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Classification of Lytic Bacteriophages Attacking Dairy *Leuconostoc* Starter Strains

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A set of 83 lytic dairy bacteriophages (phages) infecting flavor-producing mesophilic starter strains of the *Leuconostoc* genus was characterized, and the first in-depth taxonomic scheme was established for this phage group. Phages were obtained from different sources, i.e., from dairy samples originating from 11 German dairies (50 *Leuconostoc pseudomesenteroides* [*Ln. pseudomesenteroides*] phages, 4 *Ln. mesenteroides* phages) and from 3 external phage collections (17 *Ln. pseudomesenteroides* phages, 12 *Ln. mesenteroides* phages). All phages belonged to the *Siphoviridae* family of phages with isometric heads (diameter, 55 nm) and noncontractile tails (length, 140 nm). With the exception of one phage (i.e., phage ΦLN25), all *Ln. mesenteroides* phages lysed the same host strains and revealed characteristic globular baseplate appendages. Phage ΦLN25, with different Y-shaped appendages, had a unique host range. Apart from two phages (i.e., phages P792 and P793), all *Ln. pseudomesenteroides* phages shared the same host range and had plain baseplates without distinguishable appendages. They were further characterized by the presence or absence of a collar below the phage head or by unique tails with straight striations. Phages P792 and P793 with characteristic fluffy baseplate appendages could propagate only on other specific hosts. All *Ln. mesenteroides* and all *Ln. pseudomesenteroides* phages were members of two (host species-specific) distinct genotypes but shared a limited conserved DNA region specifying their structural genes. A PCR detection system was established and was shown to be reliable for the detection of all *Leuconostoc* phage types.

Lactic acid bacteria of the genus *Leuconostoc* are Gram-positive, ellipsoidal to spherical bacteria (1) and are important components of dairy mesophilic starter cultures. In nature, leuconostocs preferably grow on vegetables (e.g., cabbage), pasture, and silage and are also part of the microflora of raw milk (2–5). Milk, however, is not an optimal growth medium for leuconostocs, since they do not possess efficient proteolytic systems (1, 6). Leuconostocs are present as minor components in complex (undefined) mixed-strain starter cultures consisting mainly of *Lactococcus lactis* (*L. lactis*) starter strains. In these complex cultures, the flavor-producing microbiota consists of either leuconostocs exclusively or a blend of leuconostocs and *L. lactis* subsp. *lactis* bv. diacetylactis strains (7). *Leuconostoc mesenteroides* (*Ln. mesenteroides*) or *Ln. pseudomesenteroides* strains represent the predominant flavor-producing population (8), while *Ln. lactis* strains are less frequently used (9). These bacteria are important flavor producers, metabolizing the citrate in milk into aroma compounds like diacetyl or acetoin (6). Diacetyl represents the most important flavor compound in semihard Dutch-type cheeses (e.g., Gouda, Tilsiter), in acid cream butter, and in fresh cheeses (3, 10). Leuconostocs produce significant amounts of CO_2 in fermented milk products, resulting from their citrate metabolism and heterofermentative lactic acid fermentation, and CO_2 is important for eye formation and the texture of semihard cheeses and for the opening of the matrix of white- and blue-molded cheeses (11). Hence, bacteriophages (phages) attacking flavor-producing *Leuconostoc* starter cultures may result in undesirable variation of these technologically important functions.

Bacteriophages infecting *L. lactis* and *Streptococcus thermophilus* cultures have been studied extensively (12–14), while only limited knowledge exists for dairy *Leuconostoc* phages. Since *Leuconostoc* strains do not contribute significantly to lactic acid production in milk, phage infections of *Leuconostoc* cultures may remain unnoticed during processing but will be detected in the final products. Phages infecting dairy *Leuconostoc* strains were first reported in 1946 (15), and these phages were studied only scarcely in the following 4 decades (16–19). Preliminary classifications of *Leuconostoc* bacteriophages based on DNA-DNA hybridization were published afterwards (20, 21). The complete genomic sequences of the lytic *Ln. mesenteroides* phage Φ1-A4 isolated from a sauerkraut fermentation (22), the temperate *Ln. pseudomesenteroides* phage ΦMH1 induced from a kimchi strain (23), and the lytic dairy *Leuconostoc* phage ΦLmd1 (24) have been communicated recently.

The dissemination of *Leuconostoc* phages in German dairies has previously been determined (8). In the present work, we propose the first comprehensive phage taxonomy standard for lytic dairy *Leuconostoc* phages based on an in-depth characterization of 83 *Leuconostoc* phage isolates. Furthermore, a reliable universal PCR tool was established for the detection of all lytic *Ln. mesenteroides* and *Ln. pseudomesenteroides* phages included in this study.

MATERIALS AND METHODS

Phages and strains. *Leuconostoc pseudomesenteroides* phages and their host strains were isolated from dairy samples obtained from 3 large German dairies (i.e., dairies L1 to L3, 12 phages) and 7 small to medium-size German dairies (dairies S1 to S7, 38 phages). A set of 17 *Ln. pseudomesenteroides* phages and 11 *Ln. mesenteroides* phages was provided by two external phage collections, as described above (8).

Received 9 January 2013 Accepted 28 March 2013
Published ahead of print 5 April 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00076-13.

doi:10.1128/AEM.00076-13

TABLE 1 Dissemination, maximal titers, and characterization of 54 *Leuconostoc* phages in samples from 11 dairies and characterization of 29 supplemental dairy phages from 3 phage collections[a]

Dairy or phage	Maximum phage titer (phage isolates) from the indicated phage sources[b]			Phage characteristic					
				Lysis of the indicated host (host range)[c]					
				Ln.pseudomesenteroides		*Ln.mesenteroides*			
	Whey	Product	Brine	15L1 (A1)	BM2 (A2)	C008 (B1)	LN25 (B2)	Morphotype	Genotype
Large dairies									
L1	7×10^6 (P790, *P791*)	6×10^6 (**P812**,[d] P813[d])	3×10^6 (P796, P797)	●	—	—	—	IIb	II
L2	>10	>10	6×10^5 (P822, P824)	●	—	—	—	IIb	II
L3	>10	5×10^3 (P839,[d] P840[d])	5×10^4 (P816, P817)	●	—	—	—	IIa	II
L4	NS	1×10^2 (P841,[e] *P842*,[e] P843,[e] P844[e])	NS	—	—	●	—	Ia	I
Small to medium-size dairies									
S1	>10	NS	5×10^6 (P798, P799)	●	—	—	—	IIb	II
S2	>10	>10	2×10^2 (P825, P826, P829, P830)	●	—	—	—	IIa	II
S3	NS	4×10^5 (P792,[d] P793[d])	NS	—	●	—	—	IIc	II
	NS	NS	3×10^4 (P800, P801, P802, P803, P804, P805)	●	—	—	—	IIb	II
S4	>10	>10	2×10^5 (P806, P807, P808, P809)	●	—	—	—	IIb	II
S5	1×10^3 (P794, P795)	3×10^4 (P814,[d] P815[d])	8×10^5 (P810, P811)	●	—	—	—	IIa	II
S6	2×10^3 (P831, P832)	6×10^3 (P835,[f] P836,[f] P837,[d] P838[d])	NS	●	—	—	—	IId	II
S7	3×10^5 (P818, P819)	6×10^1 (P827,[g] P828,[g] P833,[d] P834[d])	2×10^6 (P820, P821)	●	—	—	—	IIb	II
Phage collection 1[h,i]									
P698, P699 P701, P768, P776, P784, P785, P787, P788, P789				●	—	—	—	IIb	II
P700, P702, P772, P775, P786				●	—	—	—	IIa	II
P767, (P769), P770, (P771), P773, **P774**				—	—	●	—	Ia	I
Phage collection 2[h,i]									
P777, P783				●	—	—	—	IIa	II
(P778), P779, P780, P781, P782				—	—	●	—	Ia	I
Phage collection 3,[h] ΦLN25				—	—	—	●	Ib	I

[a] The four underlined phages were either used as probes for Southern blot analysis (P791 and P842, in italics) or for DNA sequence analysis (P812 and P774, in bold).
[b] Titers are in numbers of PFU ml^{-1} or g^{-1}. NS, no sample(s) obtained.
[c] ●, lysis of host bacteria; —, no lysis.
[d] Hard cheese.
[e] Butter milk.
[f] Butter cream.
[g] Acid curd cheese.
[h] Dairy phages of unspecified origin.
[i] Phages in parentheses had separated baseplate globular appendages attached to their tails.

In addition, 4 *Ln. mesenteroides* phages from another dairy (L4) and unique *Ln. mesenteroides* phage ΦLN25 obtained from a third external phage collection were also included in this study. Hence, an extensive collection of 67 *Ln. pseudomesenteroides phages* and 16 *Ln. mesenteroides* phages was studied, as listed in Table 1. All 83 phages were propagated on selected host strains, as indicated in Table 1 and as described earlier (8).

Propagation of host bacteria and phages. The four host strains (Table 1), isolated in a former study from dairy starter cultures (8), were grown at 30°C in MRS broth (Merck, Darmstadt, Germany). Phages were isolated from dairy samples as indicated in Table 1. Phage titers were determined on MRS agar (supplemented with 0.1% glycine [Merck, Germany] and 10 mM calcium chloride [Roth, Karlsruhe, Germany]) using the double-layer method (plaque assay) with MRS soft agar (0.8%) and MRS bottom agar (1.5%). Phages (10 μl of serial dilutions) were spotted on freshly prepared bacterial lawns in MRS top agar. The plates were incubated overnight for 16 h at 30°C.

All phages were concentrated and purified by CsCl gradient centrifu-

gation for electron microscopic analysis and for isolation of phage DNA (25–27).

TEM. Negative staining of phage samples with 2% (wt/vol) uranyl acetate (Plano, Wetzlar, Germany) on freshly prepared carbon films (Plano) and transmission electron microscopy (TEM; Tecnai 10; FEI Company, Eindhoven, The Netherlands) at an acceleration voltage of 80 kV were performed as described earlier (25, 26).

Phage DNA manipulations. Isolation of phage DNAs was performed as described by Sambrook and Russell (27). Labeling of phage DNAs, Southern blot hybridization assays, and restriction endonuclease analysis were performed according to the suppliers' recommendations (Boehringer Mannheim GmbH, Mannheim, Germany [digoxigenin {DIG} DNA labeling kit]; Fermentas, St. Leon-Rot, Germany).

Phage DNA cloning, sequencing, and PCR primers for phage detection. *Ln. mesenteroides* phage P774 DNA was digested with XbaI according to the supplier's (Fermentas, St. Leon-Rot, Germany) recommendations and was subsequently purified by a PCR purification kit (Macherey-Nagel, Düren, Germany) (phage P774 XbaI profile; see Fig. 2B). Fragments were cloned into pSTBlue-1 (Novagen/Merck Millipore, Nottingham, United Kingdom). *Escherichia coli* Easy pore (Eurogentec, Cologne, Germany) was used for transformation. Transformants were grown in LB broth (27). A pSTBlue-1 transformant with a 1.1-kb insert was selected, and DNA sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany). Subsequent DNA sequencing of flanking DNA regions was done by a primer-walking strategy encompassing the flanking 1.2- and 2.7-kb XbaI DNA fragments. Phage P774-specific primers were also tested with phage *Ln. pseudomesenteroides* P812 DNA in order to identify the region of high DNA homology. Phage P774-specific primers capable of amplifying the phage P812 DNA region revealing DNA homology were used for phage P812 DNA sequencing. Subsequent sequencing of the flanking nonhomologous phage P812 DNA region was also done by primer walking (Eurofins MWG Operon).

For the simultaneous detection of *Ln. mesenteroides* and *Ln. pseudomesenteroides* phages, the following universal primers were selected: LnP_f (5′-TCAACNGGTGTNCAAAAGTTT-3′) and LnP_r (5′-CTTCG TCTTCATCGTCACTTTC-3′) (size of amplicon, 322 bp). Conditions of PCRs (DreamTaq Green PCR; Fisher Scientific, Slangerup, Denmark) were (i) initial denaturation for 3 min at 95°C, (ii) 30 cycles of 30 s at 95°C, 30 s at 56°C, and 15 s at 72°C, and (iii) a final elongation for 5 min at 72°C.

Pairwise alignment and dot plot analysis. The sequences of the genes putatively coding for the major tail protein (MTP) of phages Φ1-A4, ΦLmd1, and ΦMH1 were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). The sequences of the *mtp* gene in phages P774 and P812 were predicted using Genemark.hmm with a heuristic approach (28). Alignment of the *mtp* sequences was performed in CLC Main Workbench (version 6.7). The dot plot analysis of the *mtp* sequence of P774 and P812 phages was done using CLC Main Workbench (version 6.7) and a window length of 20.

Nucleotide sequence accession numbers. Partial nucleotide sequences of *Ln. mesenteroides* phage P812 (6,194 bp) and of *Ln. pseudomesenteroides* phage P774 (6,230 bp) have been deposited in GenBank under accession numbers KC153026 and KC153025, respectively.

RESULTS AND DISCUSSION

Dissemination of *Leuconostoc* phages in dairies. Data on the dissemination of 77 dairy *Leuconostoc* phages have recently been published (8). Here, an extended set of phage isolates was analyzed, and in total, 83 virulent phages infecting dairy *Leuconostoc* starter strains were characterized according to host range, morphotype, genotype (restriction patterns), and genetic relationship (Southern hybridization). Samples were obtained from 26 German dairy plants from different sources: whey, brine, and products (hard cheese, buttermilk, butter cream, acid curd cheese). Among these, samples from 4 large and 7 small and medium-size enterprises were found to be contaminated with phage infecting *Leuconostoc* starter strains. Phage titers varying from 6×10^1 PFU per g of hard cheese (dairy S7) to 7×10^6 PFU per ml of whey (dairy L1) were documented, as indicated in Table 1. These low to medium phage titers reflect the small number of *Leuconostoc* strains in undefined mixed-strain starter cultures (i.e., <10%) (6). Lactococcal phages, however, commonly accumulate in dairy samples at high numbers of approximately 10^9 PFU per ml of whey (25).

***Leuconostoc* phage morphotypes.** All phages were analyzed by TEM and showed the same basic morphotype of *Siphoviridae* phages (29) with noncontractile tails (length, approximately 140 nm) and isometric heads (diameter, approximately 55 nm) (Fig. 1), but distinct differences were noticed with regard to baseplate appendages.

***Ln. mesenteroides* phages.** As shown in Fig. 1, all except one phage infecting *Ln. mesenteroides* exhibited discernible, clearly defined, slightly prolonged globular appendages at their baseplates (size, 8 × 12 nm; maximum number at baseplate, 6). Hence, the whole baseplate complex, including these globular structures, exhibited a large diameter of approximately 40 nm. *Ln. mesenteroides* phages with these morphological characteristics were designated morphotype Ia phages (i.e., phages P842 and P770; Fig. 1). For 3 phages (phages P769, P771, and P778; Table 1), many of these globular structures were also found detached from the baseplate (instead adsorbing randomly on the phage tails, e.g., phage P778 in Fig. 1). Apparently, these appendages are not rigidly linked to the central baseplate structure, as they were frequently observed detached from the baseplates. Similar globular baseplate appendages characteristic for type Ia phages have also been described before for *Ln. mesenteroides* phages por2 (19) and PWL-2 (16) (see also Fig. S1 in the supplemental material).

Ln. mesenteroides phage ΦLN25 was designated a distinct morphotype Ib phage, as it was unique and did not reveal distinct globular structures at its baseplate but contained elongated Y-shaped structures with sizes of approximately 9 by 13 nm (total diameter of baseplate, 40 nm) (Fig. 1).

***Ln. pseudomesenteroides* phages.** Globular or Y-shaped baseplate structures were not detected on phages infecting *Ln. pseudomesenteroides*, which, with the exception of 2 phages (i.e., P792, P793; see below), had a simple slightly enlarged baseplate with a diameter of approximately 25 nm (Fig. 1). All *Ln. pseudomesenteroides* phages revealing a distinct collar below the phage head were designated morphotype IIa phages (e.g., phages P839 and P829 in Fig. 1 and Table 1), while all phages without a collar were classified as morphotype IIb phages (e.g., phages P791 and P822 in Fig. 1 and Table 1). The *Ln. pseudomesenteroides* phages P792 (Fig. 1; Table 1) and P793 (Table 1) were grouped as morphotype IIc phages showing undefined fluffy baseplate appendages that differed from the *Ln. mesenteroides* baseplate structures. All *Ln. pseudomesenteroides* phages of morphotype IId isolated from whey and product samples of small dairy S6 (Table 1) exhibited, on average, 9 peculiar tail striations (diameter, 15 nm; thickness, 4 nm; e.g., phage P832 in Fig. 1), which appeared to be evenly distributed over the whole tail length. Similar tail decorations of unknown function are also known from other dairy *Siphoviridae* phages, i.e., the *L. lactis* type phage 1358 (30) and the *Lactobacillus delbrueckii* subsp. *lactis* phage JCL 1032 (31).

The two morphotype IIc phages P792 and P793 were isolated from cheese samples in dairy S3. Phages isolated from brine taken on the same day in dairy S3, however, contained morphotype IIb

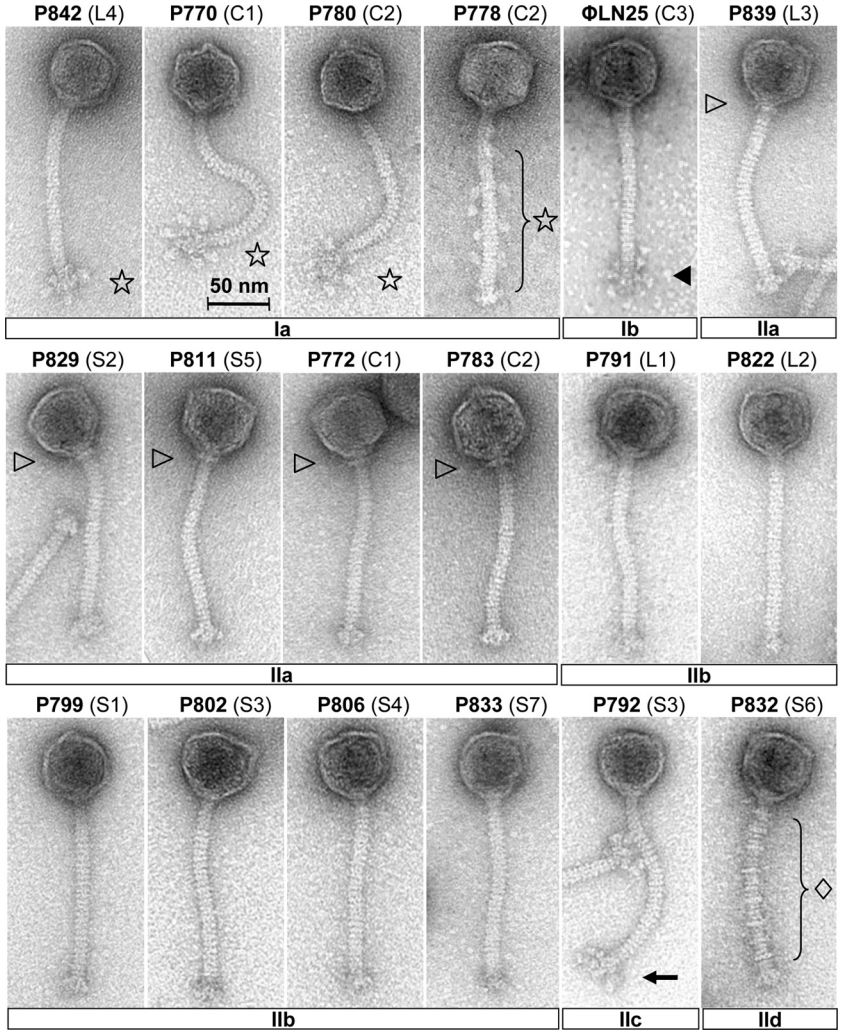


FIG 1 Transmission electron micrographs of representative *Leuconostoc* phages isolated from large dairies (L1 to L4) or from small to medium-size dairies (S1 to S7) or obtained from three phage collections (C1 to C3). The morphotypes of *Ln. mesenteroides* phages (Ia and Ib) and of *Ln. pseudomesenteroides* phages (IIa to IId) are indicated, as specified in Table 1. Morphological details are marked as follows: ☆, globular baseplate appendages (these are also detached from the baseplate but attached to the tail; see phage P778); ◀, nonglobular (Y-shaped) baseplate appendages; ◁, collar; ←, fluffy baseplate appendages; ◇, tail striations.

phages exclusively (Fig. 1 and Table 1). Plain baseplates characteristic for *Ln. pseudomesenteroides* phages of morphotypes IIa, IIb, and IId were documented before by electron microscopy of dairy *Leuconostoc* phages (17, 21).

It is remarkable that we did not find *Ln. lactis* starter strains, and consequently, phages infecting *Ln. lactis* were not isolated during our extensive study. However, *Ln. lactis* phages have occasionally been detected in dairy samples (32) but so far have not been examined in detail.

***Leuconostoc* phage host ranges.** Since phages attach to their host cells by their specific receptor binding proteins located in their baseplate structures (33–36), we analyzed a representative set of phages for their host range by testing plaque-forming ability on representative *Ln. pseudomesenteroides* and *Ln. mesenteroides* strains (Table 1). As expected, morphotype I and II phages exhibited unique host ranges. In addition, the type Ia phages and the sole type Ib phage, ΦLN25, were characterized by separate host ranges (i.e., B1 and B2, respectively; Table 1). Among morphotype II *Ln. pseudomesenteroides* phages, the two type IIc phages P792 and P793 showed a host range (i.e., host range A2) differing from that of all other morphotype IIa, IIb, and IId phages (host range A1). Thus, the differences in baseplate structures correlated perfectly with the observed host range profiles, an observation that is consistent with findings relating to lactococcal phages (34–36). Grouping of a few dairy *Leuconostoc* phages on the basis on their distinct, nonoverlapping host ranges has previously been documented (17, 18, 32), but no correlation with morphological data was provided.

Restriction enzyme analysis of *Leuconostoc* phages. To assess the genetic biodiversity of the *Leuconostoc* phages, restriction enzyme analysis was performed with the endonuclease XbaI (*Ln. mesenteroides* phages) or HaeIII (*Ln. pseudomesenteroides* phages) (Fig. 2). Notably, *Ln. mesenteroides* phage DNAs presented very few (i.e., 1 to 2) HaeIII recognition sites, whereas *Ln. pseudomesenteroides* phage DNAs possessed very few, if any, XbaI sites (data not shown). For the *Ln. mesenteroides* phages, just six different

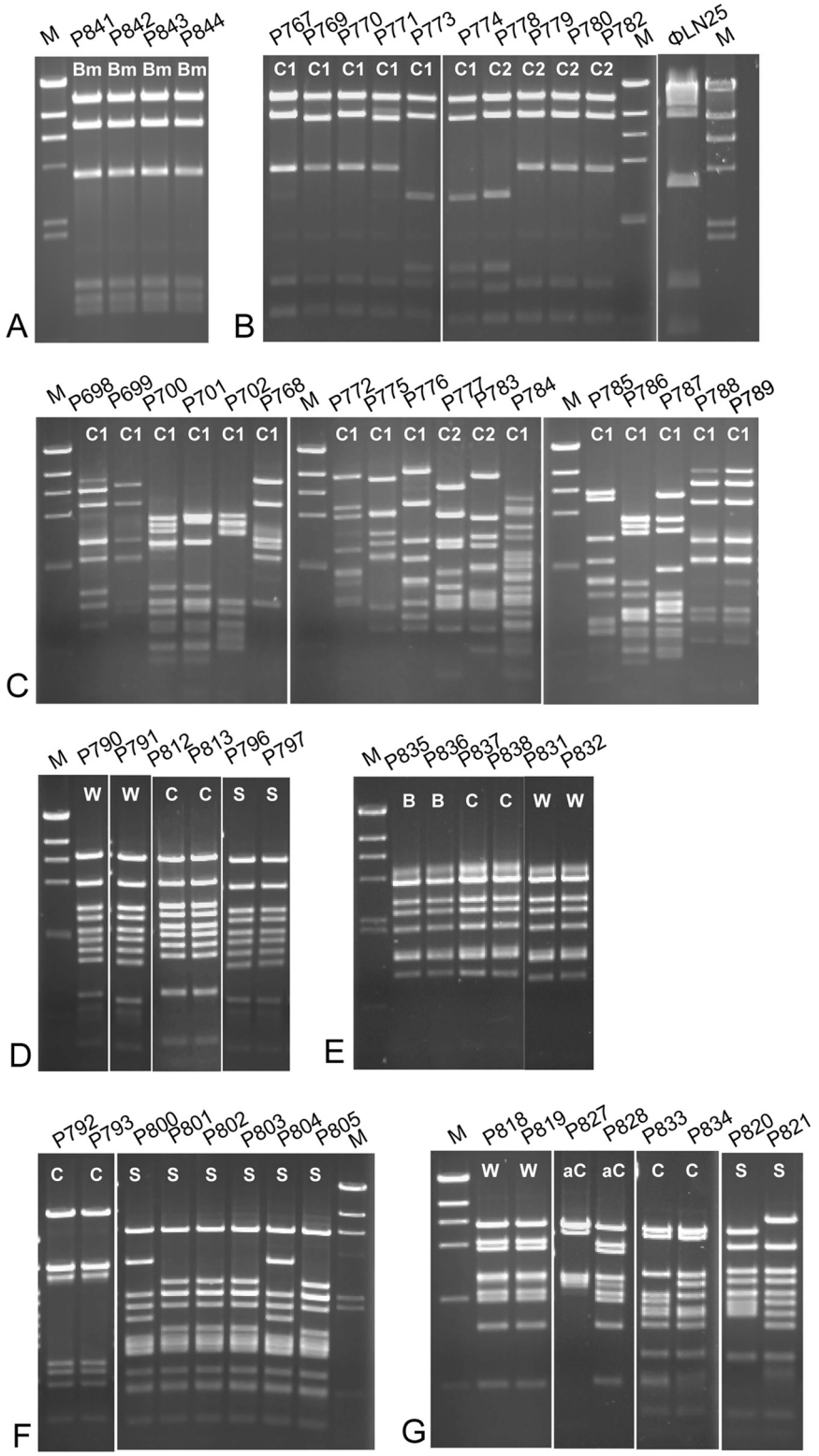


FIG 2 Restriction enzyme analysis of the DNA from *Ln. mesenteroides* phages using XbaI (A and B) and *Ln. pseudomesenteroides* phages using HaeIII (C to G). (A and B) *Ln. mesenteroides* phages were isolated from buttermilk samples (Bm) obtained from dairy L4 (A) or 3 phage collections (C1 to C3) (B). (C to G) *Ln. pseudomesenteroides* phages were obtained from phage collections C1 and C2 (C) or isolated from various dairy products: whey (W), hard cheese (C), brine bath (S), butter cream (B), and acid curd cheese (aC). Phages were isolated from dairies L1 (D), S6 (E), S3 (F), and S7 (G). HindIII digests of phage λ DNA were used as size references (lanes M).

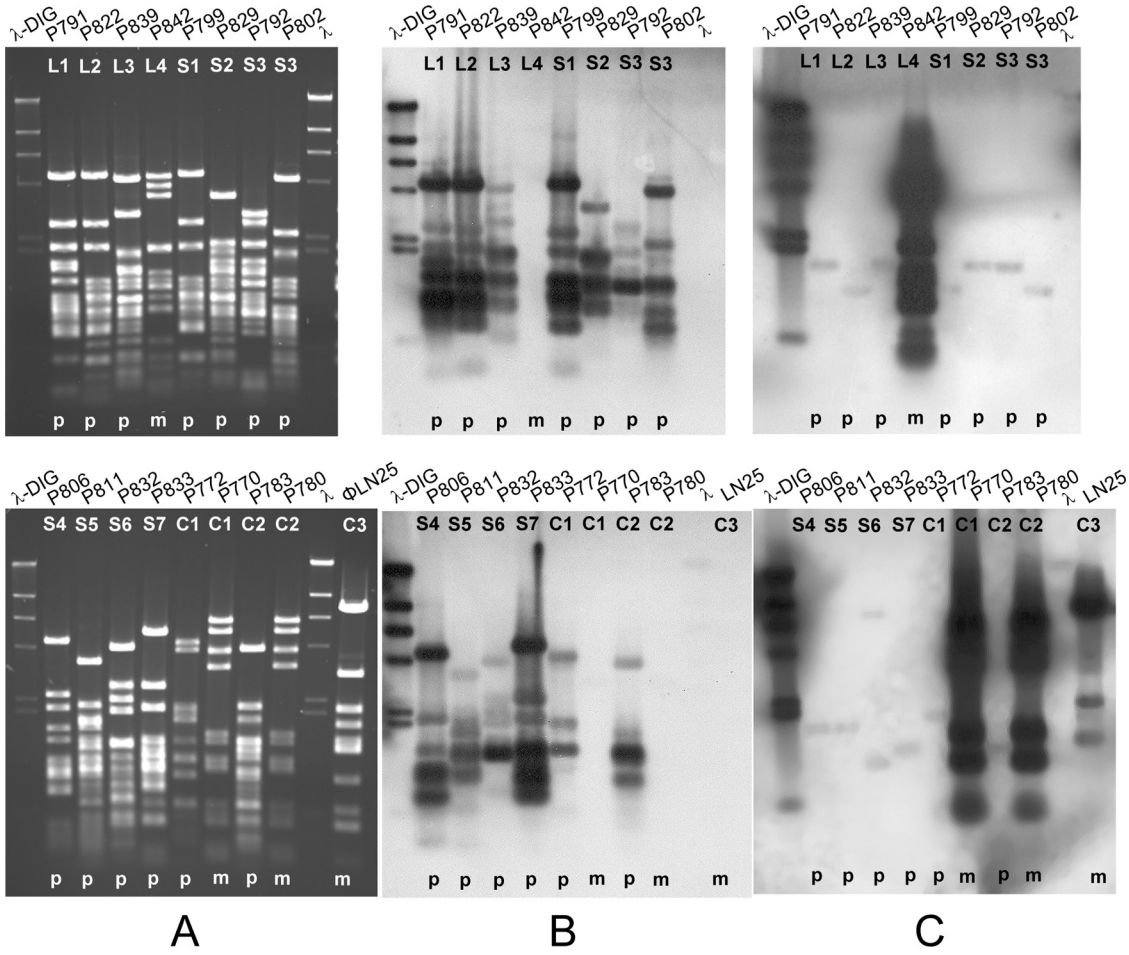


FIG 3 HinfI restriction enzyme analysis (0.8% agarose) (A) and Southern blot analysis of selected *Leuconostoc* phages isolated from 4 large dairies (L1 to L4), from 7 small to medium-size dairies (S1 to S7), and 3 phage collection (C1 to C3) with DNA probes of *Ln. pseudomesenteroides* phage P791 (B) and of *Ln. mesenteroides* phage P842 (C). Phage origins and their host species (p, *Ln. pseudomesenteroides*; m, *Ln. mesenteroides*) are indicated. Size references were unlabeled or DIG-labeled phage λ DNA cut with HindIII.

XbaI patterns were obtained (Fig. 2A and B), indicating that these phages represent a phage population of low biodiversity. The four phages isolated from buttermilk at dairy L4 had identical restriction patterns (Fig. 2A). The *Ln. mesenteroides* phages from collections C1 and C2 displayed two distinguishable XbaI restriction patterns, represented by P767 and P773, respectively (Fig. 2B). However, an additional subgroup represented by P769 exhibiting a smaller XbaI fragment of approximately 9 kb was identified in external phage collection C1. In phage collection 2, the XbaI pattern of phage P778 was different from that of P773 in small DNA fragments of approximately 1 kb (Fig. 2B). Finally, the XbaI profile of ΦLN25 DNA from phage collection C3 showed the greatest deviation within this group of phages (Fig. 2B). This corresponds with the unique host range and the distinct morphotype of phage ΦLN25, differing from those of all other phages (Table 1).

Ln. pseudomesenteroides phages revealed a broader extent of genetic heterogeneity (Fig. 2C to G). No identical HaeIII patterns were obtained from 17 phages in collections C1 and C2 (Fig. 2C). Notably, none of the HaeIII-generated DNA restriction profiles obtained from the external phage collections (Fig. 2C) matched any of the patterns generated from phages isolated from dairy plants shown in Fig. 2D to G. Conversely, a considerable homogeneity among phages isolated from different sources within the same dairy plant was also documented: all phages analyzed from whey and from different products and brine samples taken from dairies L1 (Fig. 2D) and S6 (Fig. 2E), respectively, were identical. This indicates a stable phage population within these dairies and application of probably just one *Ln. pseudomesenteroides* strain in the starter culture(s). All multiple phage isolates from samples from dairies L2, S1, and S5 (Table 1) also showed dairy-specific identical HaeIII restriction profiles (data not shown). A low degree of variation in the restriction enzyme profiles was observed for six phages isolated from brine from dairy S3 (Fig. 2F), with two related but nonidentical HaeIII patterns being found. However, these patterns were clearly different from the two identical HaeIII profiles obtained from phages P792 and P793, which were derived from a hard cheese sample of dairy S3 (Fig. 2F). These data clearly show that a new phage population had emerged during cheese production in dairy S3. The highest degree of biodiversity was observed for the 6 phages isolated from brine in dairy S7, where identical HaeIII patterns were documented for only two phages, P818 and P819, isolated from whey (Fig. 2G).

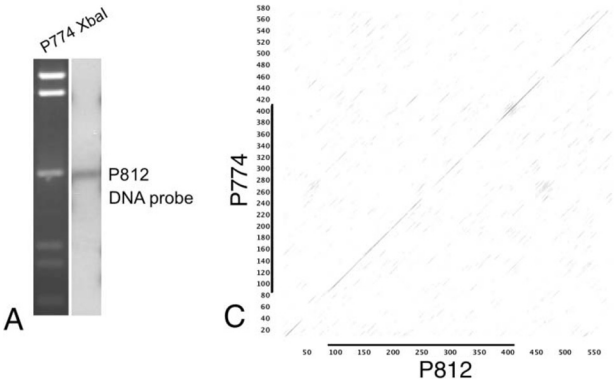
A low degree of variation in the HaeIII restriction profiles af-

fecting the sizes of 1 to 2 DNA fragments was, furthermore, documented for phages isolated from dairies L3, S2, and S4 (Table 1; data not shown).

Southern blot analysis. In order to analyze the DNA homology between *Ln. mesenteroides* and *Ln. pseudomesenteroides* phages, Southern blot analyses were conducted, as shown in Fig. 3 for a representative set of 12 *Ln. pseudomesenteroides* and 4 *Ln. mesenteroides* phages. From all 11 dairies, one phage isolate and one or two phages from the three external phage collections were selected. HinfI restriction digests of phage DNAs were blotted and probed with DNA of *Ln. pseudomesenteroides* phage P791 and of *Ln. mesenteroides* phage P842. Strong hybridization signals between *Ln. pseudomesenteroides* phage P791 and all other *Ln. pseudomesenteroides* phages were obtained (Fig. 3B). Similarly, the probe for *Ln. mesenteroides* phage P842 hybridized with all other *Ln. mesenteroides* phages (Fig. 3C). However, intensity variations between hybridizing bands within specific phages indicated diversification between phage DNA sequences. The hybridization data corroborate the presence of two separate (host species-specific) genotypes for dairy phage populations infecting *Ln. mesenteroides* and *Ln. pseudomesenteroides* starter strains. It should be noted that the biodiversity of lytic phages infecting dairy *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains is much lower than that of lactococcal phages (37). However, considering also *Leuconostoc* phages from nondairy fermentations, the variability of these phage populations may be significantly larger, as 6 DNA homology groups were reported earlier for *Leuconostoc* phages (20).

Partial genome sequencing of phages P812 and P774. Albeit the *Ln. mesenteroides* and *Ln. pseudomesenteroides* phages included in our study represent 2 distinct genotypes, 1 to 2 weak cross-hybridization signals among phages of these two host species were documented (Fig. 3), indicating the presence of limited DNA homology among all *Ln. mesenteroides* and *Ln. pseudomesenteroides* phages. With a phage P842 DNA probe, fragments with faint hybridization signals appeared in the HinfI restriction patterns of all *Ln. pseudomesenteroides* phages (sizes, approximately 1.6 kb [e.g., phage P791], approximately 1.3 kb [e.g., P822], and approximately 1 kb plus 5 kb [P832]).

In order to identify the conserved DNA region with limited DNA homology among all dairy *Leuconostoc* phages, the hybridizing DNA fragments of *Ln. mesenteroides* phage P774 and *Ln. pseudomesenteroides* phage P812 (Table 1 and Fig. 4A) were cloned and sequenced together with their flanking DNA regions (lengths, 6,230 bp of phage P774 DNA and 6,194 bp of phage P812 DNA). These areas encompassed the structural genes specifying the phage tail proteins. Significant nucleotide identity was found in genes putatively encoding the major tail protein (MTP) of phages P774 (582 bp) and P812 (585 bp). Figure 4B summarizes the pairwise comparison between these two *mtp* genes and between the corresponding genes of *Ln. mesenteroides* phage Φ1-A4 (22), *Ln. mesenteroides* subsp. *dextranicum* phage ΦLmd1 (24), and the 2 phages ΦLN25 and P793 (unpublished data). The MTP-encoding genes of *Ln. pseudomesenteroides* phages P812, P793, and ΦLmd1 revealed high sequence identity (95.4 to 99.2%). Similar high sequence similarity was also documented for the *mtp* genes of the *Ln. mesenteroides* phages P774, ΦLN25, and Φ1-A4. When phages of different host species were compared, the sequence similarity of the corresponding *mtp* genes was significantly lower (63.9 to 65.4%) (Fig. 4B). The high *mtp* sequence similarity of *Ln. mesenteroides* subsp. *dextranicum* phage ΦLmd1 to the *mtp* genes of all



B

	ΦLmd1	P812	P793	P774	ΦLN25	Φ1-A4
ΦLmd1		99.15	95.38	65.37	64.53	63.85
P812	0.01		94.87	65.54	64.70	63.85
P793	0.05	0.05		65.71	65.20	63.85
P774	0.46	0.46	0.46		93.30	88.32
ΦLN25	0.48	0.48	0.47	0.07		89.52
Φ1-A4	0.49	0.49	0.49	0.13	0.11	

min. max.

FIG 4 (A) Localization of the limited semiconserved DNA region which was used as a DNA probe in a Southern blot analysis of *Ln. pseudomesenteroides* phage P812 and *Ln. mesenteroides* phage P774. (B) Pairwise comparison of the putative major tail protein genes (*mtp*) of different *Leuconostoc* phages. The upper diagonal comparison indicates the percent identity between two phage DNA sequences. The lower diagonal comparison shows the Jukes-Cantor-corrected distance between the two sequences. (C) Dot plot analysis of the *mtp* of phages P774 (*y* axis) and P812 (*x* axis). The nucleotide positions from the start of the genes are indicated. The thick black lines on the two axes indicate the locations of the PCR target DNA fragments of phages P774 and P812 (see the text for details).

Ln. pseudomesenteroides phages indicated that its host strain also belongs to the latter species. When this phage was tested with the four host strains in Table 1, it could lyse only *Ln. pseudomesenteroides* strain 15L1 (data not shown).

Development of a PCR detection system for dairy *Leuconostoc* phages. On the basis of the conserved sequences of the putative *mtp* genes of phages P812 and P774, a primer set for targeting both *Ln. mesenteroides* and *Ln. pseudomesenteroides* phages, producing a 322-bp fragment, was developed. Figure 4C shows the dot plot alignment of the two *mtp* gene sequences, and the black lines along the *x* and *y* axes indicate the amplified regions. PCR detection of representative *Ln. mesenteroides* and *Ln. pseudomesenteroides* phages from all 11 dairies and from the 3 external phage collections is shown in Fig. 5. The PCR was also successfully validated with all other phages listed in Table 1 (see Fig. S2 in the supplemental material). Reference phages from other species were also included and did not produce PCR signals (*L. lactis* phages P008 [phage species 936] and P001 [phage species c2], *S. thermophilus* phage P53) (38, 39).

Conclusion. In this communication, 83 virulent phages infecting dairy *Leuconostoc* starter strains were characterized with respect to host species, morphotype, genotype, genetic relatedness, and host range. Based on these characteristics, we propose a classification scheme, presented in Fig. 1. Phages infecting *Ln. mesen-*

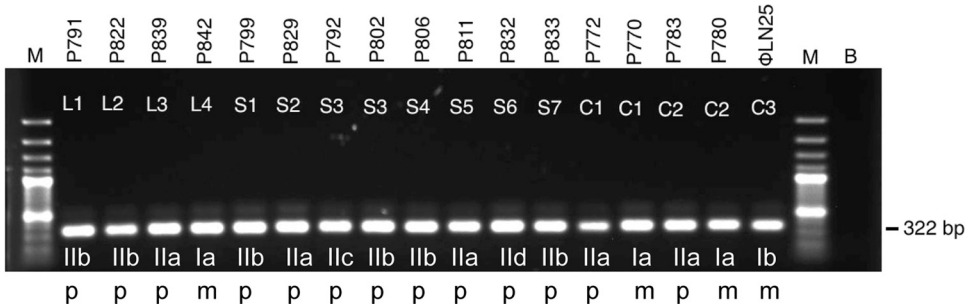


FIG 5 Agarose (1%) gel electrophoresis of the 322-bp PCR products derived from selected dairy *Ln. pseudomesenteroides* (p) and *Ln. mesenteroides* (m) phages. Phages were obtained from 4 large dairies (L1 to L4), 7 small to medium-size dairies (S1 to S7), and 3 phage collections (C1 to C3). The various morphotypes of the *Ln. mesenteroides* phages (Ia and Ib) and of the *Ln. pseudomesenteroides* phages (IIa to IId) are indicated (Table 1 and Fig. 1). Lane B, negative control without DNA; lanes M, 100-bp Plus DNA ladder (Fermentas).

teroides belonged to morphotype I and genotype I, whereas phages infecting *Ln. pseudomesenteroides* were classified as morphotype II and genotype II phages. All phages characterized showed an overall uniform morphology of isometrically headed *Siphoviridae* phages. All *Ln. mesenteroides* phages of morphotype I were characterized by clearly defined appendages surrounding their baseplates with either globular (morphotype Ia) or Y-shaped structures (morphotype Ib). These differences in baseplate structure correlated with different host ranges. *Ln. pseudomesenteroides* phages of morphotype II could be differentiated into four distinct subtypes: morphotypes IIa and IIb were differentiated by the presence and absence of a collar, respectively, and type IIc phages showed undefined baseplate appendages, while type IId phages were characterized by tail striations over the whole tail length. Morphotype IIc phages were unique among these four morphotypes, since they showed a host range different from that of the other three morphotypes. On basis of the presence of limited conserved DNA regions in the putative major tail structural genes of lytic *Ln. mesenteroides* and *Ln. pseudomesenteroides* phages, a reliable and universal conventional PCR detecting tool was established.

ACKNOWLEDGMENTS

We thank the dairy companies for their cooperation and acknowledge the generous gifts of various phage samples from the three external phage collections. Helge Holo (Norwegian University of Life Sciences, Aas, Norway) is acknowledged for providing phage ΦLmd1, and Jennifer Mahony (University College Cork) is acknowledged for editorial assistance. We thank Inka Lammertz, Angela Back, and Petra Horn (Max Rubner-Institut [MRI], Kiel, Germany) and Bashir Aideh (University of Copenhagen [UoC], Copenhagen, Denmark) for their expert technical assistance.

W. Kot was the recipient of a Ph.D. grant from UoC. The work at UoC was partially financed by MetaPhageLAB (FTP project no. 11-106991), and the work at MRI and at the University of Hohenheim was supported by the Forschungskreis der Ernährungsindustrie e.V. (Bonn, Germany), the AiF, and the Ministry of Economics and Technology (project no. AiF-FV 15886N).

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