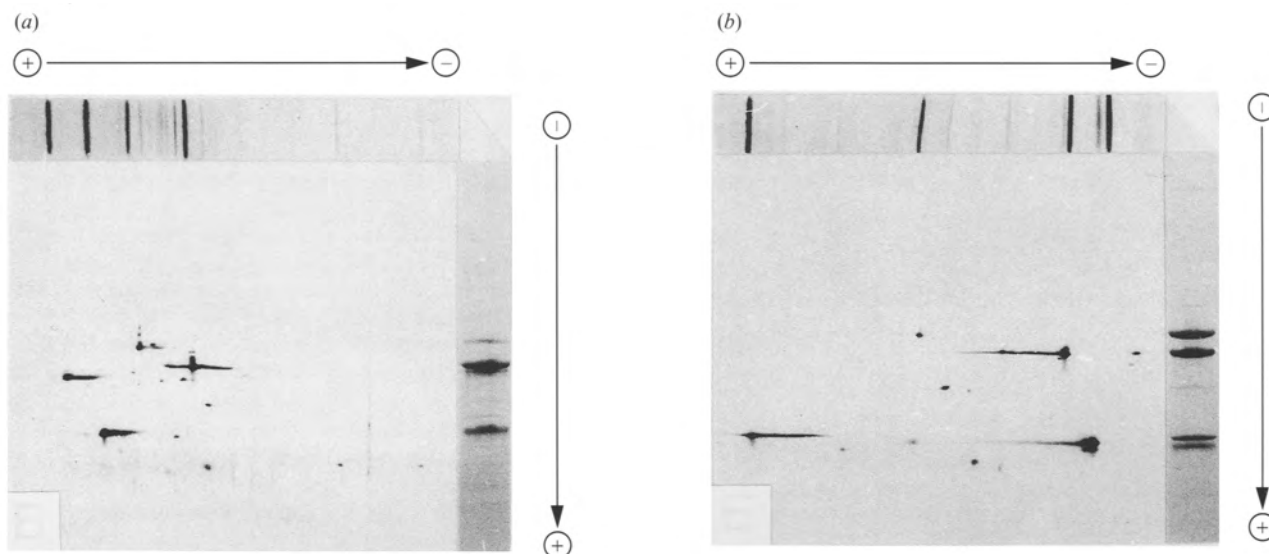
Fig. 2. Two-dimensional separation of phage proteins using IEF in immobilized pH gradient gels pH 4·5 to 6·5 in the first dimension (left to right), and molecular sizing in pore gradient (8 to 18% T) SDS gels in the second dimension (top to bottom). The respective results are given at the top and right side of each panel. (*a*) Listeriaphage A500, (*b*) B054.

Table 2. *Amino acid compositions of major capsid and tail proteins of four selected phages (in mol percent), and estimated numbers of amino acid residues for each protein (in parentheses)*

Amino acid	A511 49·5K Capsid	A511 65·5K Tail	A502 31·0K Capsid	A502 20·5K Tail	A006 31·5K Capsid	A006 26·0K Tail	B025 30·5K Capsid	B025 23·5K Tail
Asx	11·95 (54)	11·05 (66)	10·28 (29)	11·66 (22)	12·89 (37)	9·69 (24)	8·96 (26)	9·36 (20)
Thr	6·58 (30)	6·06 (36)	8·37 (23)	8·60 (17)	7·64 (22)	11·44 (29)	6·59 (19)	5·10 (11)
Ser	5·97 (27)	5·72 (34)	4·08 (11)	5·01 (10)	5·09 (15)	9·47 (24)	5·96 (17)	3·42 (7)
Glx	9·92 (45)	10·20 (61)	9·75 (27)	11·53 (22)	8·36 (24)	9·04 (23)	7·26 (21)	10·29 (22)
Pro	4·42 (20)	2·14 (13)	4·06 (11)	3·64 (7)	2·02 (6)	4·58 (12)	4·76 (14)	4·10 (9)
Gly	6·20 (28)	6·65 (40)	4·51 (13)	7·45 (14)	6·46 (19)	9·63 (24)	10·63 (31)	7·45 (16)
Ala	11·31 (51)	11·06 (66)	13·21 (37)	15·39 (30)	11·44 (33)	9·94 (25)	12·08 (35)	13·61 (29)
Val	8·45 (38)	7·92 (47)	6·75 (19)	6·52 (13)	9·55 (27)	11·49 (29)	9·04 (26)	8·36 (18)
Met	2·42 (11)	0·92 (5)	2·40 (7)	0·97 (2)	1·85 (5)	0·46 (1)	0·70 (2)	1·34 (3)
Ile	6·42 (29)	7·89 (47)	6·77 (19)	7·41 (14)	5·75 (17)	7·37 (19)	5·57 (16)	4·92 (11)
Leu	7·48 (34)	6·05 (36)	4·89 (14)	1·54 (3)	6·70 (19)	3·90 (10)	7·60 (22)	4·20 (9)
Tyr	3·05 (14)	3·59 (21)	3·48 (10)	3·72 (7)	5·03 (14)	1·99 (5)	3·60 (10)	3·74 (8)
Phe	4·05 (18)	4·79 (29)	5·01 (14)	3·61 (7)	4·92 (14)	2·71 (7)	3·92 (11)	5·27 (11)
His	1·91 (9)	2·34 (14)	1·94 (5)	1·17 (2)	1·31 (4)	0·00 (0)	2·42 (7)	3·28 (7)
Lys	5·86 (27)	8·13 (49)	9·94 (28)	8·76 (17)	8·42 (24)	7·85 (20)	8·35 (24)	11·03 (24)
Arg	4·02 (18)	5·49 (33)	4·56 (13)	3·00 (6)	2·56 (7)	0·44 (1)	2·56 (7)	4·55 (10)
Total	(453)	(597)	(280)	(193)	(287)	(253)	(288)	(215)

digestion with nuclease and the application of the sample at the anodic side. However, in some cases, undigested DNA can still be seen as a silver-stained smear at the anodic side of sample application wells (Fig. 1). All investigated listeriaphages showed two to four dense bands, which agrees well with the proposed number of major structural proteins as determined from SDS gels (Zink & Loessner, 1992). From three to 19 minor protein species could be identified per phage. The pI values for the two major protein species of the siphovirid phages ranged from 4·70 to 5·92, whereas one of the myoviruses

studied (B054) showed additional proteins with pIs between 6·1 and 6·3. These latter data most probably reflect the more complex structure of B054, since contractile tails are composed of more than a single major protein species.

Two-dimensional electrophoresis

As shown in Fig. 2, the combination of high-resolution IEF in immobilized pH gradients with molecular sizing in pore gradient SDS gels rendered it possible to correlate
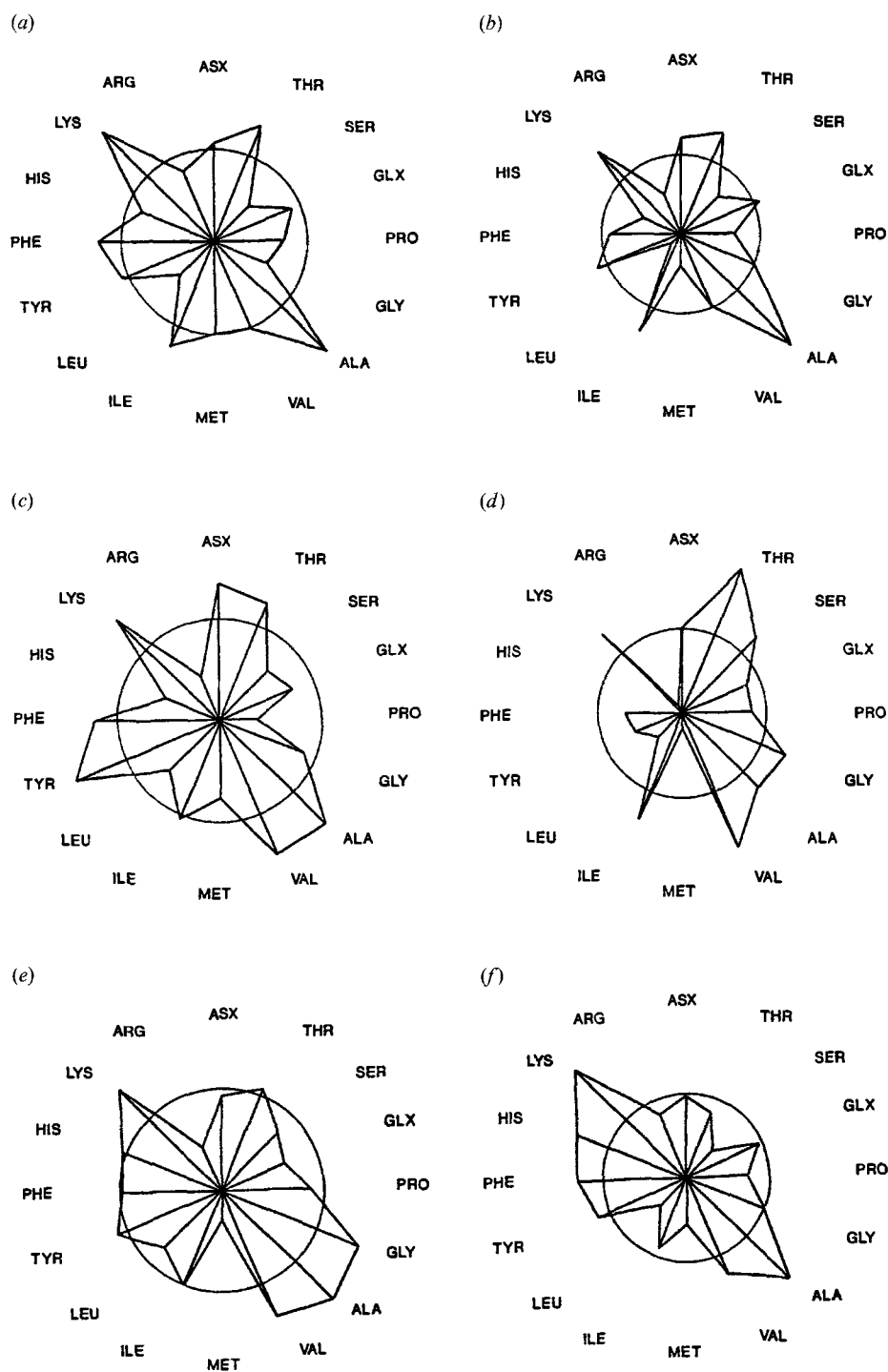
Fig. 3. Amino acid profiles of major structural proteins of the capsid and tail of three listeriaphages were plotted in relation to an 'average' protein (Shaw, 1992; see text), symbolized by a circle. Phage proteins are expressed as irregular polygons; vertices for each amino acid indicate their amount relative to the average. (*a*) Listeriaphage A502 capsid protein; (*b*) A502 tail protein; (*c*) A006 capsid protein; (*d*) A006 tail protein; (*e*) B025 capsid protein; (*f*) B025 tail protein.

M_r and pI of the major structural phage proteins (Table 1). The identity of capsid and tail proteins (except for those of phage B054) had already been determined (Zink

& Loessner, 1992). It is concluded from these data that the tail tube proteins of our phages are generally smaller and, with one exception (B110), more acidic than the

corresponding capsid elements. The structural function of the identified proteins of the myovirus B054 (morphotype A1) remains yet to be determined. With respect to the morphologically different myovirus A511, the three protein bands resolved in isoelectric focusing were not identical with the major structural proteins seen on SDS gels, as determined by 2-D electrophoresis analysis. We therefore conclude that the pIs of these proteins are either below 4·2 or above 6·8.

Amino acid composition from PVDF blots

Preliminary experiments indicated that the protein fractions separated by 2-D electrophoresis and transfer onto PVDF membranes were not sufficient for amino acid analysis. We therefore used one-dimensional (1-D) SDS gels, and obtained a very satisfactory transfer of all desired proteins (data not shown). Confirmation that only a single protein species was isolated came from a comparison of 1-D SDS gels with 2-D gels. Good transfer could also be obtained from 1-D IPG–CA gels, although the low acrylamide concentration (4% T) makes the ultrathin gels rather sticky and difficult to handle.

The amino acid composition of major proteins of four representative phages is presented in Table 2. Cysteine and tryptophan were destroyed during acid hydrolysis and are therefore not listed. Data are arithmetic means from two independent determinations, with average variations below 10%. Between 5 and 50 picomoles of each amino acid could be detected during a single analysis. Data obtained for major capsid proteins of bacteriophages λ and T7 (not shown) were compared to actual compositions derived from nucleotide sequences of these proteins (GenBank release 75.0), and indicate that this method yields a reasonably accurate amino acid composition.

As expected from the results of IEF, these phage proteins are fairly rich in acidic and hydrophobic amino acids, and some proteins show unusually high contents of alanine (e.g. A502, B035 and B110 tail proteins). Moreover, capsid proteins always contained more leucine than their corresponding tail counterparts. In contrast, the latter generally showed more glutamine and glutamic acid, which corresponds well to the data obtained by IEF.

We also used a computer program to compare our amino acid profiles with that of a database (GenBank)-derived 'average' protein (Shaw, 1992). Fig. 3 shows amino acid profiles of capsid and tail proteins of three phages plotted in relation to the GenBank average. It is evident that these proteins are unusually rich in short-

Table 3. *Sequences of six N-terminal amino acids of capsid and tail proteins of four listeriaphages*

Phage	Protein*	N-terminal amino acids
A511	Capsid	Ser–Phe–Thr–Thr–Gly–Tyr
	Tail	Ala–Ile–Glu–Ile–Tyr–Pro
A502	Capsid	Gly–Phe–Asn–Pro–Asp–Thr
	Tail	Ala–Arg–Ile–Lys–Asn–Ala
A006	Capsid	Ala–Ile–Asn–Tyr–Val–Asp
	Tail	Ala–Thr–Tyr–Ala–Val–Lys
B025	Capsid	Asp–Ile–Asn–Lys–Glu–Val
	Tail	Pro–Ile–Thr–Thr–Ile–Gly

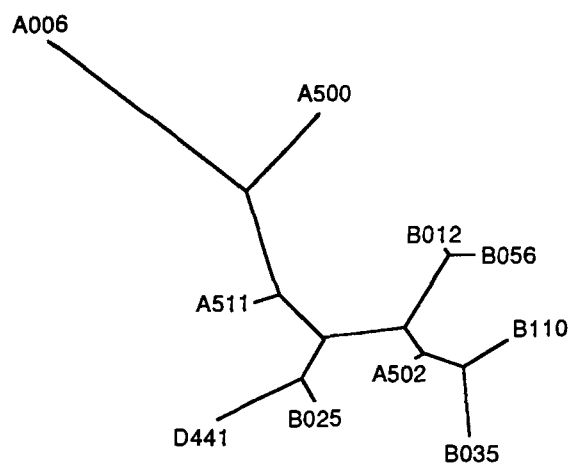* See Tables 1 and 2 for characteristics of these proteins.

Fig. 4. Amino acid composition data (mol percent) of the tail proteins of 10 listeriaphages computed with the aid of a continuous-character maximum-likelihood method (Felsenstein, 1989), and grouped in an unrooted tree according to relative similarities.

chain hydrophobic amino acids. Alanine and lysine are over-represented, whereas leucine is somewhat rare.

N-terminal amino acid sequences of structural virion proteins

Sequence comparison of the six N-terminal amino acids of capsid and tail proteins of four selected phages (Table 3) showed that all sequences are unique. This clearly indicates their unrelatedness and corresponds well to the results obtained by computer-aided analysis of amino acid compositions (see Fig. 4).

Base composition of listeriaphage DNAs

G + C percentages were relatively low and ranged from 37·7 to 39·4 (Table 4) reflecting the base composition of their *Listeria* hosts, determined to be 37 to 39% (Stuart & Welshimer, 1973). Only minor differences were

Table 4. *Characteristics and level of DNA similarity among the investigated phage DNAs*

Phage	DNA G+C (%)	DNA Size (kb)	Hybridization with digoxigenin-dUTP-labelled DNA from phage A511	A500	A006	B054	B025	B056
A511	37·7	116	+ + +*	–	–	–	–	–
A500	ND†	38	–	+ + +	–	–	–	+
A502	39·4	39	–	+ +	+	–	–	+
A006	37·3	34	–	+	+ + +	–	+	+
B054	38·3	42	–	–	–	+ + +	–	–
B025	38·1	39	–	–	+	–	+ + +	+
D441	37·9	37	–	–	–	–	+ + +	+
B024	ND	37	–	+	–	–	+ + +	+
B053	ND	37	–	–	–	–	+ + +	+
B012	38·5	38	–	+ +	+	–	–	+ +
B035	ND	35	–	+ +	–	–	+	+ +
B101	38·5	43	–	+ +	+	–	+	+ +
B110	ND	42	–	+ +	+	–	+	+ +
B056	38·8	35	–	+ +	+	–	+	+ + +

* + + +, 80 to 100% hybridization; + +, 50 to 80% (most fragments and/or strong signals); +, 20 to 50% (one or two fragments and/or weak signals); –, below 20% (no signal).
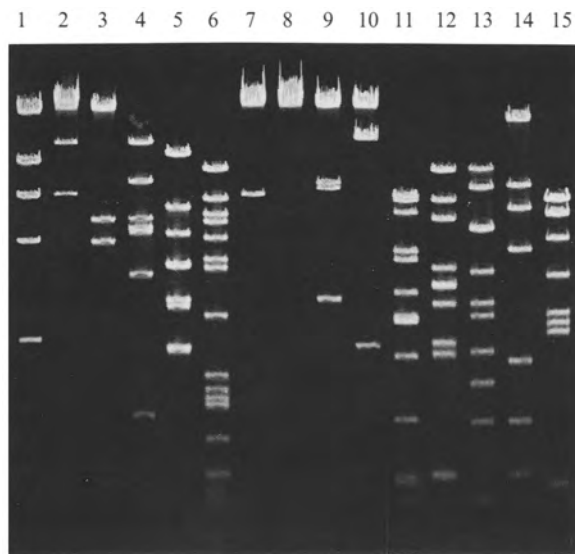† ND, Not determined.

Fig. 5. Gel electrophoresis patterns of listeriaphage DNAs digested by *Cla*I. Lane 1, molecular size standard (λ *Hin*dIII digest). Other lanes are as follows: 2, A511; 3, A500; 4, A502; 5, A006; 6, B054; 7, B025; 8, D441; 9, B024; 10, B053; 11, B012; 12, B035; 13, B101; 14, B110; 15, B056.

observed among the various DNAs, and no modified bases could be detected with our method.

Restriction enzyme digestion patterns

Fig. 5 shows the fragment pattern obtained by electrophoretic separation of *Cla*I-digested listeriaphage DNAs. The individuality of the investigated phages was confirmed by digestion with several enzymes. Although no

identical fragment patterns could be observed, some enzymes (e.g. *Pst*I and *Pvu*II) yielded somewhat similar patterns for phages B025, D441, B024 and B053 (data not shown). This may indicate their close relatedness at the nucleotide sequence level, which is further supported by DNA–DNA hybridization (see below). As expected from their base composition, the number of fragments generated by enzymes with recognition sites composed of A and T only (e.g. *Ase*I, *Dra*I and *Ssp*I) was very high (more than 25 cuts/DNA), whereas enzymes such as *Apa*I, *Ksp*I, *Kpn*I and *Sma*I had very few or no recognition sites.

Approximate total genome sizes of the phages (Table 4) were calculated by combining the fragment sizes obtained with three to four selected enzymes yielding between five and 15 fragments per DNA.

DNA–DNA hybridization

Digoxigenin-labelled DNAs from six phages were hybridized to membrane-bound *Cla*I fragments of all phages. Homologies that were detected with labelled DNA of phages A500 and A006 are presented in Fig. 6 and a summary of results is given in Table 4. The two myoviruses (A511 and B054) showed no similarity to other phages, whereas different degrees of homology were detected among the 12 siphoviridae that were investigated. Overall, the 14 phages may be clustered into five groups: I, A511; II, B054; III, A006; IV, B025, D441, B024 and B053; V, A500, A502, B012, B035, B101, B110 and B056. Only a little cross-hybridization (single bands and/or weak signals) was observed between the siphoviridae groups III, IV and V.
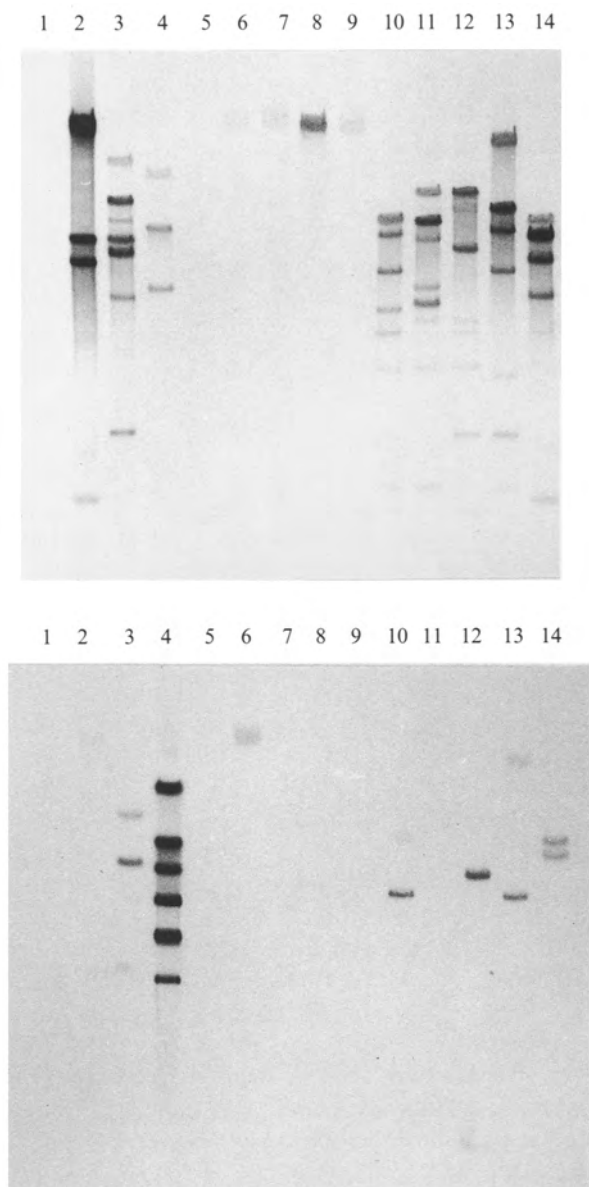
Fig. 6. Southern blots of *Cla*I fragments of phage DNAs were hybridized with digoxigenin-labelled DNA from phages A500 (top) and A006 (bottom). Lanes are as follows: 1, A511; 2, A500; 3, A502; 4, A006; 5, B054; 6, B025; 7, D441; 8, B024; 9, B053; 10, B012; 11, B035; 12, B101; 13, B110; 14, B056.

Discussion

We characterized the major structural proteins of 14 listeriaphages by IEF in immobilized pH gradients, 2-D electrophoresis and amino acid analysis. Regarding IEF, we initially observed a tendency of the phage proteins to agglomerate and precipitate, so that they would not enter the gel matrix. This was overcome by using high concentrations of urea in the aqueous solvents (sample buffer and gel), and adding surfactants as well as carrier ampholytes to both sample buffer and gel rehydration

solution. The latter increases solubility especially of hydrophobic proteins, perhaps by forming mixed, zwitterionic CA-surfactant micelles, or by directly complexing with the protein itself (Righetti, 1990). The insolubility of some particular proteins could also be overcome by disrupting the phage particles (in the presence of surfactant and thiol reagent) by heating to 95 °C for 5 min prior to addition of sample buffer. The use of SDS for treatment of phage proteins prior to IEF (Gersten *et al.*, 1981) should, in general, be avoided. SDS may severely interfere with focusing and determination of true pI values, at least when conventional CA IEF is used. However, recent results from our laboratory indicate that the addition of 0·2% SDS to phage suspensions, followed by short boiling for 2 min and addition of IPG–CA sample buffer yields identical pI values, while substantially improving the solubilization of particular proteins. Protein pIs are apparently unaffected by low SDS concentrations (final concentration 0·02%) when, at the same time, other surfactants (NP40 and CHAPS) are present at much higher concentrations (final concentration 1·8%).

As compared to 1-D SDS gels (Zink & Loessner, 1992), ultrathin-layer IPG–CA and 2-D electrophoresis improved resolution of proteins and, unlike SDS–PAGE, permitted discrimination of very closely related phages (e.g. B025 and D441). However, phages cannot be grouped in characteristic protein clusters, as they can in SDS–PAGE pherograms. Therefore, with respect to phage classification, IEF should be used in conjunction with SDS–PAGE.

The structural elements of some phages (e.g. B025, B024, B053 and B012, B101) were nearly identical in both dimensions (IEF and SDS–PAGE), indicating that they are very closely related, i.e. they could be mere variants of a single phage. Nevertheless, these phages have distinct host ranges (Loessner, 1991) and DNA restriction patterns. Their protein profiles do however suggest a common origin of at least those genetic elements which encode the structural components of capsid and tail. When phage D441 (isolated from *L. seeligeri*) is compared to phages B025, B024 and B053 (all from *L. innocua*), they are indistinguishable by SDS electrophoresis. Here we show that even though the tail proteins of these phages are identical, the D441 capsid protein differs from the others with respect to pI and amino acid composition. This could be interpreted as evidence for 'horizontal transfer' of the encoding genes or exchange of 'gene modules' as proposed for a number of other bacteriophages (Casjens & Hendrix, 1988).

Since the amino acid composition of related proteins is supposed to be a fairly reliable indicator of actual sequence similarities (Cornish-Bowden, 1979), we analysed our amino acid data with a computer program of

Felsenstein (1989) and constructed unrooted maximum likelihood trees of the capsid and tail proteins of the 10 phages studied. Both analyses yielded similar trees, one of which is depicted in Fig. 4. Although these clusters are not suggested to represent a phylogenetic system, they indicate possible sequence similarities on the structural protein level. Most importantly, they correspond well to a classification of these listeriaphages by host ranges, phage-induced host resistance patterns, particle morphology, SDS–PAGE profiles (Loessner & Busse, 1990; Loessner *et al.*, 1991; Zink & Loessner, 1992) and DNA–DNA hybridization (this study).

Restriction enzyme digestion patterns appeared different for each phage, and are therefore most useful for distinction of very closely related virions. However, as a low-level criterion, they seem to be unsuitable for establishing a taxonomic system (Ackermann *et al.*, 1992).

Listeriaphage A511 is a large myovirus and the only known virulent listeriaphage, with an extremely broad host range (Loessner & Busse, 1990). The unique position of this virus among the other listeriaphages is further supported here by its unusually large genome (approximately 116 kb), and unrelatedness at the DNA homology level. Phages of quite similar ultrastructure occur in a number of Gram-positive genera related to the listeriae, e.g. *Brochothrix, Bacillus, Staphylococcus, Enterococcus* and *Lactobacillus* (H.-W. Ackermann, personal communication). It would be very interesting to compare these viruses with respect to structural proteins and DNA homology. We intend to do this in the near future.

Classification of listeriaphages into species is currently based on essentially morphological criteria. While there are two different myovirus species, the siphoviruses are classified into five species on the basis of tail length. Rocourt *et al.* (1986) investigated 10 listeriaphages of three species by DNA–DNA cross-hybridization, and reported an excellent agreement between DNA homology and morphology. In general, their findings were confirmed in this study. However, phage A006 shared only very little homology (see Fig. 6) with the other investigated members of species 2671 (A500, A502, B012, B035, B101, B110, and B056; Zink & Loessner, 1992). Since we feel that actual DNA sequence homologies offer a greater taxonomic potential than particle dimensions alone, this phage should be excluded from species 2671 and regarded as a new species, A006. Species of tailed phages can be defined by a combination of morphology and DNA homology, as recently proposed by the ICTV Bacterial Virus Subcommittee (Ackermann *et al.*, 1992). The deviating character of phage A006 is further supported by its protein composition and, in particular, the characteristics of its tail protein (see Table 1 and Fig. 4).

The different methods applied for analysis and comparison of listeriaphages yielded a comprehensive set of data and enabled us to improve our knowledge on the biochemistry and genetics of listeriaphages. Moreover, the results obtained provide a good basis for establishing a final system for differentiation and classification of *Listeria* bacteriophages.

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