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Abstract

Bacteriophages attacking *Leuconostoc* species may significantly influence the quality of the final product. There is however limited knowledge of this group of phages in the literature. We have determined the complete genome sequences of nine *Leuconostoc* bacteriophages virulent to either *Leuconostoc mesenteroides* or *Leuconostoc pseudomesenteroides* strains. The phages have dsDNA genomes with sizes ranging from 25.7 to 28.4 kb. Comparative genomics analysis helped classifying the 9 phages into two classes, which correlates with the host species. High percentage of similarity within the classes on both nucleotide and protein level was observed. Genome comparison also revealed very high conservation of the overall genomic organization between the classes. The genes were organized in functional modules responsible for replication, packaging, head and tail morphogenesis, cell lysis and regulation and modification, respectively. No lysogeny modules were detected. To our knowledge this report provides the first comparative genomic work done on *Leuconostoc* dairy phages.

Keywords: bacteriophages, lactic acid bacteria, *Leuconostoc,* comparative genomics

1. Introduction

Phages cause large problems in dairy industry, resulting in significant losses during production (Brøndsted et al., 2001; Lubbers et al., 1995; Moineau et al., 2002). Until now, most work regarding dairy phages attacking mesophilic starter cultures has focused on phages of *Lactococcus lactis* (Moineau et al., 2002). However, recent reports suggest that a number of the commercial starter cultures might be affected by *Leuconostoc* (*Ln.*) phages (Kleppen et al., 2012). *Leuconostoc* species are minor components of L- (*Leuconostoc* strains as flavor producers) and DL- (*Leuconostoc* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* strains as flavor producers) mesophilic starter cultures (Høier et al., 2010). Despite only being a minor component of a starter, *Leuconostoc* species are responsible for producing a variety of flavor compounds that are important for the final quality of various dairy products (Parente and Cogan, 2004). Three *Leuconostoc* species have been reported as components of dairy starters. The majority of isolates has been classified either as *Ln. mesenteroides* or *Ln. pseudomesenteroides*. More rarely, members of *Ln. lactis* have been isolated from the dairy environment (Zamfir et al., 2006). As *Leuconostoc* species are marginally responsible for the acidification process during cheese-making and because these bacteria are present in much lower numbers than *Lactococcus lactis,* phage attack on *Leuconostoc* strains remains undetected by standard acidification tests (Davey et al., 1995). A drop in number of *Leuconostoc* cells, due to phage attack during fermentation, may change the concentration of certain flavor compounds. Due to the heterofermentative nature of *Leuconostoc* eye formation of the cheese may also be compromised (Atamer et al., 2011; Hemme and Foucaud-Scheunemann, 2004).
In addition to being present in cheese, the same *Leuconostoc* species can be found in other fermented dairy products such as butter, sour cream, villi, and buttermilk

(Atamer et al., 2011; Johansen and Kibenich, 1992; Nieto-Arribas et al., 2010; Olsen et al., 2007). Some of these *Leuconostoc* species are also associated with fermentation of plant-derived foods e.g. kimchi or sauerkraut and feeds e.g. silage (Johanningsmeier et al., 2007; Jung et al., 2011; Yang et al., 2010).

The first report regarding *Leuconostoc* dairy phage was communicated in 1946 by Mosimann and Ritter (Mosimann and Ritter, 1946). For many years characterization of such isolates were however limited to electron microscopy analysis of morphology and to host-range (Neve et al., 1988; Shin and Sato, 1979; Sozzi et al., 1978). Few reports have characterized *Leuconostoc* dairy phages at the molecular level and these studies have been restricted to DNA hybridization experiments (Davey et al., 1995). In 2011, characterization of the thermal resistance of 77 *Leuconostoc* phages isolated from dairy products was communicated (Atamer et al., 2011). Recently, some of us were involved in further characterization of these isolates, which resulted in a classification of lytic dairy *Leuconostoc* phages based on DNA:DNA hybridization, host-range and morphology (Ali et al., 2013).

Today there are three full genomic sequences of *Leuconostoc* phages present in public databases. In 2010, Lu et al. determined and analyzed the full genomic sequence of the lytic *Ln. mesenteroides* phage Φ1-A4 isolated from a sauerkraut fermentation (Lu et al., 2010). Shortly after, the sequence of the temperate *Ln. pseudomesenteroides* phage ΦMH1 from a UV-induced bacterial lysate from kimchii was determined (Jang et al., 2010). In 2012, the first sequence of the lytic *Leuconostoc* phage ΦLmd1 isolated from a dairy product was published (Kleppen et al., 2012).

In the present study we determined the genomic sequences of nine phages of *Leuconostoc* isolated in relation to a product defect (e.g. diminished eye formation or absence of mold growth in blue cheeses) from a several European locations. We

performed the comparative genomic of the sequenced phages. In addition, we tested the phylogenetic relationship with other known LAB phages. The aim of this work was to provide insights into dairy *Leuconostoc* phages population and evaluate their diversity.

2. Materials and Methods

2.1 Phages, bacterial strains and media

The strains used in this study are listed in Table 1. The material for phage isolation was obtained from different European geographic locations (Table 2). Species designation of *Leuconostoc* strains was based on sequencing of a nearly complete 16S rRNA gene using universal primers 27F and 1492R (Macrogen Europe, Netherlands) followed by BLAST (Basic Local Alignment Search Tool) in the NCBI database (National Center of Biotechnology Information). Strains were propagated on MRS agar plates or in MRS broth (Difco, Sparks, USA) at 28^{o}C, aerobically and for 16 hours. For phage propagation MRS was supplemented with 10 mM $CaCl_2$ (MRS-Ca). Phage host-range and phage titers were determined by spotting 10 µl of serial dilutions of phage solution on bacterial lawns in MRS-Ca top agarose (MRS-Ca broth, 0,8% agarose) on MRS-Ca agar plates and incubated overnight at 28^{o}C.

2.2 Phage preparation and DNA isolation

Phage lysates were performed essentially as described for λ phage by Sambrook and Russell (Sambrook and Russell, 2001). Briefly, log-phase host cells were infected with the corresponding phage with a low multiplicity of infection (0.01), left at 28^{o}C until complete lysis occurred. Cellular debris was removed by centrifugation for 10 min at 11,000 x g. Phage particles were precipitated with 10% PEG6000 (Merck) for 12 to 16 h and after centrifugation at 11,000 x g resuspended in SM buffer (100 mM

sodium chloride, 10 mM magnesium sulfate, 50 mM Tris [pH 7.5], and 0.01% gelatin). Phages were purified by two-step centrifugation in CsCl gradients (Sambrook and Russell, 2001). The first centrifugation was a block gradient centrifugation for 2 h at 22,000 rpm using Beckman SW28 rotor followed by a second equilibrium centrifugation at 38,000 rpm for 22 h using Beckman SW55Ti rotor. Phage DNA was isolated from dialyzed phage solution using phenol-chloroform extraction as described by Sambrook and Russell for phage λ (Sambrook and Russell, 2001). The phages were stored at high titer in the CsCl solution at 4^{o}C.

2.3 Electron microscopy

Drops (10 µl) of purified phages taken from CsCl gradients were placed for 15-min on Millipore MF filter membrane discs (type VSWP 0.025 µm, Merck, Darmstadt, Germany) floating on SM-buffer. After micro-dialysis, an ultra-thin carbon film was transferred in a drop of phage solution diluted 1:50 with SM-buffer and was incubated for 10 min for phage adsorption. The carbon film was washed twice in demineralized water and stained for 30 s with 2% (w/v) uranyl acetate (Agar Scientific, Stansted, United Kingdom). Stained carbon films were transferred onto 400-mesh copper grids (Agar Scientific) and examined with a Tecnai 10 transmission electron microscope (FEI, Eindhoven, The Netherlands) at an acceleration voltage of 80 kV. Phages were photographed with a Megaview G2 CCD camera (Olympus SIS, Münster, Germany).

2.4 Library construction, sequencing and assembly of sequences

In all cases DNA from CsCl purified phages was used for library construction. Library preparation and sequencing were done using standard protocols as recommended by the manufacturers. Two different approaches were used to determine the complete genome sequences of the phages. Eight phages were sequenced using the 454 Roche Titanium platform (Life Sciences, Branford, USA)

These phages were sequenced as part of tagged pools of unrelated phages, built as MID-tagged Rapid libraries and sequenced in one region (half a picotitre plate) using the GS FLX Titanium Sequencing Kit XLR70. One phage, P793, was sequenced as 96 base reads using the Illumina HighSeq2000 (Illumina, San Diego, USA) platform, again as part of a pool of unrelated phages, tagged with an index as part of one lane of the flowcell. Custom indexing primers were used to build libraries as described earlier (Kampmann et al., 2011). Reads were assembled into contigs using CLC Genomics Workbench 5.0.1 (CLC bio, Aarhus, Denmark). The assembly process was confirmed by PCRs (Table 3). In order to obtain sequences of the *cos*-sites, primers flanking the *cos*-region were designed (Table 3). Ligation was performed prior to PCR using T4 ligase (New England Biolabs, Ipswich, USA) according to the protocol recommended by manufacturer. After ligation and amplification by PCR, fragments were sequenced using Sanger sequencing. Additional Sanger sequencing of isolated-linear phage DNA was performed using the same primers in order to analyze the *cos*-site region. All Sanger sequencing for verification and *cos*-site determination were performed at Macrogen (Macrogen Europe, the Netherlands) using customized primers (Table 3).

2.5 Sequence analysis

The obtained sequences were subjected to a two-stage ORF prediction process. First, sequences were analyzed using the Genmark.hmm program (Besemer and Borodovsky, 1999) and afterwards additional manual check was performed. Additional criteria were taken into consideration during manual check i.e. the presence of a convincing potential Shine–Dalgarno sequence with homology to the consensus AGGAGG (Mahanivong et al., 2001) in a close distance upstream from the most convincing initiation codon (preferably ATG but also GTG or TTG). Alternatively, in absence of a potential ribosomal binding site, the initiation codon

could be placed closely to the putative stop codon of the preceding gene giving a possibility for translational coupling (Brøndsted et al., 2001; Lubbers et al., 1995). Predicted ORFs were analyzed using a combination of blastp and psi-blast algorithms on the NCBI non-redundant protein sequences database. Translated ORFs were analyzed for Pfam (Protein Family) domains using the full Pfam database with a maximum E-value of 1.0 using CLC Main Workbench 6.6.2 (CLC bio, Aarhus, Denmark). Genome comparison was calculated using blastn and tblastx algorithm (BLAST 2.2.26+). Tblastx comparison was visualized using Easyfig 2.1 software (Sullivan et al., 2011) with the following blast options: minimum alignment length of 50 bp, maximum E-value of 0.0001 and minimal identity value of 30%. The phylogenetic analysis of LAB phages was calculated using Geneious 6.1.2 with the matrix cost 5.0/-3.0.

2.6 Analysis of structural proteins

CsCl-purified phages (approx. 10^{11} pfu/ml) were dialyzed against water and mixed with loading buffer (final concentrations: 50 mM Tris-HCl, 3% SDS, 13% sucrose, 0,1 M DTT, 0,2 mg/ml bromophenol blue) and boiled for 10 min. Phage structural proteins were separated on a gradient 10-20% SDS-PAGE gel (RunBlue, Expedeon, UK). Gel bands were manually excised and subjected to in-gel tryptic digestion essentially as described before (Zhang et al., 2007). Briefly, gel bands were de-stained in 40 % ethanol and dehydrated in 100% acetonitrile. Bands were rehydrated in 10 mM NH_4HCO_3 and digested with 12.5 ng μl^{-1} trypsin (Promega, porcine sequencing grade) on ice for 45 min. The digests were diluted five-fold with 10 mM NH_4HCO_3 and incubated at 37° C for 16h. The supernatant was removed from gel and stored at –20° C until analysis.

Samples were added on an Anchorchip™ (Bruker-Daltonics, Bremen, Germany) as described before (Zhang et al., 2007). Mass determinations were obtained by an Ultraflex II MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany). Spectra were externally calibrated using a tryptic digest of β-lactoglobulin. The obtained spectra were analysed using Flex-Analysis 3.0.96 and Biotools 3.1 software (Bruker-Daltonics, Bremen, Germany) before searching at in-house MASCOT (Matrix Science, Boston, USA) server against translated ORFs from sequenced *Leuconostoc* phages.

2.7 Genome accession numbers

The GenBank accession numbers for the nucleotide sequences are KC013021-KC013029.

3. Results and discussion

3.1 General description of phages

The morphology of the phages was determined by transmission electron microscopy (TEM). The obtained micrographs showed that all tested phages have a long, non-contractive tail and an isometric head thus belonging to the B1 morphotype of the *Siphoviridae* family within the order *Caudovirales* (Ackermann and DuBow, 1987). Furthermore, phages could be classified into 5 morphotypes according to the classification proposed previously (Ali et al., 2013). Phages ΦLN34, ΦLNTR2 and ΦLNTR3 belong to morphotype Ia (with defined, globular appendices), phage ΦLN25 belongs to morphotype Ib (with defined, y-shaped appendices). Phages ΦLN03 and ΦLN12 can be classified into morphotype IIa (lack of appendices, presence of the neck passage structure (NPS)). Phages ΦLN04 and ΦLN6B belong to

morphotype IIb (lack of appendices and NPS). The phage P793 is the only member of IIc morphotype (undefined base plate appendices, no NPS) (Figure 1).

The host range of phages was determined against strains of *Ln. mesenteroides* (15 strains), *Ln. pseudomesenteroides* (7 strains) or *Ln. lactis* (3 strains). The phages ΦLN25, ΦLN34, ΦLNTR2 and ΦLNTR3 propagated exclusively on *Ln. mesenteroides* strains, while the phages ΦLN03, ΦLN04, ΦLN12, P793 and ΦLN6B only propagated on *Ln. pseudomesenteriodes* strains (Table 4). None of the phages formed plaques on *Ln. lactis* strains. The phages revealed four different host-range patterns; two were exclusive for *Ln. mesenteroides* strains and two were exclusive for *Ln. pseudomesenteroides*. None of the patterns were overlapping. Limited number of hosts and high conservation of host-range patterns in *Leuconostoc* phages has been observed before (Atamer et al., 2011) and could be partially explained by a small diversity of *Leuconostoc* strains found in starter cultures (Johansen and Kibenich, 1992; Nieto-Arribas et al., 2010) or broad host-ranges of the receptor binding protein. The relatively narrow diversity of the *Leuconostoc* host strains susceptible to the phages of this study was suggested by rep-PCR (data not shown).

3.2 Genomic organization of *Leuconostoc* phages

The phages have a dsDNA genome with sizes ranging from 25.7 to 28.4 kb. The genomic G+C content was in range from 36.0% in phage ΦLN34 to 36.8% in phage ΦLN03, which is close to the G+C content of 37.7% found in *Ln. mesenteroides* ATCC 8293 (Makarova et al., 2006).

The 9 sequenced phages can be divided in two classes that differ greatly in terms of nucleotide sequence between classes but are conserved within the class. Class I is constituted of phages attacking *Ln. mesenteroides* and class II are phages attacking *Ln. pseudomesenteroides*. The high conservation regarding the genomic sequence and the

host-range patterns is noteworthy, taking into consideration that phages were isolated from different geographic locations (Table 2). Bioinformatic analysis revealed presence of 38-42 potential ORFs per genome. ORFs were named with consecutive numbers starting from the first predicted ORF in the closest proximity to the left *cos*-site (*cos*L) of the genome.

The putative functions of the genes, based on the similarities to already known sequences, are listed in Table 5 for *Ln. mesenteroides* phages and in Table 6 for *Ln. pseudomesenteroides* phages. Restriction patterns on ligated and linear phage DNA suggested that the phages utilize *cos*-type packaging system (data not shown). Comparison between Sanger reads on amplified-ligated and isolated-linear phage DNA indeed revealed the presence of 3' overhang *cos*-sites. Sequence of the conserved 12 nt *cos*-sites of the 4 *Ln. mesenteroides* phages was determined (CGGTTAGTAGTA). The *cos* sequence was shorter than 22-nucleotide *cos*-site reported for phage Φ1-A4 (Lu et al., 2010) however the beginning of the Φ1-A4 *cos*-site (GGTTAATAGTAGTCTTTTTTAA) share high similarity with the sequence of the newly sequenced *Ln. mesenteroides* phages. The 13 nt *cos*-sites of the 5 *Ln. pseudomesenteroides* phages (TCGTGCAATAGTA) were also conserved and identical to the first 13 nt of phage ΦLmd1 (TCGTGCAATAGTAGGCG TTTTAA)(Kleppen et al., 2012).

3.3 Comparative genomics

The overall composition of the modules in both classes was very similar to the ones from *Ln. mesenteroides* phages Φ1-A4 and ΦLmd1, however Φ1-A4 is more related to *Ln. mesenteroides* phages while ΦLmd1 clusters with *Ln. pseudomesenteroides* phages group (Figure 2). The similarities to the temperate phage ΦMH1 were limited, indicating that temperate *Ln. pseudomesenteroides* phages are not the source of the

lytic *Ln. pseudomesenteroides* phages. Though, Blastp analysis of all putative gpORFs from ΦMH1 versus all gpORFs of the newly sequenced phages resulted in 7 unique hits of E-value below 0.01. The similarities were found within gpORF28 from ΦLN12 and a putative methylase from ΦMH1 (e-value 1.80e-85), putative baseplate (e-value, 1.64e-36), large terminase (e-value, 2.74e-18), TMP (e-value, 1.68e-10) and putative endonuclease (e-value, 2.39e-6) and two hypothetical proteins without predicted function.

The whole genome comparison with several LAB phages revealed that phages of *Ln. mesenteroides* and *Ln. pseudomesenteroides* form separate clusters (Figure 3).

Five functional modules specific for replication, packaging, morphogenesis, cell lysis and regulation/modification were identified in all phage genomes. No lysogeny modules were detected. Comparative genome analysis showed high percentage of similarity within the classes on both nucleotide and protein level. High level of conservation within classes is present especially in the replication, packaging and structural module (Figure 2). Similarities at the nucleotide level were limited between the two different classes (data not shown). This was also previously shown by DNA:DNA hybridizations and sequencing of the *mtp* gene and flanking regions (Ali et al., 2013).

3.4 Genetic diversity of *Leuconostoc* phages

The core genome of the analyzed *Ln. mesenteroides* phages consisted of 36 ORF out of total pool of 47 ORFs. In case of *Ln. pseudomesenteroides* phages homologs of 37 ORFs were detected in all the phages out of the total pool of 44 ORFs (table 5 and 6, respectively).

In the *Ln. mesenteroides* ΦLN25 phage there is a putative gene (ORF12) between the small terminase subunit gene (ORF11) and the large terminase subunit gene (ORF13).

An additional gene located between the terminase subunits was recently reported for a number of 936 phages (Castro-Nallar et al., 2012). Two different versions of putative lysin were detected in *Ln. mesenteroides* phages. In phage ΦLN25 the putative lysin showed 98% similarity to the amidase from phage Φ1-A4. In phages ΦLN34, ΦLNTR2 and ΦLNTR3 the lysin exhibited high similarity to the lysin from *Leuconostoc citreum* KM20 (e-value, 6.47e-52). The two types of lysin did not show any significant nucleotide similarity with each other.

The transmission electron micrographs showed that two of the *Ln. pseudomesenteroides* phages, ΦLN03 and ΦLN12 had a distinct neck passage structure (NPS) (Figure 1). NPS genes are commonly found in lactococcal phages belonging to the P335, 936 and c2 phages species and are part of their structural module (Brøndsted et al., 2001; Høier et al., 2010; Rousseau and Moineau, 2009). Although the putative structural module is highly conserved in the *Ln. pseudomesenteroides* phages, two possible locations for the NPS determinant were identified by comparative genomics. The first putative location was detected as a 573 bp long in-frame insertion located in ORF20 and ORF21 in phages ΦLN03 and ΦLN12, respectively. The second putative NPS determinant was located in close proximity to the right *cos*-site of phage ΦLN03 and ΦLN12. It consisted of ORF36 and ORF37 in phage ΦLN03 and showed high similarity to an insertion element from *Lactobacillus delbrueckii* phage LL-K (e-values, 4.11e-10 and 4.11e-50, respectively) (Forsman and Alatossava, 1993). Further experiments are necessary in order to specify the actual NPS determinant. Apparently, this NPS is not involved in host-range interactions as phages lacking these structures (i.e. ΦLN04 and ΦLN6B) had the same host range as phages ΦLN03 and ΦLN012.

In the sequenced *Leuconostoc* phages two putative methyltransferase genes could be

found. One of them was encoded by ORF28 in ΦLN12 and was found only in this phage. The gene product showed significant similarity (e-value, 1.84e-143) to a putative DNA methyltransferase from bacteriophage Φ1-A4, however it was located differently. In phage Φ1-A4 this methyltransferase was located in the replication module while in phage ΦLN12 it was placed next to the putative lytic enzyme. This gene also showed high similarity with a putative methylase from temperate *Leuconostoc* phage ΦMH1 (e-value, 1.72e-16). The second putative methyltransferase was gpORF28 of phage ΦLN25. It was also located next to the putative lysis module and had significant similarity (e-value, 9.41e-172) to the methyltransferase of a type I restriction-modification system from *Ln. lactis* KCTC 3528. DNA methyltransferases are sometimes incorporated to the phage genome as a strategy of overcoming hosts restriction modification system (Labrie et al., 2010).

In *Ln. pseudomesenteroides* phages ΦLN04 and ΦLN12 an additional gene coding for the HNH endonuclease (ORF6 and ORF5, respectively) located between genes coding for DNA primase and DNA polymerase was detected. A related endonuclease is encoded by ORF6 from the ΦLmd1 phage (e-value, 1.89e-89) (Kleppen et al., 2012). HNH endonucleases have been reported to mobilize their own reading frames by generating DNA breaks at specific sites, activity of homing endonucleases may lead to site-specific recombination and may result in insertion, deletion, mutation or correction of DNA sequence (Stoddard, 2011). The majority of the identified putative endonucleases were clustered together in close proximity to the left *cos*-site of the phage genomes, being part of the regulation/modification module.

3.5 Protein identification

Two phages were selected for protein identification using mass spectrometry; phage ΦLN34 as the *Ln. mesenteroides* phages representative and ΦLN04 as *Ln.*

pseudomesenteriodes phages representative. SDS-PAGE profiles of selected phages revealed 6 and 5 major bands respectively (Figure 4). Obtained results from peptide mass fingerprints and MS/MS analysis allowed identification of the majority of the structural proteins of the two phages. The largest predicted protein with 92,1 kDa in ΦLN34 (ORF21) and actual size of 75 kDa in SDS-PAGE gel was identified as a tape measure protein (TMP). This may suggest that TMP is being processed prior to incorporation into the phage particle. Proteolytic cleavage of the C-terminal part of TMP has been recorded before in phages including the lactococcal phage Tuc2009 (Mc Grath et al., 2006). The second largest band in both phages (46,4 kDa in ΦLN04 and 42,9 kDa in ΦLN34) was identified as putative portal protein (ORF12 and ORF13, respectively).

The third band in both of the phages was identified as a receptor binding protein (RBP). The function of this gene was recently verified in *Ln. pseudomesenteroides* phages (Kot et al., 2013). RBP of ΦLN04 (ORF23, app. 30 kDa) seems to be slightly bigger than the RBP of ΦLN34 (ORF25, app. 28 kDa) and can be explained by considerably different shape in structures observed on the micrographs of those phages (Figure 1).

Identification of an approx. 21 kDa protein band in phage ΦLN04 suggested that this protein was the major tail protein (ORF19). This finding was supported by the large amount of the protein present in SDS-PAGE gel (Figure 4).

It proved to be difficult to obtain significant matches of the smallest structural proteins. The smallest identified protein was a 12,5 kDa protein band from phage ΦLN34, which showed high similarity to a putative tail protein (ORF17).

It was not possible to identify the putative head protein among bands excised from the SDS-PAGE gel in any of the phages, however it was possible to get a match for the

putative major capsid protein ($ORF14_{\Phi LN04}$) when analyzing the protein material that failed to enter the gel. This could suggest that capsids of phage ΦLN04 undergo a wholesale head crosslinking similar to phage HK97 (Jang et al., 2010; Popa et al., 1991).

4. Conclusions

Nine dairy *Leuconostoc* phages were characterized and sequenced. Phages can be divided into two different genotypes. The sequenced bacteriophages exhibit four different host patterns, two for *Ln. mesenteroides* and two for *Ln. pseudomesenteroides*. High conservation within genotypes and host range pattern is notable taking into consideration different locations from which phages were obtained. Phages of *Ln. mesenteroides* cluster together with the *Ln. mesenteroides* phage ΦA1-4, while *Ln. pseudomesenteroides* phages cluster together with the *Ln. mesenteroides* subsp. *dextranicum* phage ΦLmd1.

To our knowledge this report provides the first comparative genomic work performed on phages lytic to the *Leuconostoc* species. The phages are highly conserved within their classes both on nucleotide and protein level. Additionally, similarities on protein level were present between the classes. The sequenced phages had analogous, conserved genetic organization suggesting close evolutionary distance between them. Genome comparison between the sequenced phages provided additional information that may result in deeper understanding of phage genetics and evolutionary mechanisms occurring in phages.

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Figure Captions:

Figure 1. Transmission electron micrographs of phages. Phages were negatively stained with 2% uranyl acetate. Morphological details are indicated as follows: globular base plate appendices (O), y-shaped base plate appendices (), neck passage structure (↖), undefined, fluffy base plate appendices (❖).

Figure 2**.** Schematic representation of the genomic comparison of *Leuconostoc* phages. Genomes are indicated with thick, black lines. Arrow represents putative protein. The color of the arrows indicate the putative function of the gene product and the number indicates the number of the ORF. Each genome was compared only with the succesive genome. Gray shading connecting two genomes corresponds to the similarity level obtain from tblastx comparison. All genomes are alligned according to the position of their *cos*-sites.

Figure 3. Phylogenetic tree of a total of 29 LAB infecting phages. The distance matrix and visualization was performed in Geneious 6.1.2. Phages sequenced in this study are indicated with the bold font. Other phages of *Leuconostoc* are indicated with bold, italic font.

Figure 4. Purified proteins from *Ln. pseudomesenteroides* ΦLN04 and *Ln. mesenteroides* ΦLN34 phages. Inner lanes show the protein marker (PageRuler™ Unstained Broad Range Protein Ladder, Fermentas). Protein standard is indicated in kilodalton (kDa).

Table 1. Bacterial strains used in this study.

Bacterial strain	Relevant feature	Reference
Leuconostoc sp. strain		
LN08	*Ln. mesenteroides*	This study, Sacco
LN18	*Ln. mesenteroides*	This study, Sacco
LN25	*Ln. mesenteroides,* host for ΦLN25	This study, Sacco
LN35	*Ln. mesenteroides*	This study, Sacco
LN27	*Ln. mesenteroides*	This study, Sacco
LN05	*Ln. mesenteroides,* host for ΦLN34, ΦLNTR2, ΦLNTR3	This study, Sacco
LN34	*Ln. mesenteroides*	This study, Sacco
LN07	*Ln. mesenteroides*	This study, Sacco
LN16	*Ln. mesenteroides*	This study, Sacco
LN28	*Ln. mesenteroides*	This study, Sacco
LN29	*Ln. mesenteroides*	This study, Sacco
LN30	*Ln. mesenteroides*	This study, Sacco
LN32	*Ln. mesenteroides*	This study, Sacco
LN33	*Ln. mesenteroides*	This study, Sacco
LN02	*Ln. pseudomesenteroides,* host for ΦLN03, ΦLN04, ΦLN12, ΦLN6B	This study, Sacco
LN03	*Ln. pseudomesenteroides*	This study, Sacco
LN12	*Ln. pseudomesenteroides*	This study, Sacco
LN04	*Ln. pseudomesenteroides*	This study, Sacco
LN23	*Ln. pseudomesenteroides*	This study, Sacco
BM2	*Ln. pseudomesenteroides,* host for P793	Atamer et al., 2011
LN19	*Ln. lactis*	This study, Sacco
LN24	*Ln. lactis*	This study, Sacco
DSM 8581	*Ln. lactis*	Dicks et al., 1993
DSM 20193	*Ln. pseudomesenteroides,* type strain	Kandler, 1970
DSM 20484	*Ln. mesenteroides subsp. dextranicum* type strain	Garvie et al., 1974

Table 2. Phages used in the study.

Phage	Place of isolation	Isolation material	Supplier/ starter culture used	Year of isolation	Species attacked
ΦLN03	Belarus	Whey	Supplier A	2006	*Ln. pseudomesenteroides*
ΦLN04	England	Whey	Supplier A	2006	*Ln. pseudomesenteroides*
ΦLN12	France	Whey from Roquefort	Supplier B	2004	*Ln. pseudomesenteroides*
ΦLN6B	Denmark	Whey	Supplier C	2010	*Ln. pseudomesenteroides*
P793	Germany	Whey from hard cheese	Supplier C	2009	*Ln. pseudomesenteroides*
ΦLN25	England	Whey	Supplier A	2006	*Ln. mesenteroides*
ΦLNTR2	Sweden	Whey	Supplier D/ starter 1	2010	*Ln. mesenteroides*
ΦLNTR3	Sweden	Whey	Supplier D/ starter 2	2010	*Ln. mesenteroides*
ΦLN34	England	Whey	Supplier A	2007	*Ln. mesenteroides*

Table 3. Primers used in the study for genome verification and *cos*-site analysis.

Primer sequence (5' to 3')		Class[a]	Phage name (size in bp)
Forward	Reverse		
GCAAAATAAAAAGACCTAAC	CATTCACAACAAAAAACG	I	ΦLN34 (320), ΦLNTR2 (320), ΦLNTR3 (320), ΦLN25 (225)
TAAAAACAAAAGCAGAACG	AGAACCAACCATCATAAC	I	ΦLN34 (3627), ΦLNTR2 (3627), ΦLNTR3 (3627), ΦLN25 (3515)
ATTTGTTCAGGGAATGGT	ATGTGTGGTAAGATTGGT	I	ΦLN34 (2191), ΦLNTR2 (2512), ΦLNTR3 (2191), ΦLN25 (2788)
TCCCAATCAAAACCTAAC	CACCCCTATCTAATCAAC	I	ΦLN34 (1092), ΦLNTR2 (1092), ΦLNTR3 (1092), ΦLN25 (np)
ATCTTGCTTCTTAGTCTT	ATTTATTTGGTGTCGTTG	I	ΦLN34 (np), ΦLNTR2 (np), ΦLNTR3 (np), ΦLN25 (922)
ACAAAACTAGCAAGGCACAA	CCTCCCCTTTTACTCGTC	I	ΦLN34 (3914), ΦLNTR2 (3962), ΦLNTR3 (3962), ΦLN25 (3982)
TGGTCGTTCTTGTTTAATGG	CCAATTGTGCGTCTTCAT	I	ΦLN34 (2957), ΦLNTR2 (3278), ΦLNTR3 (2957), ΦLN25 (3551)
CTGACCTGTTACGACTTC	CGGGGTCTTTTTTTTATGCT	I	ΦLN34 (3601), ΦLNTR2 (3601), ΦLNTR3 (3601), ΦLN25 (3436)
CATCTACATCCACCACATC	CCGTCTTACCCTTTTCTTT	II	ΦLN03 (3482), ΦLN6B (2911), ΦLN04 (2911), ΦLN12 (3484) P793 (np)
AATAGTCGCCATATCCCA	GAGTAAAGTTAGACGTGAGAGA	II	ΦLN03 (2269), ΦLN6B (2878), ΦLN04 (2334), ΦLN12 (3157) P793 (np)
AGTGAAGAGCCATCTGAA	GTCTTGTTGTTTGGTGGT	II	ΦLN03 (3440), ΦLN6B (3440), ΦLN04 (3905), ΦLN12 (3902), P793 (np)
AGAAAAGTTTGGCGGTAG	GGTTGTGTCATTGGGTATT	II	ΦLN03 (2934), ΦLN6B (np), ΦLN04 (np), ΦLN12 (2934), P793 (np)
AGAAAAGTTTGGCGGTAG	CCACCCTACGAAAATACAA	II	ΦLN03 (np), ΦLN6B (np), ΦLN04 (np), ΦLN12 (3660), P793 (np)
TTTATTTGAATGGGGTTG	GTTTTATCTCGCTTTTCT	II	ΦLN03 (3983), ΦLN6B (3983), ΦLN04 (3983), ΦLN12 (3983), P793 (3983)
			Relevant feature
CATCTTAATACCTTGACGAACC	CCATTCAAAGGTACGCTAAAAG	I	*cos*-site primer set for class I phages
CACTCTTGGTTACTCCTAATACTTC	CGAACGGCTGGTACATAAATTAG	II	*cos*-site primer set for class II phages

[a] I- phages attacking *Ln. mesenteroides* strains, II- phages attacking *Ln. pseudomesenteroides* strains.
(np)- no PCR product expected.

Table 4. Host range of *Leuconostoc* phages

	Name[a]	Species[b]	Phages								
			ΦLN03	ΦLN04	ΦLN12	ΦLN6B	P793	ΦLN34	ΦLN25	ΦLNTR2	ΦLNTR3
Bacterial strains	LN25	M	-	-	-	-	-	-	+	-	-
	LN27	M	-	-	-	-	-	-	+	-	-
	LN18	M	-	-	-	-	-	+	-	+	+

LN05	M	-	-	-	-	-	+	-	+	+
LN03	P	+	+	+	+	-	-	-	-	-
LN12	P	+	+	+	+	-	-	-	-	-
LN04	P	+	+	+	+	-	-	-	-	-
LN02	P	+	+	+	+	-	-	-	-	-
BM2	P	-	-	-	-	+	-	-	-	-

Infection was determined by a spot test, + indicates infection. Only host strains susceptible to infection are presented in the table. The following strains were not attacked by these phages: LN08, LN35, LN34, LN07, LN16, LN28, LN29, LN30, LN32, LN33, LN19, LN24, DSM 8581, DSM 20193, DSM 20484.
[a]Name of the *Leuconostoc sp*. strain.
[b]Species that the *Leuconostoc* host strain was classified into. M indicates *Ln. mesenteroides*, P- *Ln. pseudomesenteroides.* Strains were typed based on similarity of 16S rRNA gene and comparing its sequence to a public database.

Table 5. Coordinates and information about putative ORFs of *Leuconostoc mesenteroides* bacteriophage ΦLN34 and information of homologues ORFS in other phages.

Strand	ORF	Start	Stop	Size (aa)	MM (kDa)	pI	SD sequence	ORF ΦLNTR2	ORF ΦLNTR3	ORF ΦLN25	Function
-	1	672	370	101	11.722	8.47	ACAAGGATAATTAATATG	1	1	1	phage HNH endonuclease
										2	hypothetical protein
-	2	1111	674	146	16.788	8.54	AAGAAGAGGTACTAAAAATG	2	2	3	phage-related protein
-	3	1254	1108	49	6.204	11.61	AGGAGCGAAGAAGAAATG	3	3	4	hypothetical protein
-	4	1625	1251	125	14.986	8.92	AACAGGAGGGTAACATATG	4	4	5	endodeoxyribonuclease
+	5	2007	3320	438	50.595	5.89	AGGAGGAAAACAGATATG	5	5	6	DNA helicase
+	6	3317	4072	252	29.175	6.51	none	6	6	7	DNA primase/polymerase
+	7	4151	5986	612	69.886	7.57	AGGAGAAAAAAGATTATG	7	7	8	DNA polymerase
+	8	6176	6748	191	21.765	5.26	ATAAGGAGAACATATATG	8	8	9	hypothetical protein
+	9	6801	7433	211	24.184	6.3	GGGAGGAATTAAAGTATG	9	9	10	hydrolase
+	10	7445	7627	61	7.317	7.16	AGGAGGATTGACTATG	10	10		hypothetical protein
+	11	7636	7962	109	12.488	5.5	ATGAGGTAATATATG	11	11	11	terminase small subunit
										12	hypothetical protein
+	12	8166	9812	549	62.999	5.45	ACGAGGAGGGTAATAGATG	12	12	13	terminase large subunit
+	13	9825	10946	374	42.982	5.21	AGGAGAAAACTATATG	13	13	14	portal protein
+	14	10906	11598	231	25.252	4.78	AGGAGACACTACGAATG	14	14	15	phage prohead protease
+	15	11650	12603	318	34.875	5.59	AGGAGACCTATAATATG	15	15	16	major capsid protein
+	16	12718	12990	91	10.394	4.77	AGGAGGTGACACAATG	16	16	17	hypothetical protein
+	17	12980	13258	93	10.692	10.4	AGAGGAGGCGATCAGATATG	17	17	18	phage tail protein
+	18	13258	13575	106	12.479	4.92	GGGAGGTAGTCATTTAATG	18	18	19	hypothetical protein
+	19	13572	13901	110	12.507	11.38	AGGTGTTAATATTATG	19	19	20	hypothetical protein
+	20	13950	14531	194	21.354	5.13	AGGAGAATTAATCAATTATG	20	20	21	major tail protein
+	21	14663	17395	911	92.883	9.92	AGAAAGGAAATGTATTATATG	21	21	22	phage tail tape measure protein
+	22	17459	18655	399	45.822	5.03	AGAATGGAGGAAATTATATG	22	22	23	hypothetical protein
+	23	18663	19633	333	36.8	5.35	AGGAGATTAATCATG	23	23	24	structural protein

		58	56		42						
-	24	202	198	134	15.7	5.05	AGGAGAATTAAAGACA**TG**	24	24	25	hypothetical protein
		64	63		33						
+	25	203	211	256	27.9	6.75	GAGGAGATTTAAAATA**TG**	25	25	26	receptor-binding protein
		44	11		61						
-	26	215	211	124	14.1	7.69	AGGAGACCCCGCATTA**TG**	26	26	27	holin
		15	44		01						
										28	hypothetical methylotransferase
-	27	217	216	55	6.53	9.52	GAGGAGAAGTA**ATG**	27	27	29	hypothetical protein
		90	26		4						
								28			hypothetical protein
										30	hypothetical protein
-	28	220	217	77	9.12	8.93	ATGGAGGTTCTATA**GTG**	29	28	31	hypothetical protein
		20	90		7						
-	29	222	220	78	9.21	6.35	AAAGGAACGAGAAA**TG**	30	29	32	hypothetical protein
		53	20		4						
-	30	225	223	73	9.14	9.1	GAGAGGTTCGCAAGTA**ATG**	31	30	33	hypothetical protein
		79	61		6						
-	31	230	225	167	18.8	9.74	AAAAGGAGATTTAAA**TG**	32	31	34	phage-related hydrogenase
		79	79		12						
-	32	233	231	57	6.11	10.1	AAGAGGAACAAAC**GTG**	33	32	35	hypothetical protein
		25	55		7	9					
-	33	242	234	282	31.1	5.37	AAAAGGAGGACAAGTA**ATG**	34	33	36	lysin
		92	47		19						
-	34	246	242	116	12.8	9.86	AGGAGGAAACAATAA**TG**	35	34	37	holin
		41	94		76						
-	35	261	246	504	56.0	5.77	AGGAGGAAATTAC**ATG**	36	35		hypotetical protein
		69	58		98						
										38	conserved protein
-	36	263	262	49	5.76	5.76	AAGGGGTATTGTAA**TG**	37	36		hypothetical protein
		77	31		8						
-	37	266	263	103	11.6	10.1	AGGAGATATAAC**ATG**	38	37	39	hypothetical protein
		85	77		2	6					
-	38	268	266	70	8.20	5.53	none	39	38		hypothetical protein
		96	87		8						
-	39	271	268	93	10.8	4.89	GAGGAAGTGAGCAA**TG**	40	39		hypothetical protein
		68	90		62						
-	40	274	271	84	9.94	5.64	GAGGAGATAACAA**ATG**	41	40	40	hypothetical protein
		16	65								
-	41	277	275	57	6.57	8.97	AGGAATAATATATGAC**ATG**	42	41	41	repressor
		32	62		1						

Table 6. Coordinates and information about putative ORFs of *Leuconostoc pseudomesenteroides* bacteriophage ΦLN04 and information of homologues ORFS in other phages.

Strand	ORF	Start	Stop	Size (aa)	MM (kDa)	pI	SD sequence	ORF ΦLN03	ORF ΦLN12	ORF ΦLN6B	ORF P793	Function
-	1	733	422	104	12.523	9.15	none	1	1	1	1	phage HNH endonuclease
-	2	1239	730	170	19.077	10.15	AAGGGGGCTAAAAACAAAA**ATG**			2		HNH endonuclease
-	3	1618	1232	129	15.205	9.23	ACTTGGCTT**ATG**	2	2	3	2	endodeoxyribonuclease
+	4	2198	3592	465	52.896	5.43	AGGAGGCCTAAAAAAC**ATG**	3	3	4	3	DNA helicase
+	5	3582	4352	257	29.924	6.26	CGGAGGTGCTTTCT**ATG**	4	4	5	4	DNA primase/polymerase
+	6	4411	4890	160	18.595	9.7	AAGGAGGACAGAA**ATG**		5			putative HNH endonuclease
+	7	4874	6691	606	68.793	6.12	AGCTGGAGGTTATACTT**TTG**	5	6	6	5	DNA polymerase
+	8	6749	7306	185	21.125	8.85	AAGGAAGTGTAACA**ATG**	6	7	7	6	hypothetical protein
+	9	7376	7999	207	24.343	6.54	AAGAGAAGATAATC**ATG**	7	8	8	7	hydrolase
+	10	8018	8368	117	13.488	5.06	TTGAGGTAATAACCAAT**ATG**	8	9	9	8	terminase small subunit
+	11	8371	10054	547	63.084	5.51	CGGAGAATTGAGT**ATG**	9	10	10	9	terminase large subunit
+	12	10002	11243	413	46.473	5.29	none	10	11	11	10	portal protein
+	13	11194	11924	242	26.136	4.84	AGGGAGCACGGCTA**ATG**	11	12	12	11	phage prohead protease
+	14	11977	12945	323	34.723	6.12	GTGAGGAAAATATTATA**ATG**	12	13	13	12	major capsid protein
+	15	13020	13298	93	11.124	4.61	AGGAAACCGACTATT**ATG**	13	14	14	13	hypothetical protein
+	16	13215	13598	94	10.523	10.1	AGGTGGTGGCAAGA**ATG**	14	15	15	14	phage tail

		95	76		92	37	**G**					protein
+	17	135	138	10	11.8	5.3	GGGTGGTAATCGCTA**AT**	15	16	16	15	hypothetical
		76	87	4	37		**G**					protein
+	18	138	142	11	13.2	10.	ATGTGGTGGTTCTCTA**A**	16	17	17	16	hypothetical
		87	43	9	55	83	**TG**					protein
+	19	142	148	19	21.5	5.2	GAGGATAACAAAAAT**AT**	17	18	18	17	major tail
		94	78	5	8		**G**					protein
+	20	150	174	81	83.4	10.	AAAAGGAGCTTTTAA**AT**	18	19	19	18	phage tail tape
		28	84	9	78	53	**G**					measure protein
+	21	175	186	37	41.7	4.9	AAAATGTGATATAATCG	19	20	20	19	hypothetical
		28	43	2	79	9	TAGT**ATG**					protein
+	22	186	195	29	32.4	5.3	TGGAGACTAGAG**ATG**	20	21	21	20	structural
		46	15	0	39	7						protein
+	23	195	204	31	33.3	9.0	AGAAAGGTAATAAT**ATG**	21	22	22	21	receptor-binding
		29	61	1	64	1						tail protein
-	24	206	204	55	6.23	9.8	AGGATAATAGCCTTTCT	22	23	23	22	hypothetical
		46	82		1	9	C**ATG**					protein
-	25	210	207	12	14.7	4.8	AAAAGGACGAACCAAC	23	24	24	23	hypothetical
		88	08	7	34	6	**ATG**					protein
-	26	215	211	12	14.3	6.5	GGAAACGTAATA**ATG**	24	25	25	24	holin
		36	53	8	21	8						
-	27	217	216	56	6.23	7.1	CGGAGATAAAAATC**ATG**	25	26	26	25	hypothetical
		83	16		3	1						protein
-	28	222	217	15	16.2	4.5	AAAAGGAGACTTTTA**AT**	26	27	27	26	lysin
		28	76	1	83	7	**G**					
									28			phage-related methyltransferase
-	29	225	222	92	10.7	9.6	AAAAGGAAAATAACTA	27	29	28	27	hypothetical
		03	28		75		**ATG**					protein
-	30	227	225	90	10.1	7.7	ATAAGGAGCCAGATAG**A**	28	30	29	28	hypothetical
		72	03		86	6	**TG**					protein
-	31	229	227	73	8.17	5.3	AAGGAGATAACACATT**G**	29	31	30	29	hypothetical
		87	69		5		**TG**					protein
-	32	232	230	83	9.84	4.8	TGGAGTGAGTG**ATG**	30	32	31	30	hypothetical
		91	43		2	1						protein
-										32		hypothetical protein
-	33	235	232	70	8.26	9.6	AGAGGATTTTCAAAAAA**A**	31	33	33	31	transcriptional
		00	91		6	1	**TG**					regulator
-	34	236	234	49	6.22	11.	AAGGGGTAAATTGGT**AT**	32	34	34	32	hypothetical
		43	97		9	6	**G**					protein
-	35	239	236	90	10.8	10.	AGGAGCAATTATAA**TG**	33	35	35	33	response
		09	40		29	25						regulator
-	36	241	239	84	9.86	9.6	AGGAGGTCACAAAAG**A**	34	36	36	34	hypothetical
		60	09		3		**TG**					protein
-	37	250	242	26	29.3	6.2	ATAAGGAGGTAAGT**ATG**	35	37	37	35	lysin
		62	71	4	64	2						
-								36	38			phage infection protein
-											36	hypothetical protein
-								37	39			hypothetical protein
-	38	255	250	14	15.6	9.1	AGAAGAGGATTAACAC**A**	38	40	38	37	holin
		10	73	6	01		**TG**					
-	39	256	255	49	5.69	7.2	AGGAACGATAAGACT**AT**	39	41	39	38	repressor
		60	14		8	3	**G**					

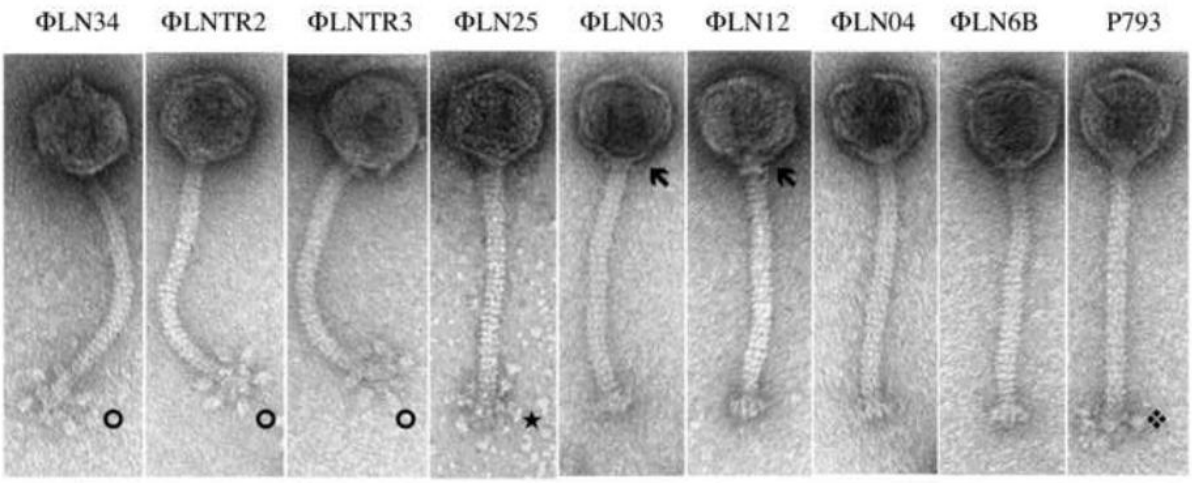

Figure 1

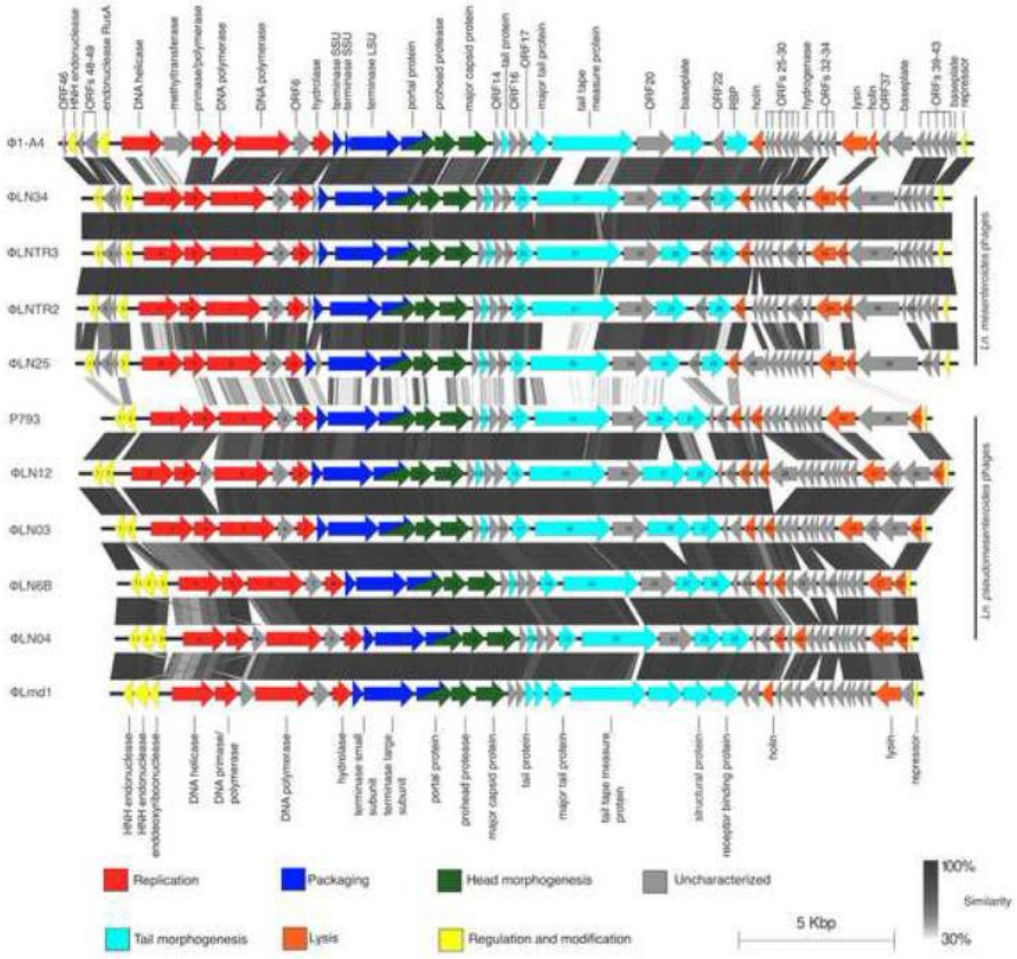

Figure 2

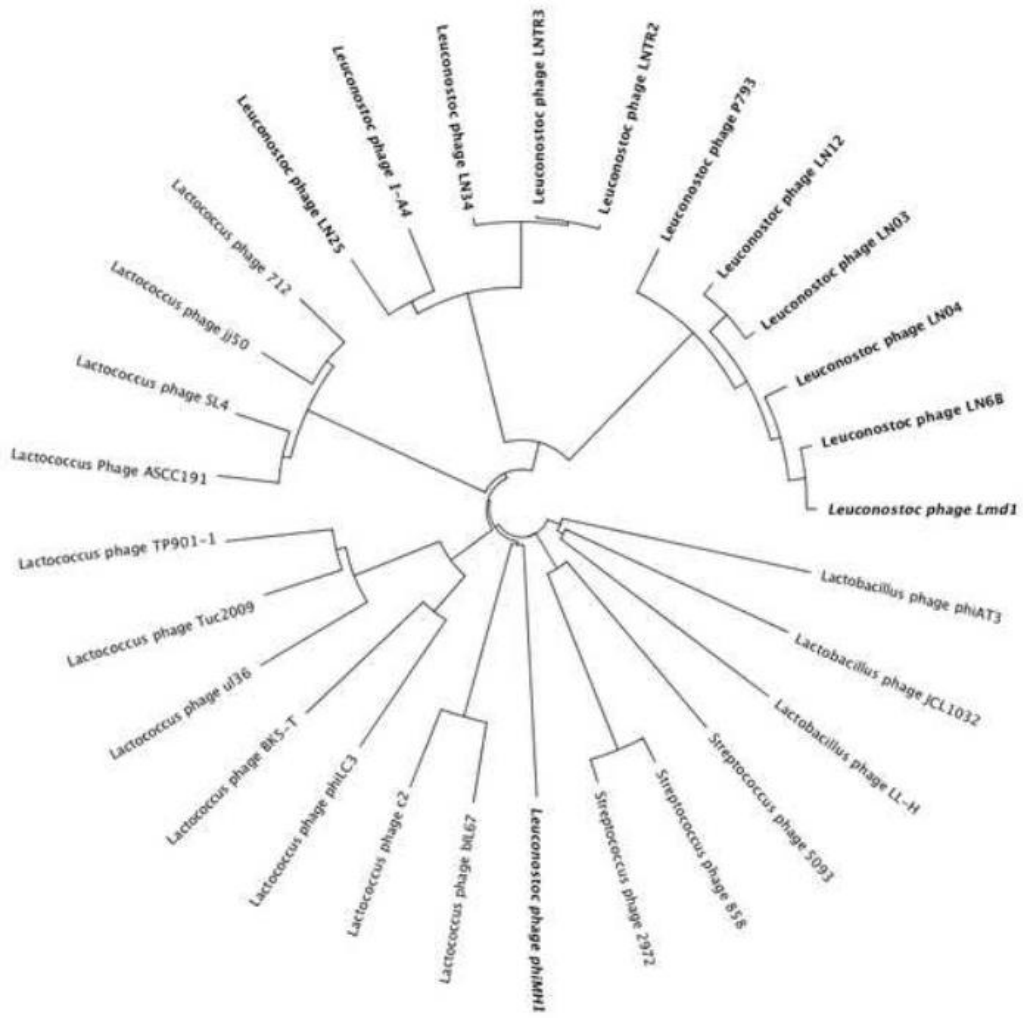

Figure 3

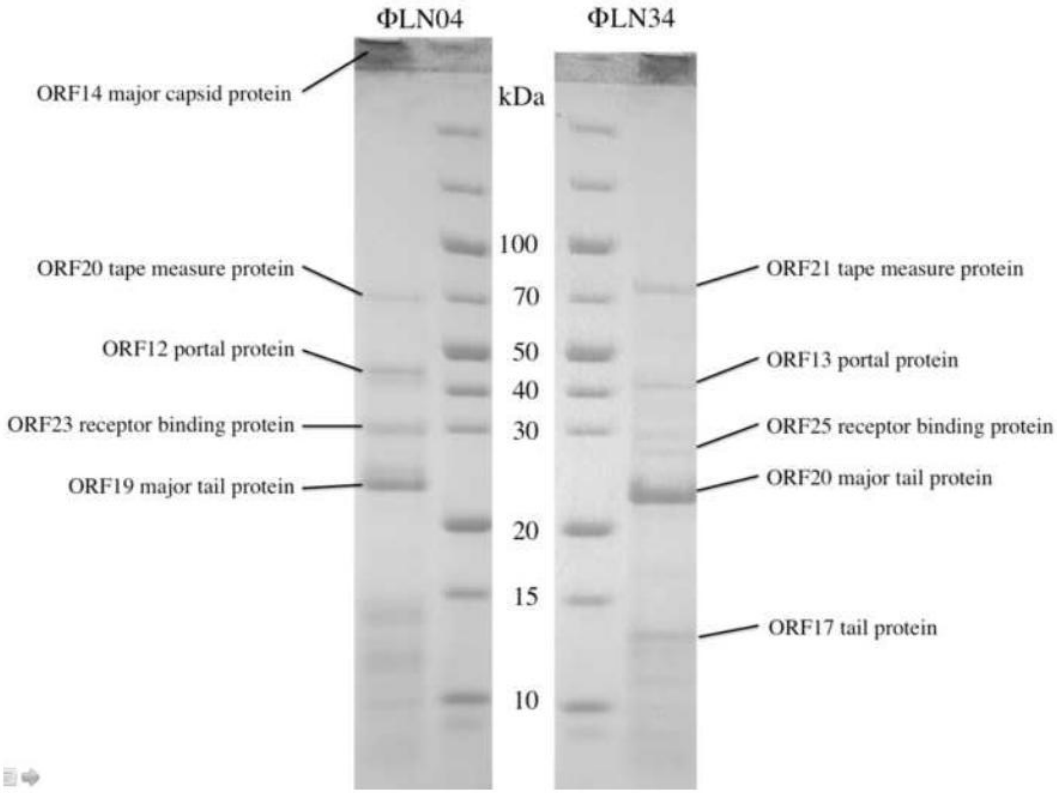

Figure 4

Research Highlights:

- Nine dairy *Leuconostoc* phages were characterized and sequenced
- Phages were isolated in relation to a fermentation problem
- Sequenced phages can be grouped in two classes that correlate with the host species
- Comparative genomic work revealed high conservation within the classes