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Philosophiae Doctor (PhD) Thesis 2012:17

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Studies on bacteriophages infecting lactic acid bacteria of industrial food fermentations

Studier av bakteriofager som infiserer melkesyrebakterier i industriell næringsmiddelfermentering

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SUMMARY

Industrial food fermentations by lactic acid bacteria (LAB) are of great practical and economic importance. Bacteriophages (phages; bacterial viruses) infecting LAB can negatively affect quality, safety and value of the fermented product. This problem is especially pronounced in the dairy industry, where bacteriophages are recognized as the most common cause of fermentation failure.

In the study on bacteriophages infecting *Lactococcus lactis* of starter cultures used in the production of Dutch-type cheese, virulent 936-like phages were identified as the predominant phage group. P335-like prophages were detected but not phages of the c2 group. Bacteriophage biodiversity was assessed by DNA sequencing and a growth inhibition assay employing arrays of starter culture isolates as indicators. Large day-to-day variation in diversity was found within a dairy plant and a peak in the biodiversity coincided with a fermentation failure. The effect of starter culture rotation was reflected by a radical change in phage diversity, but phages infecting the original starter culture rapidly re-emerged. Phage levels in raw milk were low and the phage contamination most probably came from within the plant. The assay used for bacteriophage analyses may have potential for predicting a fermentation failure.

Two new bacteriophages were characterized, and their genomes sequenced and analyzed. Bacteriophage Lmd1 represents the first dairy *Leuconostoc* phage to be characterized on a genomic level. Its closest relative was found to be a *Leuconostoc* phage, ϕ1-A4, isolated from sauerkraut fermentation. The two phages share homologous proteins and near complete conservation of gene order in the functional modules involved in replication, packaging and morphogenesis. However, corresponding genes show little conservation at the DNA level. Bacteriophage Lmd1 grows fast and can reach high titers. It was found capable of inhibiting all *Leuconostoc* isolates of one starter culture, and sensitive leuconostocs were detected in 3 out of 4 commercial starter cultures tested. These results emphasize the importance of including strains with different phage sensitivity in starter cultures and the importance of verifying that starter cultures used in starter culture rotation are unrelated with respect to phage sensitivity.

The other bacteriophage characterized in this study, ϕYS61, was isolated from kimchi, a traditional Korean fermented vegetable product. Phage YS61 belongs to the *Podoviridae* family and infects *Weissella cibaria*. It differs from the majority of phages

isolated from industrial food fermentations since it is difficult to propagate and very susceptible to resistance development by the host bacterium. Results indicate strongly that ϕYS61 replicates via a protein-primed mechanism, similar to that of phages belonging to the *Picovirinae* subfamily, but differences in morphology and genome size led to the conclusion that ϕYS61 probably represents a new subfamily. This is the first genomic characterization of a phage infecting the genus *Weissella*.

This study has provided deeper understanding of how bacteriophages affect the production of Dutch-type cheese in Norway. Determination of high phage quantities and diversities in the dairy environment and during milk fermentations stresses the importance of stringent hygiene measures, protection of bulk starter milk from contaminating phage and the use of phage-unrelated starter cultures in starter culture rotation. The knowledge gained in this study may also be useful for other industrial fermentations.

SAMMENDRAG

Fermentering av næringsmidler ved hjelp av melkesyrebakterier har blitt brukt i årtusener i produksjon av ost, spekepølser, surkål og en lang rekke andre produkter. At fermenteringsprosessen fungerer som den skal er helt avgjørende for kvaliteten, sikkerheten og verdien til sluttproduktet. Bakteriofager (virus som infiserer bakterier) kan hindre riktig fermentering gjennom å drepe essensielle melkesyrebakterier. Dette problemet er særlig utbredt i meieriindustrien der en regner bakteriofager som den viktigste årsaken til fermenteringssvikt. I produksjon av gulost blir starterkulturer som inneholder *Lactococcus lactis* og bakterier av genus *Leuconostoc* benyttet til å fermentere melk. *Lactococcus lactis* er viktig for å danne melkesyre og *Leuconostoc* for dannelsen av smaksemner. Starterkulturen blir først dyrket opp for å danne brukssyre, eller bulk-starter, og denne blir så brukt til inokulering av melk i ystekarene.

En del av denne studien omhandlet bakteriofager som infiserer *Lactococcus lactis* i norske meierier. Det ble funnet at virulente bakteriofager av typen 936 var dominerende i alle meieriene som ble undersøkt. Ingen virulente bakteriofager ble påvist i kommersielle starterkulturer. Diversiteten av bakteriofager i norske meierier ble kartlagt ved hjelp av DNA sekvensering og gjennom vekstinhiberingsforsøk der bakterieisolater fra starterkulturer ble benyttet til å indikere tilstedeværelse av bakteriofager. Gjennom testing av myse- og brukssyreprøver som ble tatt ut daglig i et meieri, ble det funnet stor variasjon i bakteriofagdiversitet fra dag til dag. En topp i bakteriofagdiversitet sammenfalt med en fermenteringssvikt. Bytte av starterkultur i meieriet førte til en dramatisk endring i bakteriofagsammensetningen i meieriet, men effekten av dette var begrenset siden bakteriofagene som infiserte den originale kulturen raskt vendte tilbake etter at kulturene igjen ble byttet. Bakteriofagnivåene i gårdsmelk levert til meieriet var svært lavt og det ble funnet at smittekilden mest sannsynlig er å finne inne i meieriet.

I denne studien ble det også karakterisert to nye bakteriofager. Genomene deres ble sekvensert og analysert. Den ene bakteriofagen, ϕLmd1, infiserer *Leuconostoc* isolert fra en starterkultur brukt til osteproduksjon. Den nærmeste slektningen til ϕLmd1 er ϕ1-A4, en *Leuconostoc*-bakteriofag isolert fra surkålproduksjon. Bakteriofag Lmd1 hemmet veksten til samtlige *Leuconostoc*-isolater fra en starterkultur og den var i stand til å vokse på 3 av de 4 starterkulturene som ble testet. Dette viser at det er viktig å inkludere bakteriestammer med ulik bakteriofagsensitivitet i starterkulturer, samt viktigheten av å teste at ulike

starterkulturer som skal benyttes til starterkulturrotasjon ikke er følsomme for de samme bakteriofagene.

Den andre bakteriofagen som ble karakterisert, ϕYS61, ble isolert fra kimchi, en tradisjonell koreansk rett som lages gjennom fermentering av grønnsaker. Bakteriofagen tilhører familien *Podoviridae* og vertsbakterien dens er *Weissella cibaria*. Den skiller seg ut fra andre bakteriofager isolert fra industriell næringsmiddelfermentering siden den svært hyppig gir resistensdannelse hos vertsbakterien. Det ble funnet at ϕYS61 mest sannsynlig har proteiner kovalent knyttet til endene av genomet, og at disse benyttes til igangsetting av DNA-replikasjon. Denne mekanismen benyttes av bakteriofager tilhørende underart *Picovirinae*. Bakteriofag YS61 skiller seg fra *Picovirinae* i morfologi og genomlengde og bør derfor sannsynligvis danne grunnlaget for en ny underart av *Podoviridae*.

Dette arbeidet har ført til en bedre forståelse av hvordan bakteriofager påvirker osteproduksjonen i norske meierier. Resultatene fremhever viktigheten av gode hygienetiltak, streng beskyttelse av brukssyretanken fra bakteriofag-rike deler av meieriet, samt viktigheten av å benytte starterkulturer med ulik bakteriofagfølsomhet når det roteres mellom ulike starterkulturer. Erfaringer fra dette arbeidet vil trolig også være relevant for annen industriell fermentering som er utsatt for bakteriofagangrep.

LIST OF PAPERS

List of papers included in this thesis:

Paper I:

Kleppen, H.P., T. Bang, I.F. Nes, H. Holo. (2011) Bacteriophages in milk fermentations; Diversity fluctuations of normal and failed fermentations. *International Dairy Journal*, 21: 592-600.

Paper II:

Kleppen, H.P., I.F. Nes, H. Holo. Characterization of a *Leuconostoc* bacteriophage infecting flavor producers of cheese starter cultures. (Submitted manuscript)

Paper III:

Kleppen, H.P., H. Holo, S-R. Jeon, I.F. Nes, S-S Yoon. A novel bacteriophage of the *Podoviridae* family infecting *Weissella cibaria* isolated from kimchi. (Submitted manuscript)

1. INTRODUCTION

1.1 General introduction

For millennia people have used fermentation by lactic acid bacteria (LAB) to preserve various foods. Lactic acid bacteria are gram-positive bacteria that share certain morphological, metabolic and physiological characteristics: they are nonsporulating, nonrespiring but aerotolerant cocci or rods, and they produce lactic acid as one of the main fermentation products from carbohydrates. They are catalase negative and are devoid of cytochromes (Wright and Axelson 2011). The LAB genera of importance in the microbial ecology of foods, including food production and spoilage, are *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Vandamme, Pot et al. 1996; Stiles and Holzapfel 1997). LAB traditionally used in food fermentations include certain species of the genera *Lactobacillus*, *Lactococcus, Streptococcus*, *Leuconostoc*, *Pediococcus, Enterococcus* and *Weissella* (Stiles and Holzapfel 1997; Ross, Morgan et al. 2002; Park and Kim 2011). LAB contribute to food preservation by the production of organic acids like lactic- and acetic acid. Other anti-microbial agents produced by LAB include hydrogen peroxide, carbon dioxide and bacteriocins. Fermented foods are produced from vegetables, fruits and meats, or from animal products like milk and honey. Foods such as sauerkraut, kimchi, fermented sausage, rakfisk (traditional Norwegian fermented fish), yoghurt and cheese have greatly improved shelf-life compared to the raw materials from which they are derived. Moreover, characteristics like flavor, taste and texture are significantly altered and the digestibility improved. LAB alter food texture by breaking down proteins and carbohydrates, and the resulting metabolites can improve the taste and flavor of fermented foods.

Industrialized food fermentation appeared as a result of the introduction of bacterial starter cultures for cheese and sour milk production in 1890. With the advent of industrial scale food fermentation the problem of bacteriophage (phage) infection became apparent. Whitehead and Cox studied the strange and sudden loss of acid production from cheese starter cultures, and were in 1935 the first to report bacteriophages affecting dairy fermentation (Whitehead and Cox 1934; Whitehead and Cox 1936). Since then, it has become well recognized that phage infection is the principal cause of starter culture failure in many food fermentations carried out by LAB. The economic importance of food fermentation has led to numerous studies aimed at gaining knowledge on bacteriophages and their interactions with host LAB.

The following sections give a short overview of bacteriophages in general and of research performed on bacteriophages affecting industrial food fermentation. Special emphasis is placed on milk fermentation in the production of Dutch-type cheese, vegetable fermentation relating to kimchi

production and the counter-measures applied to overcome phage infections in industrial food fermentation.

1.2 Bacteriophages

Bacteriophages are viruses that depend on bacterial hosts for proliferation. Bacteriophages consist of a genome, comprised of single or double stranded DNA or RNA, encapsulated by a protein capsid. In addition, bacteriophages have structures to mediate the injection of the phage genome into a bacterial host, thereby initiating a new infective cycle. Phages are widely distributed in all habitats populated by bacterial hosts. They are probably the most abundant biological entity on the planet (approximately 10^{31} phages), and they have a major role in important environmental processes such as carbon and energy cycling in the oceans (Brüssow and Hendrix 2002). Their ubiquity in nature is well reflected by the constant threat they pose to industrial food fermentation involving LAB. All known phages infecting LAB belong to the order *Caudovirales*, which counts for about 96 % of all characterized phages (Ackermann 1998; Ackermann 2001). Phages belonging to order *Caudovirales* have linear double stranded DNA (dsDNA) genomes, they are not enveloped, and are easily recognizable by their characteristic "head-tail" morphology (**Figure 1**). According to the current classification by the International Committee on Taxonomy of Viruses, order *Caudovirales* is comprised of three phage families, *Myoviridae*, *Siphoviridae* and *Podoviridae*. These families are distinguished by tail morphology, *Myoviridae* having contractile tails, *Siphoviridae* long non-contractile tails and *Podoviridae* having short non-contractile tails. According to the nature and size of the phage head, phages of each family can be grouped into three distinct morphotypes. Morphotype 1 includes phages with an isometric head structure, type 2 includes phages with small prolate heads and morphotype 3, phages with large or elongated prolate heads. The morphological characteristics of each family and morphotype are shown in **Figure 1**.

Bacteriophage infections are initiated by the attachment of phages to specific receptors on the bacterial surface. This attachment is carried out by receptor binding proteins located at the tip of the phage tail, which upon binding, confers a conformational change in the virion leading to the injection of the phage genome into the host bacterium. Phages with a virulent life-style will then go on to replication of the phage genome, intracellular assembly of progeny phages, and finally cell lysis with the release of progeny phages (**Figure 2**). This is known as the lytic pathway. The number of progeny phages released from one bacterial burst can vary from phage to phage or with different host bacteria. Often the number of phages released (burstsize) is between 20 and 100.

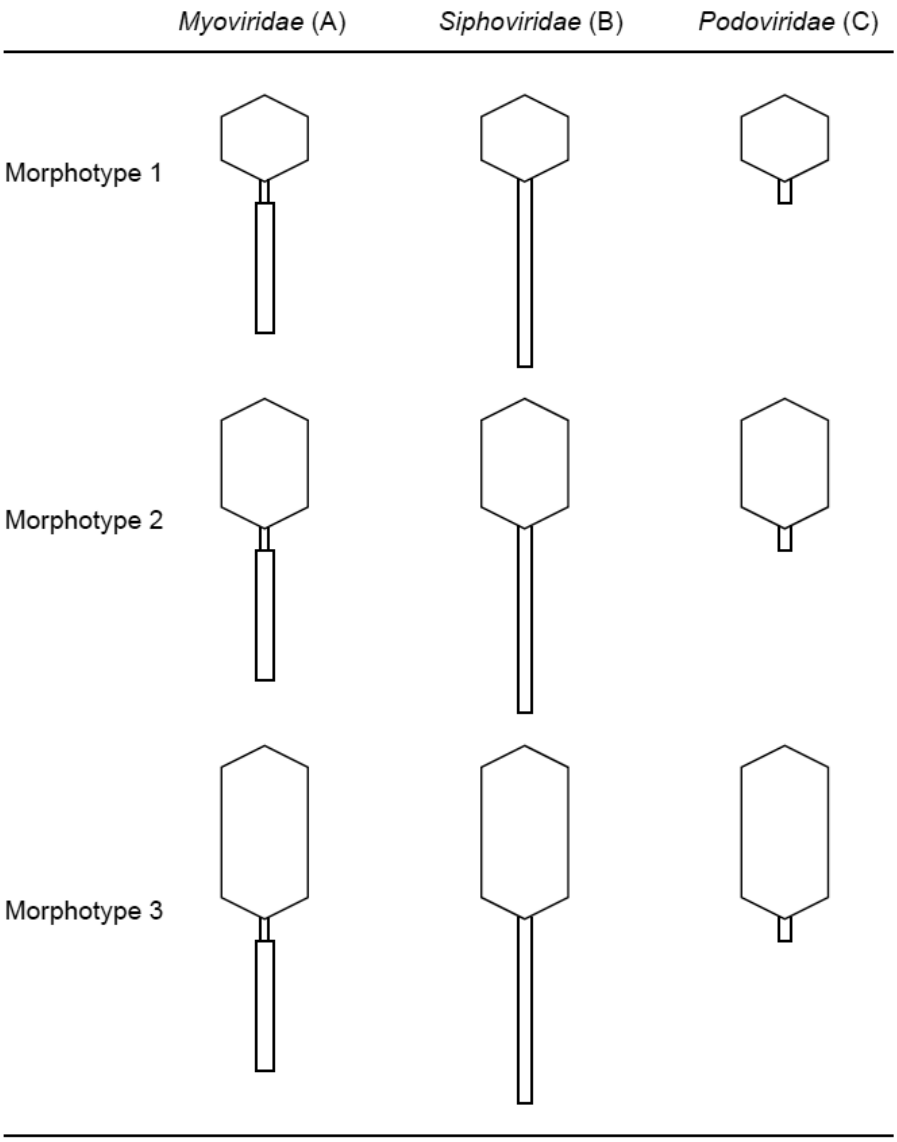


Figure 1. *Morphotypes of bacteriophage families belonging to the order Caudovirales. Figure adapted from Ackermann (2001).*

Two lifestyles of bacteriophages are well characterized: virulent phages are restricted to the lytic pathway, and are strictly reliant on repeated cycles of infection, propagation and release for their survival. In environments less densely populated by suitable hosts, this life-style demands that phages survive potentially long timespans before a new infection cycle can occur. This puts selective pressure on phage structural components favoring those resistant to environmental stress factors. The temperate phages have the ability to reproduce by the lytic cycle, but in addition they can follow the lysogenic pathway. During lysogeny the phage genome is stably maintained as a prophage inserted into the chromosome of the host bacterium, and is thus replicated along with its host's DNA. The prophage can be induced to exit its lysogenic state and to leave its host by switching to a lytic life-style. Such induction

can occur spontaneously or following host cell stress induced (intentionally) by DNA-damaging agents such as UV-light and mitomycin C. The two different lifestyles are illustrated in **Figure 2**.

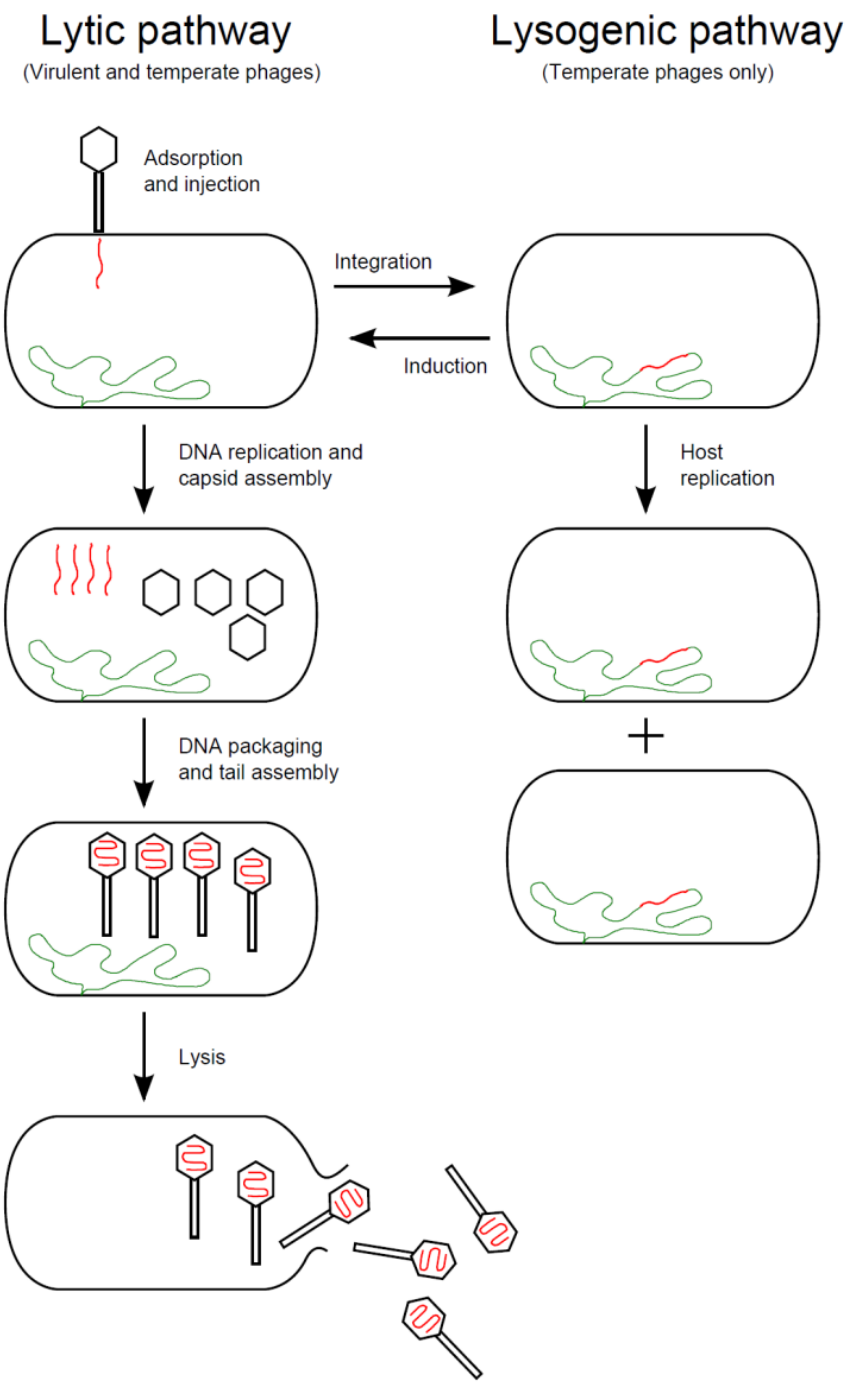


Figure 2. *Illustration of the two different propagation strategies employed by virulent and temperate phages. Bacteriophage DNA is shown in red and the bacterial chromosome in green.*

The genomes of LAB bacteriophages are often organized in distinct functional modules. Genetic modules are clusters of genes that act together to achieve some function, for instance DNA replication, DNA packaging, head- and tail morphogenesis, transcription, lysis or lysogeny. If improved fitness is gained from the co-transcription of functionally related genes, they will by random translocation or duplication events tend to migrate to form a genetic cluster, and genes that need to be transcribed in a definite sequence will tend to arrange themselves in transcriptional order (Newth and Green 2007). In small populations, this mechanism would lead to all individuals in a population having the same genetic makeup, but with respect to bacteriophages, populations are vast and they face ever changing selective pressure, including shifts in host cell populations and the emergence of new phage resistance mechanisms. Homologous recombination of genetic modules between phage genomes or between phage genomes and bacterial chromosomes and prophages provides bacteriophages with a way to rapidly adapt to changes in selective pressure. This mechanism is known as modular evolution (Botstein 1980). A major advantage of modular evolution is that it provides phages access to a large array of functional specificities by means of homologous recombination. According to the original theory (Botstein 1980), phages would exchange complete functional modules, however, exchange reactions can also occur within modules or even within genes (Neve, Zenz et al. 1998). In their recent review article Veesler and Cambillau discussed evolution of phage morphogenesis modules in light of the extensive conservation of protein folds among homologous structural proteins (Veesler and Cambillau 2011).

The genome plasticity of bacteriophages was demonstrated in several studies on the virulent lactococcal phage ul36. Phage ul36 belong to the P335 group of phages that also include temperate lactococcal phages. Sequence similarity to P335-like prophages makes ul36 prone to homologous recombination during infection in hosts carrying P335-like prophages. Following infection of the host strain *Lactococcus* (*Lc.*) *lactis* SMQ-88, two new recombinant phage mutants that were resistant to abortive infection mechanism AbiK and one phage exhibiting a reduced burst size and new origin of replication was discovered (Bouchard and Moineau 2000). A bacteriophage, ul37 that evolved from ul36 during infection of host *Lc. lactis* NCK203 had gained a longer tail, a different baseplate and a new origin of replication. It was also resistant to abortive infection mechanism of the host strain (Moineau, Pandian et al. 1994). Moreover, as much as 79 % of the genome was exchanged in one mutant phage evolving from ul36 during infection with host strain *Lc. lactis* SMQ-86. (Labrie and Moineau 2007).

By modular evolution phage genomes become mosaics of functional genetic elements taken from a pool of available genes or functional modules. Compared to evolution by linear descent, this makes taxonomic classification of bacteriophages challenging. Classical methods for taxonomic differentiation,

like electron microscopy, DNA-DNA hybridization and protein profiles (Braun, Hertwig et al. 1989), have in addition to analysis of replication and packaging strategies been used to classify LAB bacteriophages (Maniloff and Ackermann 1998). With an increasing number of available genome sequences and a realization of the extent of homologous recombination between phages, the classical taxonomic criteria and the use of hierarchical taxonomic grouping have been challenged (Lawrence, Hatfull et al. 2002). Instead, Lawrence and co-workers have suggested a novel classification system, based on characteristic genomic modules, which does not allow a hierarchical branching phylogeny (Lawrence, Hatfull et al. 2002). Others have suggested classification methods based on protein sequence similarities between all predicted proteins of phage genomes (Rohwer and Edwards 2002) or between proteins encoded by sets of core genes (Lavigne, Seto et al. 2008; Lavigne, Darius et al. 2009). Both of the latter methods predicted phage groups that were largely in accordance with the established taxonomy of the International Committee on the Taxonomy of Viruses (ICTV), suggesting that the classical taxonomic system can be unified with taxonomic classification based on whole genome proteomics. Lavigne and colleagues also stated that the increased probability of recombinational exchange hypothesized for temperate phages, does not necessarily complicate phage classification (Lavigne, Seto et al. 2008). A method for predicting functional relationship between bacteriophages has also been described (Lu, Altermann et al. 2010; Altermann 2012).

The functional modules commonly encountered in bacteriophage genomes include modules involved in replication, packaging, head- and tail morphogenesis, transcription, lysis and lysogeny. Proteins encoded by genes in different replication modules are responsible for phage DNA replication. There are several different strategies for DNA replication, executed by different replicative proteins. For an overview of phage replication modules, see Weigel and Seitz (2006). DNA packaging, the process of inserting the phage genome into a preformed capsid or prohead, is performed by ATP-driven packaging machines against significant force. The key components of the packaging machine are the packaging enzyme (terminase or motor) and the portal protein that forms the DNA entrance of the prohead. A recent series of reviews covers aspects from DNA packaging to DNA ejection in different tailed bacteriophages (Black and Thomas 2012; Chemla and Smith 2012; Feiss and Rao 2012; Morais 2012; Tavares, Zinn-Justin et al. 2012). The head, or capsid, morphogenesis module of bacteriophages encode proteins involved in prohead formation and maturation, and in some cases proteins or protein domains involved in receptor recognition and immune system evasion. The following reviews covers general principles of virus structural organization (Prasad and Schmid 2012), the function of scaffolding proteins during phage morphogenesis (Prevelige and Fane 2012) and the mechanism of capsid formation in *Escherichia coli* phage HK97 (Hendrix and Johnson 2012). Tail morphogenesis and function in

Siphoviridae (Davidson, Cardarelli et al. 2012), *Podoviridae* (Casjens and Molineux 2012), and *Myoviridae* (Leiman and Shneider 2012) have also recently been reviewed. Lysogeny modules are found in temperate phages and the genes encoded are involved in integration of phage genomes to form prophages in the host chromosome, or excision from the chromosome following induction. Host lysis is achieved by the combined function of holin and lysin. Lysin is the term for muralytic enzymes that degrade the cell wall of the host bacterium. Holins are small membrane proteins that form pores in the inner membrane giving lysins access to the cell wall. Several different kinds of protein inhibitors regulate holins and ensure lysis at an optimal time (Wang, Smith et al. 2000; Dewey, Savva et al. 2010).

1.3 LAB of cheese starter cultures and their bacteriophages

Starter cultures

Cheese production is one of the most economically important fermentation processes, second only to brewing (Coffey, Stokes et al. 2001). Starter cultures are used to convert milk sugar (lactose) into lactic acid and milk citrate into metabolites important for flavor and texture. *Lactococcus* (*Lc.*) *lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* are the major acid producers of cheese starter cultures due to their capability of fast growth. Bacteria of the genus *Leuconostoc* (*Ln.*) produce insignificant amounts of acid but are incorporated into dairy starter cultures since they are able to produce metabolites such as diacetyl, acetaldehyde and CO_2 from citric acid (Cogan and Jordan 1994; Björkroth and Holzapfel 2006). Diacetyl is the primary source of aroma and flavor compounds in a variety of fermented milk products including buttermilk, cultured butter and various cheese types (Björkroth and Holzapfel 2006). The different leuconostocs associated with dairy starters include *Ln. mesenteroides* subsp. *cremoris*, *Ln. mesenteroides* subsp. *dextranicum*, *Ln. lactis* and *Ln. pseudomesenteroides* (Daly 1983; Atamer, Ali et al. 2011). Depending on the composition, these cultures can be used in the production of a variety of cheeses.

Mesophilic starter cultures always include lactococci for acid production (Daly 1983). Different types of starter cultures are designated based on the type of flavor producing bacteria it includes. Leuconostocs are used as flavor producers in L-type and DL-type mesophilic starter cultures, in the latter together with *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*. D-type starters only include *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* for flavor production, and O-type cultures have no flavor producers included. The number of flavor producers in different starter cultures varies between 1-10 % (Cogan and Jordan 1994).

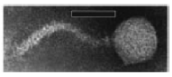
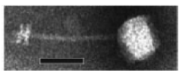
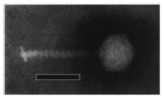
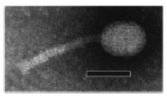
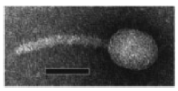
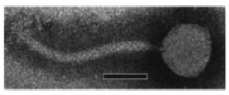
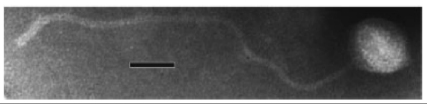
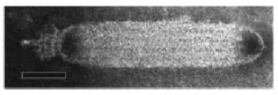
Starter cultures for cheese production are divided into mixed or defined cultures. Most defined cultures are blends of a low number of well characterized strains. The strains are selected based on their individual and combined performance in fermentations, as well as their phage sensitivity. Starter culture strains should ideally be phage-unrelated (not infected by the same phages). Mixed starter cultures are composed of an undefined number of strains. These starters have evolved naturally in industrial practice and are often less susceptible to complete fermentation failure (Boucher and Moineau 2001). This is probably due to a high number of phage-unrelated strains that have evolved together with bacteriophages. Accordingly, virulent bacteriophages have on several occasions been isolated from commercial starter cultures (Lodics and Steenson 1990).

Cheese starter cultures can be used to produce a bulk starter culture that is subsequently used to inoculate the cheese fermentation vats, or it can be added directly to the fermentation vat. The use of direct inoculation in cheese production vats reduces the risk of phage problems, since phages are given less time to multiply. Still, many prefer bulk starters for sensory reasons and for the higher initial fermentation speed.

Bacteriophages of *Lactococcus lactis*

Bacteriophages negatively affect quality, safety and value of the fermented milk product. Lactococcal phages have long been recognized as the primary cause of failed (slow) fermentation. Reduced acidification rates can lead to unwanted growth of spoilage bacteria, or worse, pathogenic bacteria. The more common problem resulting from slow fermentation vats is stalling of the production flow. This can be serious enough in large cheese factories which rely on continuous production and tight production schedules. Due to their economic importance dairy phages infecting *Lactococcus lactis* are among the best studied groups of bacteriophages to date. The current classification of lactococcal phages includes

Table 1. Overview of phage groups infecting *Lactococcus lactis*.

	Group	Representative phage	Virion dimensions (nm) (capsid diameter / tail width / tail length)	Electron micrograph [A]	Genome sequences [B]
Siphoviridae	936	936	50 / 11 / 126		12
	P335	P335	49 / 7 / 104		11
	1358	1358	45 / 10 / 93		1
	c2	c2	54x41 / 10 / 95		2
	Q54	Q54	56x43 / 11 / 109		1
	P087	P087	59 / 14 / 163		1
	949	949	70 / 12 / 490		1
	1706	1706	58 / 11 / 276		1
Podoviridae	P034	P369	57x40 / 5 / 19		1
	KSY1	KSY1	223x45 / 6 / 32		1

[A] *Bars represent 50 nm.*

[B] *Genome sequences of lactococcal phages assigned to phage groups available in GenBank (February 2012). Table 1 is adapted from Deveau, Labrie et al. (2006).*

10 groups, sometimes referred to as species (Deveau, Labrie et al. 2006) (**Table 1**). Of these, three groups, 936, P335 and c2, are predominant and responsible for most dairy fermentation breakdowns (Prevots, Mata et al. 1990; Moineau, Fortier et al. 1992). Phages of the P335 group can be temperate or virulent whereas members of the 936 and c2 groups are virulent. It is not clear why these three groups of lactococcal phages are dominating dairy environments, but the continued use of selected *Lc. lactis* strains in dairy starter cultures might influence which phages are able to propagate. Moreover, phages belonging to these groups are capable of rapid growth and can reach high titers compared to the lesser known lactococcal phages, a fitness parameter likely to contribute to their widespread distribution (Deveau, Labrie et al. 2006).

Phage genomics is well developed, especially for phage groups 936, P335 and c2. To date, 39 lactococcal phage genomes have been sequenced (www.ncbi.nlm.nih.gov). Among these are 32 genomes of phages that have been assigned to one of the phage groups infecting *Lc. lactis* (**Table 1**), 3 unassigned phages and 4 recombination mutants of P335-like phage ul36 (Labrie and Moineau 2007). For an updated review on phage genomics, see Mills, Ross et al. (2011).

As a consequence of advances in comparative genomics, new molecular methods based on PCR have been developed for the detection and identification of lactococcal phages (Labrie and Moineau 2000; Deveau, Labrie et al. 2006; del Rio, Binetti et al. 2007). Other new technologies for bacteriophage detection includes the use of flow cytometry (Michelsen, Cuesta-Dominguez et al. 2007) and detection of the changes in impedance occurring when phages infect bacteria on a microelectrode surface (García-Aljaro, Muñoz-Berbel et al. 2009).

Bacteriophages affecting dairy fermentations can come from various sources. Raw milk is well known to harbor phages and is considered an important source of phage contamination (Jarvis 1987; Bruttin, Desiere et al. 1997). In a study of 900 milk samples from various regions in Spain it was found that 9 % of the samples contained lactococcal phages (Madera, Monjardin et al. 2004). In another study from Spain, 36 % of milk samples contained DNA form P335-like phages. 936-like DNA was detected in 27 % of the samples (del Rio, Binetti et al. 2007). Some phages contained in raw milk can survive pasteurization and can thus gain entrance to milk fermentations. Several studies have investigated thermal resistance of lactococcal phages, focusing especially on the temperatures used to sanitize milk in the dairy industry (63, 72 and 90°C), reviewed in 2011 (Guglielmotti, Mercanti et al.). Atamer and co-workers screened a collection of lactococcal phages of dairy origin for phages with high thermal resistance and found that 40 % of the isolates resisted treatment at 80°C for 5 minutes when they were heated in milk. The most resistant isolate survived 97°C for 5 minutes (Atamer, Dietrich et al. 2009). Milk proteins have been shown have a protective effect on bacteriophages during thermal inactivation

(Atamer, Dietrich et al. 2010). Madera, Monjardín and Suárez postulated that a rich supply of diverse bacteriophages enter dairies via raw milk and survive pasteurization, and that some of these phages become prevalent in a particular dairy if susceptible host strains are present in the starter cultures in use (Madera, Monjardin et al. 2004). Prevalent phages are likely to be found in high titers in whey and on equipment and surfaces that have been in contact with whey. Dairies generally apply stringent hygiene measures to avoid contaminating bacteria. Some of these measures include washing production premises and fermentation vats with large amounts of water, processes inevitably producing aerosols rich in bacteriophages. Lactococcal phages have been found in high titers in air samples and on surfaces of dairy environments, thus contamination of new production batches from in-house phages is likely (Neve, Berger et al. 1995; Verreault, Gendron et al. 2011). Also the reuse of whey proteins and whey cream might be a source of phage contamination, through aerosol formation during handling (e.g. centrifugations), and through the use of whey proteins to standardize milk before fermentation (Atamer, Dietrich et al. 2009). Rousseau and Moineau (2009) showed that a bacteriophage can survive in a cheese factory for more than a year.

Many starter culture strains contain inducible prophages which have the potential to influence starter culture performance (Reyrolle, Chopin et al. 1982). In recent years, however, focus on prophages has shifted towards the role they serve as a genetic pool in the evolution of new virulent phages emerging within dairies (Labrie and Moineau 2007).

Bacteriophages infecting dairy leuconostocs

Bacteriophages infecting dairy leuconostocs were first described in 1978 (Sozzi, Poulin et al.). One year later Shin and Sato (1979) demonstrated that *Leuconostoc* phages are widely distributed in dairy products. They identified phages infecting *Ln. mesenteroides* subsp. *cremoris*, *Ln. mesenteroides* subsp. *dextranicum* and *Ln. mesenteroides* subsp. *mesenteroides* in raw milk and a large variety of cheeses: Blue cheese, Camembert, Cottage cheese, Cream cheese, Edammer and Gouda.

Natural habitats of bacteria of the genus *Leuconostoc* include green vegetation and silage (Stirling and Whittenbury 1963). It is thus possible that *Leuconostoc* phages regularly contaminate milk and thereby providing a constant supply of *Leuconostoc* phages to dairies, similar to the situation postulated for lactococcal phages (Madera, Monjardin et al. 2004). Atamer and co-workers studied the thermal resistance of 77 *Leuconostoc* phages and found that commonly applied pasteurization conditions were insufficient to ensure complete inactivation of dairy *Leuconostoc* phages (Atamer, Ali et al. 2011). Phages infecting dairy *Leuconostoc* have previously been characterized (Davey, Ward et al. 1995; Atamer, Ali et al. 2011). The four *Leuconostoc* phages belonged to the family *Siphoviridae* and

resembled dairy lactococcal phages with respect to morphology. Hybridization experiments, however, showed no homology between DNA from the *Leuconostoc* phages and DNA from two lactococcal phages of the c2-like and 936-like groups. The genome sequences of a virulent *Ln. mesenteroides* phage and a temperate *Ln. pseudomesenteroides* phage, both isolated from vegetable fermentations have been characterized (Jang, Hwang et al. 2010; Lu, Altermann et al. 2010). The genomic analysis of a *Leuconostoc* phage of dairy origin could provide new knowledge on phage biology and possibly be used to devise improved phage control methods.

1.4 Bacteria and bacteriophages in kimchi fermentation

Kimchi is manufactured by fermentation of vegetables such as Chinese cabbage and radish, and different seasonings, such as scallions, powdered chili peppers, garlic, ginger and fermented seafood. Lactic acid produced during fermentation contributes to preservation and gives kimchi its characteristic sour taste. Proper ripening and preservation is ensured by 2-5 % (wt/vol) salt content and anaerobic fermentation at low temperatures. Traditionally, kimchi is prepared by spontaneous fermentation by lactic acid bacteria (LAB) indigenous to the vegetable ingredients, but starter cultures have been developed in order to better control fermentation and thus improve the safety and shelf-life of the kimchi product (Choi, Jung et al. 2003; Eom, Park et al. 2008; Chang and Chang 2010; Chang and Chang 2011). Lee and Kim (1988) used a mixture of Lactobacillus (Lb.) plantarum, Lb. brevis, Pediococcus cerevisiae and Ln. mesenteroides strains isolated from kimchi as a starter culture for kimchi fermentations. Fermentation time was shortened and production batches were consistent in quality. Moreover, kimchi flavor was improved (cited in (Park and Kim 2011)). Probiotic starter cultures have been developed to improve health benefits of kimchi (Lee, Yoon et al. 2011).

Due to the influence of bacteria present on the raw materials, the microbial composition of kimchi fermentation is complex. Generally the fermentation process is characterized by an initial heterofermentative phase followed by a homofermentative phase. The microbial composition of kimchi fermentations has been investigated by culture dependent (Lee, Chun et al. 1997; Kim, Chun et al. 2000; Choi, Cheigh et al. 2002; Lee, Lee et al. 2002; Bae, Rhee et al. 2005; Cho, Lee et al. 2006; Lee, Kim et al. 2008; Shin, Han et al. 2008) and culture independent methods (Kim and Chun 2005; Lee, Heo et al. 2005; Nam, Chang et al. 2009; Park, Shin et al. 2010; Jung, Lee et al. 2011). Several LAB species have been identified as likely contributors in kimchi fermentations, including the *Leuconostoc* species *Ln. mesentereoides, Ln. kimchii, Ln. citreum, Ln. gasicomitatum* and *Ln. gelidum*, the *Lactobacillus* species

Lb. brevis, Lb. curvatus, Lb. plantarum and *Lb. sakei, Lactococcus lactis, Pediococcus pentosaceus*, and the *Weissella* species *W. confusa, W. kimchii* and *W. koreensis*. The species *Weissella kimchii*, first described in 2002 (Choi, Cheigh et al. 2002), was reclassified as *Weissella cibaria* in 2004 (Bjorkroth, Schillinger et al. 2002; Ennahar and Cai 2004).

Several studies have been carried out to investigate the microbial population dynamics in kimchi fermentations (Bae, Rhee et al. 2005; Cho, Lee et al. 2006; Chang, Kim et al. 2008; Lee, Kim et al. 2008; Nam, Chang et al. 2009; Park, Chang et al. 2009; Jung, Lee et al. 2011). Reportedly, successions of various LAB species and their metabolic activities are responsible for the quality and safety of these products. Which bacterial species dominates the different stages of kimchi fermentation varies with the ingredients used and fermentation conditions such as temperature, salinity etc. Generally, *Weissella* species are abundant in the late fermentation and can continue to grow during storage at low temperatures (-1°C). *Weissella* species have thus been associated with the excessive acidic taste of over-ripened kimchi products.

Metagenomic analysis of kimchi microbial dynamics revealed high abundance of phage DNA, indicating that bacteriophages affect kimchi fermentation and might be an important determinant of kimchi microbial dynamics (Jung, Lee et al. 2011). Bacteriophage involvement in bacterial community succession has also been reported in sauerkraut fermentation (Lu, Breidt et al. 2003). Phages are apparently responsible for the variability observed in such vegetable fermentations (Barrangou, Yoon et al. 2002). A virulent bacteriophage infecting six out of 11 *Lactobacillus plantarum* strains tested was isolated from kimchi (Yoon, Kim et al. 2001). No kimchi bacteriophages infecting *Weissella cibaria* have been characterized, but recently, a bacteriophage infecting a *Weissella cibaria* starter culture strain used in the fermentation of Thai Nham sausage was reported (Pringsulaka, Patarasinpaiboon et al. 2011). Bacteriophages likely infecting the genus *Weissella* was also reported in a sauerkraut fermentation, but the bacterial isolate was not conclusively identified (Lu, Breidt et al. 2003).

1.5 Bacteriophage counter-measures in industrial food fermentations

Bacteriophages are ubiquitous in nature and all industrial fermentations that rely on bacteria to carry out metabolic processes are in theory susceptible to phage inhibition. Phages can gain access to food fermentations through raw materials, since the food fermentation substrates in most cases cannot be sterilized prior to fermentation. The liquid nature of milk allows for rapid dispersal of bacteriophages from the initial site of infection, thereby making dairy fermentations especially vulnerable to infection.

Dairy microbiologists have since the first discovery of dairy phages nearly 80 years ago attempted to control bacteriophages interfering with milk fermentation. As a result of this, dairy phages are among the best studied bacteriophages and a wide array of phage counter-measures has been devised. Most of these can probably be applied in other industrial fermentation settings. The various anti-phage strategies can be grouped into factory design, improved sanitation, production routines, specific culture media, and the use of phage-resistant strains.

Factory design

Milk fermentation can be compromised when phage titers are higher than a critical threshold of 10^4 plaque forming units per mL (pfu/mL), and when titers increase beyond 10^5 – 10^6 pfu/mL, a fermentation failure is likely (Moineau and Lévesque 2005). Bacteriophages can reach titers above 10^9 pfu/mL in whey (Neve, Berger et al. 1995), and it is therefore of great importance to avoid contact between whey and the milk used for dairy production. Strict compartmentalization of dairy production units is therefore common in dairies operating on industrial scale. Compartmentalization should also include separate entrances, separate work clothing for dairy staff, separate air ventilation systems and elevated air pressure in compartments handling fermentation substrate, starter cultures and bulk starter production facilities.

Sanitation

A good sanitation procedure is the most efficient way to reduce spread of bacteriophages within a dairy plant. Several sanitizers including ethanol, isopropanol and sodium hypochlorite have been tested for efficiency in disabling dairy phages (reviewed in (Guglielmotti, Mercanti et al. 2011)). Generally, isopropanol showed little effect and ethanol had to be used at near 100 % concentrations to produce phage inactivation to a considerable degree. Some dairy phages have been shown to survive in 100 % ethanol for more than 45 minutes (Guglielmotti, Mercanti et al. 2011). Phage resistance to sodium hypochlorite mimicked the results found for ethanol, with most phages inactivated by exposure to 100

ppm hypochlorite. Some phages required up to 400 ppm for 45 minutes and one phage, Lactobacillus delbruckii phage Ib_3 needed 1200 ppm for at least 45 minutes for complete inactivation (Quiberoni, Guglielmotti et al. 2003). New biocides are constantly entering the commercial market. Few however, are tested for activity against bacteriophages. There is a need for a sanitizer that is efficient against several LAB phages, that is cost effective, eco-friendly and no adverse effect on production equipment or the fermentation process (Garneau and Moineau 2011).

Inactivation of phages in raw materials have been addressed in several studies and was recently reviewed by Guglielmotti and co-workers (2011). Thermal inactivation generally requires temperatures above 90°C for reliable phage inactivation (Atamer, Dietrich et al. 2010), however some phages of the 936-group can withstand temperatures of up to 97°C for 5 minutes (Atamer, Dietrich et al. 2009). This kind of heating can have undesirable effects on milk and can be unsuited in the production of dairy products. Dynamic high pressure was shown to be effective for phage inactivation (Moroni, Jean et al. 2002), and a synergistic effect between thermal and high pressure inactivation have been documented (Müller-Merbach, Rauscher et al. 2005).

Production routines

Production routines aimed at reducing bacteriophage impact on dairy fermentations include the use of starter culture rotation. Rotation of different phage-unrelated starter cultures is done to avoid recurrent amplification of the same phages over consecutive fermentation processes. The importance of using phage-unrelated starter cultures in starter culture rotations have been demonstrated (Neve, Kemper et al. 1994). Another strategy to reduce phage impact on dairy fermentations is to omit production of bulk starter cultures, since this procedure provides bacteriophages with an opportunity to multiply prior to fermentation in the production vats. Starter cultures for direct vat inoculation (DVI) have been developed and can be used as the only inoculum in fermentation vats or in response to slow acidification rates caused by bacteriophages in the bulk starter.

Phage-inhibiting culture media

Different components can be used to inhibit the function of bacteriophages in fermentations. The addition of phage derived peptides to culture media was shown to delay the infection of lactococcal phage c2 (Hicks, Clark-Safko et al. 2004). Removal of calcium ions by phosphates with the intention of inhibiting phages dependent on Ca^{2+} for infection, have also been explored, and it was found that phosphate levels sufficient to inhibit the function of bacteriophages also affected the stability of caseinate particles (Suarez, Capra et al. 2007). The use of phosphates might be useful in synthetic bulk

starter media. Hultberg and co-workers (2007) demonstrated efficient inhibition of lactococcal phages by *Lactobacilli* expressing llama heavy-chain antibody fragments directed against the receptor binding protein. Another approach based on gene modified organisms (GMO) is the use of a combinatorial library of ankyrin repeat proteins (DARPins) to selectively bind and occupy receptor binding proteins by specific DARPins (Veesler, Dreier et al. 2009). Several approaches based on GMOs have been developed and have been reviewed by Sturino and Klaenhammer (2006).

Bacteriophage resistance

When bacteriophages inhibiting starter culture strains appear, one possibility is to isolate bacteriophage insensitive mutants (BIM) of the affected strain. BIMs are spontaneous mutants selected through standard phage challenge assays and might differ from the original strain only in the phage receptor. However, many BIMs also have altered physiology and might not be usable in fermentations (Moineau and Lévesque 2005). Another strategy is to develop phage insensitive strains based on the phage resistance mechanisms naturally found in lactic acid bacteria. These are often plasmid-encoded and can thus be transferred to starter culture strains via conjugation, minimizing the risk of altering properties important for starter culture performance (Moineau and Lévesque 2005). Natural bacteriophage resistance mechanisms interfere with different stages of phage infections, and are grouped accordingly. They include:

(1) Prevention of phage adsorption.
(2) Preventing phage DNA entry.
(3) Cutting phage nucleic acids.
(4) Abortive infection mechanisms, Abi.

For detailed reviews on bacteriophage resistance, see Coffey and Ross (2002), Labrie, Samson and Moineau (2010) and Mills, Ross, Neve and Coffey (2011).

2. AIM OF STUDY

Bacteriophages have long been recognized as the major cause of fermentation failure in dairy fermentations and are probably affecting starter culture strains and naturally occurring LAB in other food fermentations as well. A wide range of phage counter-measures have been devised to reduce phage impact on industrial food fermentations, but more knowledge on phage biology and ecology is needed in order to better control bacteriophages in industrial food fermentations.

The main focus of this study was on bacteriophages infecting cheese starter cultures used in the production of Dutch-type cheese in Norwegian dairies. One aim of this study was to find out what bacteriophages are present in Norwegian dairies, and to figure out how bacteriophages inhibit the function of a mixed starter culture presumably containing several phage-unrelated strains with overlapping functional properties. This knowledge could possibly enable us to develop a method to predict fermentation failure. In order to advice better phage control strategies to avoid phage contamination in dairy fermentations, we wanted to find out where the phages entering cheese production come from.

Bacteriophages infecting important flavor producing leuconostocs have received relatively little attention compared to the lactococcal phages, and a genome sequence of a dairy *Leuconostoc* phage have not been available. We wanted to characterize a bacteriophage infecting leuconostocs of cheese starter cultures and to analyze its complete genomic sequence.

We were also lucky enough to get involved with the characterization of a bacteriophage of kimchi fermentation through Professor Sung-Sik Yoon who visited our lab for six months in 2007. The collaboration with Professor Yoon and his group at the Yonsei University in Seoul, Korea, was aimed at characterizing ϕYS61 with respect to growth parameters, morphology and genome sequence.

3. MAIN RESULTS AND DISCUSSIONS

3.1 Bacteriophages in milk fermentations: Diversity fluctuations of normal and failed fermentations.

In order to advice better phage control measures for Norwegian dairies producing Dutch-type cheese, more knowledge on the bacteriophage situation in these dairies was required. The quantities of the three most common groups of lactococcal phages (P335, 936 and c2) were assessed in bulk starter and whey samples collected from eight large cheese factories. No c2 DNA was detected in any of the samples. Large amounts of 936 DNA, corresponding to 10^8-10^{10} pfu/mL, were detected in all bulk starter and whey samples. DNA of temperate P335-like bacteriophages was found in quantities corresponding to the expected levels of prophage DNA released by lysis of lysogenic starter culture bacteria. P335 DNA could not be detected after DNase treatment, showing that the DNA was not packaged in viral particles. Based on these findings, it was concluded that virulent 936-like bacteriophages are probably responsible for starter culture inhibition experienced in these dairies. In one of the productions analyzed, a failed (slow) fermentation was reported, but neither bulk starter nor whey sampled from this fermentation differed from other productions in levels of 936-DNA. DNA from 936-like phages were not detected in any of the four commercial starter cultures analyzed, showing that contaminating bacteriophages entered cheese production via the milk or came from the dairy environment.

Bacteriophage diversity in 6 Norwegian and 1 US dairy plants was assessed by DNA sequencing. The sequenced 2.4 to 3.1 kb region comprised genes corresponding to *l13*, *l14*, *l15* and part of *l16* in lactococcal phage bIL170. These genes are conserved among 936-like phages and encode structural proteins. Twenty six unique sequence types were found among the 136 sequenced bacteriophage isolates. No correlation between sequence type and geographical origin of the phages were seen, probably because the sequenced region was too conserved to show any real correlation. In about half of the 26 sequence types, one of four different large (557-671 bp) insertion sequences were identified between the genes corresponding to *l13* and *l14*. The gene *l13* putatively encodes the major structural protein (MSP). All phages carrying a large insertion had a +1 translational frameshift motif in the 5' terminus of the putative *msp* gene, leading to an extension of *msp* by an open reading frame spanning each of the insertions. The four different C-terminal extensions of MSP each carried a variable domain that in some protein settings have been shown to be involved in host recognition. Whether the variable domains functions in host recognition in this protein setting remains to be elucidated.

Since PCR primers used for phage group/species determination did not allow for discrimination between different phages within the 936 group, phage biodiversity was measured as the host range of the phages present in a sample by use of growth inhibition assays performed in microplates. By this

method, the inhibitory spectrum of each dairy sample was tested on 96 lactococcal isolates from each of three different cheese starter cultures (B, C and D), as well as 88 strains of *Lactococcus lactis* from our laboratory collection. The different starter culture arrays showed no overlap in phage sensitivity, except for a possible overlap between starters B and D. The arrays were used to study the day-to-day development of bacteriophage diversity in one cheese plant. Generally, great daily variation in phage diversity was observed. The sample material included samples taken when a fermentation failure (slow acidification) was experienced. The fermentation failure coincided with an increased host range of phages in the bulk starter, however, the number of inhibited isolates did not differ much from other fermentations when normal acidification rates were reported.

In response to the fermentation failure starter culture B, which had been in use in cheese production for about three months, was replaced by starter culture D for five days, before production resumed with starter culture B. This starter rotation had a dramatic effect on the bacteriophage flora. No phage could be detected by array B after starter cultures had been changed. On the other hand, phages infecting strains of culture D were readily detected from the first day it was used. Moreover, after returning to starter B, phages against this culture rapidly reappeared. These finding clearly demonstrates the potential of starter culture rotation, however, the effect of the rotation was short lived, emphasizing the ubiquity of phages in dairy environments and the importance of supplementary phage counter-measures.

In order to better understand how bacteriophages cause fermentation failure, and to differentiate between normal and failed fermentations, phage growth during cheese production was determined by comparison of phage titers in bulk starter and corresponding whey samples. No or only little phage growth indicates that the host bacterium has been incapacitated already in the bulk starter, and is thus unable to perform in the fermentation. *Lactococcus lactis* IL1403 is sensitive to a wide variety of bacteriophages and was thus used as an indicator in plaque quantifications. Phage titers varied greatly between production days. Notably, in the bulk starter samples collected on the day of the fermentation failure, elevated phage titers were found in the bulk starter, and no growth of phages had occurred during cheese production. Interestingly, the same result was found for productions with starter D, but normal starter activity was reported for these fermentations. It is possible that strains with overlapping phage sensitivity to IL1403 are important for starter B performance but are less important in starter culture D. In a further characterization of bacteriophage growth during the failed cheese production, bacteriophage growth was determined using a selection of starter B isolates as plaque assay indicators. When the fermentation failure was experienced, as many as 30 out of 32 isolates were inhibited.

Although other, normal, fermentations with starter culture B also showed high levels of inhibition, these results show that bacteriophage growth analyses can reflect fermentation failure.

One of the main aims of this study was to develop a tool that could be used to predict fermentation failure and the results showed that fermentation failure coincided with a high number of inhibited starter culture isolates. There was however a very fine line between successful and failed fermentations. In order to include starter culture strains important for fast acidification, an array comprised of isolates from a successful fermentation was set up. The rationale behind this selection of isolates was that isolates predominating immediately after a successful fermentation were fast growing and major contributors to acidification. By growth inhibition assays with this array of isolates it was possible to show a clear difference between successful and failed fermentations, and the results indicated that growth inhibition assays including key starter culture strains might be used to predict fermentation failure.

The origin of lactococcal bacteriophages contaminating a cheese production process was studied by PCR and growth inhibition assays. An entire milk delivery chain consisting of 112 farm tanks, 7 tanker trucks and one dairy silo tank was studied in addition to bulk starter preparation and cheese production using the same milk. Bacteriophage levels in the milk samples were too low for PCR detection but 936-like phages were readily detected after an enrichment step with starter culture B, the starter culture in use at the dairy in the sampling period. Interestingly, phage enrichment using starter culture C did not result in detectable levels of virulent phage DNA, suggesting that phage contamination in the milk delivery chain might come from the dairy itself. Forty-one array B isolates were inhibited by the dairy silo tank milk after phage enrichment with starter culture B, however only six isolates were inhibited by whey from cheese production using the same milk and starter culture B. Of these six isolates only two were among the 41 isolates inhibited by silo tank phages and four came from elsewhere. Thus, phages against 39 isolates did not multiply to detectable levels during cheese production, even in the presence of suitable hosts. These results indicate that phage titers in raw milk are too low to have an impact on the milk fermentation. Moreover, considering the thorough sterilization scheme for bulk starter milk, it is unlikely that phage contamination in raw milk is the source of high bacteriophage levels found in the bulk starter samples. This indicates that bacteriophages contaminating the bulk starter vat most probably came from in-house sources.

The results of this study have shown the robustness of multi-strain starter systems, demonstrated the importance of using phage unrelated starter cultures in rotation schemes and led to a deeper understanding of the thin line separating successful fermentations from failed. Growth inhibition assays involving key starter culture strains as indicator bacteria have been identified as a possible tool for

prediction of fermentation failure. Moreover, the finding that phage contamination came from within the dairy plant itself emphasize the importance of bulk starter hygiene, strictly aseptic inoculation, and physical separation from fermentation vats and other production facilities that are bound to be teeming with bacteriophages.

3.2 Characterization of a *Leuconostoc* bacteriophage infecting flavor producers of cheese starter cultures.

Bacteria of the genus *Leuconostoc* are important flavor producers in fermented milk products such as buttermilk, cultured butter and various cheeses, including Dutch-type. Compared to lactococcal phages, relatively little has been known about phages infecting dairy leuconostocs. The aim of this study was to characterize a bacteriophage inhibiting cheese starter culture leuconostocs, and to sequence and analyze its genome.

A microplate array containing isolates tentatively identified as *Leuconostoc* spp. from commercial starter cultures commonly used in the production of Dutch-type cheese was set up. Forty-eight isolates from each of two starter cultures were included in the array. A brine sample from a Norwegian cheese plant contained phages inhibiting the growth of all 48 isolates of one starter culture, but it did not inhibit isolates of the other starter culture. One bacteriophage, ϕLmd1, was isolated from the growth inhibition assay, and its host, isolate A1, was determined to be a *Leuconostoc mesenteroides* subsp. *dextranicum* based on its 16s rRNA gene sequence, sugar fermentation pattern (API50 CHL) and its ability to grow in the presence of high salt concentrations.

Bacteriophage Lmd1 belongs to the *Siphoviridae* family, as do most dairy bacteriophages and, to our knowledge, all described phages infecting *Leuconostoc*. The lytic cycle of ϕLmd1 was completed within 30 minutes, with the release of approximately 50 progeny phages. Thermal inactivation studies revealed that ϕLmd1 is unaffected by pasteurization, but its titer was reduced by more than 7 log when exposed to a thermal inactivation scheme resembling commonly employed bulk starter vat sterilization schemes. Since pasteurization does not affect ϕLmd1, there is no barrier for the bacteriophage to enter cheese fermentation vats through contaminated milk, thus, infection of bulk starter vats would require contamination during or after cooling of the bulk starter milk.

Phage ϕLmd1 is able to inhibit the growth of all *Leuconostoc* isolates of one starter culture. Moreover, it is able to propagate on 3 out of 4 commercial DL starter cultures tested. This identifies ϕLmd1 as a potential threat to flavor producing leuconostocs in dairy fermentation, and emphasize the

importance of selecting phage unrelated strains with overlapping metabolic activities for starter cultures. In this, bacteriophage Lmd1 could be a valuable selection tool.

The linear 26,201 bp genome ϕLmd1 was found to have cohesive ends. It has a 36.4 % G+C content, very close to the G+C content of *Ln. mesenteroides* subsp. *mesenteroides* ATCC 8293. Forty open reading frames (ORFs) were predicted, covering 91.7 % of the genomic sequence. Half of the ORFs are located on one strand and the other half on the complementary strand. Twenty four of the 40 ORFs were assigned putative functions based on homology to previously characterized proteins. Similar to most characterized bacteriophage genomes, the genome of ϕLmd1 is organized in functional modules. Four distinct modules were identified: the DNA replication module, DNA packaging module and the head and tail morphogenesis modules, all located on the positive strand. The putative DNA replication module is found downstream of the predicted origin of replication, and comprises six predicted genes encoding proteins putatively involved in DNA replication. One predicted protein is most probably a type B DNA polymerase (ORF4). Type B polymerases are only rarely found in phages of the *Siphoviridae* family. Three ORFs found upstream of the origin of replication and encoded on the negative strand were assigned the putative functions of HNH homing endonucleases and one endodeoxyribonuclease of the RusA family. Phage encoded RusA-like endonucleases are thought to be involved in triggering replication restart in the case of replication fork stalling (Weigel and Seitz 2006). Based on their putative DNA binding domains and the similarity of ORF40 to RusA, it is possible that these three ORFs are also involved in DNA replication. No significant similarity in sequence or gene order was found to any of the well characterized phage replication modules described by Weigel and Seitz (2006). It would be very interesting to experimentally elucidate the mechanism of replication of ϕLmd1. The DNA packaging module have ORFs encoding putative terminase small and large subunits, which in other phages have been found to function as DNA packaging machines during phage assembly. Eight proteins were identified as structural proteins of the ϕLmd1 virion based on SDS-PAGE and mass spectrometric analysis. These proteins are encoded by ORFs in the tail morphogenesis module, and include a putative portal protein, major tail protein, tape measure protein, baseplate protein and receptor binding protein. Three predicted proteins with no assigned function were identified as structural proteins. Most of the ORFs identified on the negative strand produced no significant hits to database entries, however, the putative lysin and holin genes were found interspersed in this region.

The modular organization of ϕLmd1 is very similar to that of *Ln. mesenteroides* subsp. *mesenteroides* phage 1-A4, which was isolated from industrial vegetable fermentation. The two phages showed homology in about half of the predicted proteins. Most of the homologous genes are located on the positive strand, and the predicted gene order in this region is nearly completely conserved between

the two phages. In their characterization of ϕ1-A4, Lu and co-workers did a functional distribution analysis including 112 phage genomes (Lu, Altermann et al. 2010). They showed that ϕ1-A4 cluster most closely with lactococcal phages including Q54-like, c2-like and 936-like phages, but they suggested that ϕ1-A4 should form a separate functional cluster based on the relatively large distance between it and its closest relatives. This is in agreement with the relatively low number of significant BLAST hits found to phage sequences other than ϕ1-A4. Even though ϕLmd1 and ϕ1-A4 are highly similar, sharing both homologous gene products and conserved gene order, corresponding genes are not sufficiently conserved at the DNA level to allow design of PCR primers specific for both phages without extensive use of degenerate nucleotide positions. This suggests that, despite extensive similarities, the two phages are relatively distant relatives.

The characterization of bacteriophage Lmd1, infecting *Ln. mesenteroides* subsp. *dextranicum*, represents the first analysis of a dairy *Leuconostoc* phage genome. This study contributes to a better understanding of *Leuconostoc* phages and might help development of detection methods and better phage control strategies in the dairy industry.

3.3 A novel bacteriophage of the *Podoviridae* family infecting *Weissella cibaria* isolated from kimchi.

In this study a novel bacteriophage, ϕYS61, isolated from kimchi fermentation was characterized. This work represents the first analysis of a complete genome sequence from a bacteriophage infecting the genus *Weissella*. *W. cibaria* (*W. kimchii*) is one of many LAB species involved in the production of kimchi, a traditional Korean fermented vegetable dish. Bacteriophages are recognized as a serious problem in industrial food fermentations however, ϕYS61 differs from many virulent phages associated with food fermentations since it is difficult to propagate and also very susceptible to resistance development.

By electron microscopy, ϕYS61 was identified as belonging to the *Podoviridae* family, with a moderately elongated capsid (85 by 36 nm) and a short non-contractile tail. This identifies ϕYS61 as having a C2 morphotype. The morphology of ϕYS61 is similar to *W. cibaria* phage ϕ22 isolated from Nham, a Thai fermented pork sausage.

The linear double-stranded genome of ϕYS61 is 33,594 bp long with a G+C base composition of 43.9 %. The 48 predicted ORFs comprise 86 % of the genome. Also present are 25 bp inverted terminal repeat sequences, which are characteristic for the *Picovirinae* subfamily within family *Podoviridae*. This subfamily, which includes the phi29-like phages, consists of phages with terminal proteins (TP) covalently linked their DNA (TP-DNA), small genomes, and DNA replication by a protein-primed

mechanism involving a polymerase B with additional protein domains interacting with TP during replication initiation. The TP-DNA complex of ϕYS61 could not be isolated by the methods employed in this study, however, several observations led to the conclusion that ϕYS61 also replicates by a protein-primed mechanism: proteinase K treatment is essential for DNA purification and characteristic terminal inverted repeats are present. Moreover, the conserved phi29-like DNA polymerase B features, and the conserved functional amino acids and predicted secondary structures in TP are also present. Other predicted proteins of ϕYS61 resemble proteins of phages belonging to the subfamily *Picovirinae*, including the putative DNA encapsidation protein and several structural proteins. The *Weissella* phage ϕYS61 is, however, markedly different from the *Picovirinae* with respect to genome size and morphology. *Picovirinae* are characterized by small (approximately 20 kb) genomes which are contrasted by the 33.6 kb genome of ϕYS61. The capsid dimensions of ϕYS61 are also significantly larger than those of phages belonging to the *Picovirinae*. The novelty of ϕYS61 is further emphasized by the low number of ORFs showing significant similarity to database sequences. The results of this study suggest that the bacteriophage ϕYS61 should represent a new subfamily within the family *Podoviridae*.

4. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this study, lactococcal bacteriophages affecting milk fermentation in the production of Dutch-type cheese was investigated. Lytic phages of the 936 group of phages were found both diverse and numerous in Norwegian dairies. The quantity and diversity of lactococcal phages was studied over time and correlation was found between the number of starter culture isolates inhibited and fermentation failure. Moreover, phage diversity was followed through the milk delivery chain, but the source of phage contamination in the bulk starter tank was found to be the dairy environment itself. In this study phage detection and quantification was mainly performed using microbiological methods. Phage assays based on inhibition of bacterial isolates have the quality of direct detection of phages, however, the value of such methods is limited by the number of relevant starter culture indicator strains included in the assay. When studying the diversity of phages infecting strains of a starter culture it is important that most or preferably all phage unrelated strains of a particular starter are included. This could be ensured by strain or phage-type specific identification methods like ribotyping, plasmid profiling and phagetyping.

When studying correlation between phage diversity and starter culture performance, an effort should be made to include indicator strains important for the performance parameter, e.g. acid- or diacetyl production. In this study a set of indicator isolates which might be used to predict low starter culture activity was identified. By including indicators from other productions, isolated by the same criteria it is conceivable to get an array of indicators capable of reliable prediction of slow fermentations by growth inhibition assays.

Detection of phage DNA by the use of phage specific primers or probes does not rely on selection of relevant indicator strains for phage detection. However, these methods are limited by the amount of phage DNA sequence data available for primer or probe design. Ideally, one should know the genome sequence of all phages of an environment, and preferably the correlation between genome sequence and host specificity prior to performing the study.

In recent years, a possible solution to the challenges with microbiological and PCR-based methods for phage studies has become apparent. The increasing availability of high throughput sequencing technologies has opened up for new possibilities in phage research. High throughput sequencing has obvious implications for phage genomics, but could also be successfully applied in the study of bacterial and viral community dynamics in food fermentations. Metagenomic (total DNA) sequence analysis can be employed to study bacterial and phage population dynamics at various time points during fermentation, as was done in a study of kimchi fermentation by Jung and colleagues (2011). By a metatranscriptomic (total RNA) sequencing approach coupled with *in silico* prediction of gene function it would be possible to study how expression of genes related to different metabolic activities varies

during fermentation. If this information is coupled with data on phage population dynamics, a comprehensive insight into how phages affect food fermentations could be gained.

Two new bacteriophages, *Leuconostoc mesenteroides* phage Lmd1 and ϕYS61 infecting *Weissella cibaria* were characterized. The predicted proteins of these two phages were assigned putative functions based on sequence homology to database sequences. As evolutionary distance increase between putative homologs, there is also an increasing uncertainty in the functional prediction of one sequence based on sequence similarity to the other. Thus, for phages representing separate functional or phylogenetic clusters, determination of gene function based on experimental data is of great importance. Bacteriophage Lmd1 forms, together with *Ln. mesenteroides* phage ϕ1-A4, a group of phages with little sequence homology to other known bacteriophages. Phage YS61 probably represents a new phage subfamily within the *Podoviridae* family. It would be of great interest to experimentally determine gene functions of these two phages, as a reference for prediction of gene function in other phage genomes, but also to verify the putative functions assigned in this study.

One of the high throughput sequencing technologies currently available at reasonable cost, the Illumina technology (Bentley, Balasubramanian et al. 2008; Quail, Kozarewa et al. 2008; Kozarewa, Ning et al. 2009), can provide as much as 600 gigabases of sequencing data per run. For comparison, 50 gigabases is in theory enough sequence data to get 1,000 fold sequence coverage of 1,000 phage genomes of 50 kb each. It should thus be possible to get the complete genome sequences of most, if not all different bacteriophages from an environmental sample or a fermentation vat. The vast number of phage genome sequences likely to become available in the near future will have tremendous impact on our understanding of phage biology and evolution, for the less studied phages of food fermentations as well as the well characterized lactococcal phages. If properly combined with experimental data on gene function and protein interactions, this will likely increase, if not revolutionize, our understanding of phage biology, and likely also deepen our knowledge on more complex biological systems.

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Bacteriophages in milk fermentations: Diversity fluctuations of normal and failed fermentations

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ABSTRACT

In this study on lactococcal phages in Norwegian dairies producing Dutch-type cheese, virulent 936-like phages were identified as the predominant phage group. Twenty six unique phage sequence types were identified. Interestingly, open reading frames encoding conserved variable domains were found overlapping with the putative major structural protein gene. A growth inhibition assay employing arrays of different indicator isolates was used to study phage biodiversity, and large day-to-day variation was found within a dairy plant. A peak of phage diversity coincided with a fermentation failure. The effect of a starter culture rotation was reflected by a radical change in phage diversity, but phages infecting the starter culture rapidly re-emerged in the plant. Phage levels in raw milk were low and the phage contamination came from within the plant. The assay used for bacteriophage analyses may have a potential for predicting a fermentation failure.

1. Introduction

Bacteriophages impairing dairy fermentations negatively affect quality, safety and value of the fermented product. In the production of cheese, *Lactococcus lactis (L. lactis)* is a major contributor in the milk fermentation and lactococcal bacteriophages have long been known to cause delay or disruption of fermentation processes. Three bacteriophage groups (936, c2 and P335) infecting *L. lactis* have been identified as the main bacteriophages occurring in dairy fermentations (Deveau, Labrie, Chopin, & Moineau, 2006; Moineau, Fortier, Ackermann, & Pandian, 1992; Prevots, Mata, & Ritzenthaler, 1990). Lactococcal phages occur naturally in raw milk and constitute a continuous supply of bacteriophages into dairy plants (Jarvis, 1987; Madera, Monjardin, & Suarez, 2004; McIntyre, Heap, Davey, & Limsowtin, 1991). Moreover, prophage induction from the many lysogenic starter culture strains has the potential to influence fermentations (Davidson, Powell, & Hillier, 1990; Huggins & Sandine, 1977; Reyrolle, Chopin, Letellier, & Novel, 1982).

In recent years, focus on temperate bacteriophages has shifted towards the role they serve as a genetic pool in the evolution of new virulent phages, emerging within dairies (Bouchard & Moineau, 2000; Bruttin & Brussow, 1996; Durmaz & Klaenhammer, 2000; Josephsen et al., 1994; Labrie & Moineau, 2007). PCR methods have been developed for easy detection and taxonomic determination of dairy phages (Dupont, Vogensen, & Josephsen, 2005; Labrie & Moineau, 2000; del Rio et al., 2007), and several studies have been published on the prevalence of lactococcal phages in milk and dairy environments from countries around the world (Bissonnette, Labrie, Deveau, Lamoureux, & Moineau, 2000; Josephsen et al., 1994; Madera et al., 2004; Miklic & Rogelj, 2003; Moineau et al., 1992, 1996; Neve, Berger, & Heller, 1995; Neve, Kemper, Geis, & Heller, 1994; Raiski & Belyasova, 2009; Szczepanska, Hejnowicz, Kolakowski, & Bardowski, 2007).

To reduce bacteriophage impact on dairy fermentations, phage resistant bacteria are preferred in starter cultures. Molecular and microbiological aspects as well as the applications of bacteriophage resistance have been reviewed (Allison & Klaenhammer, 1998; Coffey & Ross, 2002; Forde & Fitzgerald, 1999; Moineau, 1999), most recently by Labrie, Samson, and Moineau (2010). Other counter-measures include stringent hygiene, compartmentalization of dairy plant production units, use of multi-strain starters, and rotation of starter cultures. Rotation of phage-unrelated starter cultures is done in response to a fermentation failure or as a precaution on a regular basis. Direct inoculation of starter culture in the fermentation vat (Direct Vat Inoculation, DVI), eliminating the time available for phages to multiply in the bulk starter, is employed by many dairies. Still, many use bulk starters for sensory reasons and for the higher initial fermentation speed. A recent

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review on the control of bacteriophages in industrial fermentations was written by Moineau and Levesque (2005).

Dutch-type cheeses are typically made using mesophilic LD mixed multi-strain starter cultures. Most such cultures are derived from old starter cultures (Lodics & Steenson, 1990), and long term natural selection of starter strains surviving phage attacks gave these starters robustness against bacteriophage inhibition. Modern cheese production requires predictable performance of the starter culture. To assure this, a few well performing starter cultures are perpetually used, a practice that possibly contributes to bacteriophages becoming increasingly problematic, since new bacteriophage variants are allowed to develop on permanent hosts (Allison & Klaenhammer, 1998; Boucher & Moineau, 2001).

Neve et al. (1995) described large day-to-day fluctuations in bacteriophage quantity and diversity in the production of fresh cheese. Bacteriophage titres as high as 10^{10} plaque forming units (pfu) per mL were found in bulk starter samples. Successful fermentations were achieved due to the presence of phage insensitive strains in the mixed multi-strain starter cultures used. However, fermentation failure can occur even when mixed starters are used.

Assessing bacteriophage influence on mixed starter fermentations is difficult due to the high number of strains (many with overlapping traits) and their inter-dependencies. Plaque assays and bacterial activity assays are commonly used (Moineau & Levesque, 2005) and new technologies, like flow cytometry, are continuously being developed (Michelsen, Cuesta-Dominguez, Albrechtsen, & Jensen, 2007). These and other bacteriophage analyses that can characterize differences between failed and successful fermentations will be valuable for the continued improvement of phage countermeasures.

In this work the abundance of lactococcal phages in eight Norwegian dairies producing Dutch-type cheese was assessed, and day-to-day variation in bacteriophage diversity was studied in dairy samples collected from one cheese plant. Moreover, we characterized a fermentation failure and a starter culture rotation with respect to quantity and diversity of lactococcal bacteriophages. The origin of bacteriophage contamination was studied by comparing bacteriophage biodiversity in a milk delivery chain and a dairy plant.

2. Materials and methods

2.1. Sample collection and treatment

Dairy samples were collected in sterile containers (Bulkotest; Landteknikk AS, Oslo, Norway) by dairy plant staff, and shipped frozen to our laboratory. Samples were stored in aliquots at −20 °C. Before analysis, bacteria and cell debris were removed from whey and bulk starter samples by centrifugation at 5000 × *g* for 15 min at 4 °C (in an A-4-44 rotor; Eppendorf AG, Hamburg, Germany) and filtration (Filtropur S, 0.45 μm; Sarstedt AG, Nümbrecht, Germany). Milk samples were brought to pH 4.6 with 6 M HCl to coagulate before being processed the same way as whey and bulk starter samples. This was done to avoid disturbance of optical density (OD) measurements performed in the growth inhibition assays (Section 2.3). Brine samples were dialyzed against phage storage solution, 75 mM $MgCl_2$ (Lillehaug, Lindqvist, & Birkeland, 1991) and filtered (Filtropur S, 0.45 μm) before being used in biological assays.

2.2. Growth of bacteria and phages

All bacterial strains were grown at 30 °C in M17 (Terzaghi & Sandine, 1975) (Oxoid, Basingstoke, UK) supplemented with 0.4% (w/v) glucose (GM17). When growing or assaying for bacteriophages, GM17 was also supplemented with 5 mM calcium chloride (GM17–C). Plaque assays were performed essentially as described by Lillehaug (1997). For bacteriophage quantification, plaques were counted after overnight incubation of 5 μL phage suspension samples spotted on soft-agar overlays inoculated with $\approx 3 \times 10^7$ colony forming units mL^{-1} log-phase cells. Bacterial strains were stored at −80 °C in GM17 containing 15% (v/v) glycerol. Filtered phage lysates and bacteriophages isolated from plaques (kept in 75 mM $MgCl_2$) were stored at 4 °C for up to 12 months or at −20 °C with 20% (v/v) glycerol added. Bacteriophage enrichment in milk samples was done by the addition of 5 mL GM17-C to 3 mL untreated milk samples before inoculation with 5 μL commercial starter culture. After 16–24 h incubation at 30 °C samples were treated as described above for bulk starter and whey samples.

2.3. Growth inhibition assays

Commercial starter cultures and bulk starters were diluted in sterile water and plated on GM17 agar plates. After growth for two days at 30 °C separate colonies were picked and transferred to GM17 for further growth. Aliquots (150 μL) of the isolates were dispensed to microtitre plate wells, added 60% (v/v) glycerol to a final concentration of 15% (v/v) and stored at −80 °C. Each plate (array) contained isolates from the same starter culture. Isolates from the frozen arrays were inoculated in the respective wells of new plates containing GM17, incubated at 30 °C over night and subsequently used for inoculation of growth inhibition assays. Growth inhibition assays were performed in 96 well microtitre plates in volumes of 200 μL (180 μL GM17-C and 20 μL phage sample pretreated as described above) per well. Using a microtitre plate replicator, each well was inoculated with its respective indicator bacterium. Control plates were prepared in an identical manner except that phage containing material was omitted. The plates were incubated at 30 °C over night and the optical density at 620 nm measured in each well using a microtitre plate reader (Multiscan Ascent; Labsystems Oy, Helsinki, Finland). Before OD was read, plates were shaken for 15–30 s at 960 rpm to resuspend deposited cells and cell debris. Growth inhibition was recorded for wells with less than 50% of the OD of its corresponding control.

2.4. Polymerase chain reaction

PCR was performed as described by Labrie and Moineau (2000) including the updated c2c primer described by Deveau et al. (2006) and with reagents supplied with the Dynazyme II polymerase kit (Finnzymes Oy, Espoo, Finland). Dairy samples treated as above or phage lysates were incubated for 10 min at 94 °C before being used directly as PCR templates. For PCR detection of phages in commercial starter cultures, cells were suspended in water, boiled for 10 min, centrifuged (10,000 × *g*, 2 min) and the supernatant used as template. Template DNA for quantitative PCR was purified from bulk starter and whey samples using the QIAamp DNA Blood mini kit (QIAGEN GmbH, Hilden, Germany) in accordance with manufacturer's instructions. Real-time PCR was carried out using FastStart SYBR green master (ROX) chemistry (Roche Diagnostics GmbH, Mannheim, Germany) and species specific primers (Deveau et al., 2006; Labrie & Moineau, 2000). Amplification processes were monitored on an ABI PRISM 7700 Sequence detector (Applied Biosystems, Foster City, CA, USA). DNA purified as above from standard dilutions of bacteriophage quantified by plaque assay was used to correlate CT values to plaque forming units per mL (pfu mL^{-1}).

2.5. DNA sequencing

Isolated bacteriophages were sequenced over a 2.4–3.1 kb genomic region encoding structural proteins, as defined by primers 936-F1.1 (5′-TGAAGCTCTAATCGTAGCTG) and 936-R1 (5′-TTTAG-CACTATCTCCTAAACC). Additional sequencing primers used were 936-F5 (5′- AGGGTATGTAATGGCAAGTA), 936-R2 (5′-GATAAGCC-CAACTGCGTTTG), 936-R4 (5′-TCAGTAATTTCTCCGTCTTC), 936-R5 (5′- CCCAAATTTCGTACTCAATAG), 936-R7 (5′-CCTCCGTTACAATCTA CCCA) and 936-R8 (5′-CTTGCCATTACATACCCTTTCT). The sequenced region corresponded to position 8880–11304 in the genome of lactococcal phage bIL170 (accession no. AF009630). Primer 936-F1.1 bound near the 3′ end of the major structural protein gene (*l13*) and 936-R1 in the tail tape measure protein gene (*l16*) of bIL170. Sanger sequencing was carried out on an ABI PRISM 3700 Sequence detector with Big Dye v3.1 chemistry (Applied Biosystems, Foster City, CA, USA). Sequence- and bioinformatic analyzes were performed using CLC Main Workbench version 5.6 (CLC bio, Aarhus, Denmark). Homology searches were done using BLASTP 2.2.23+ (Altschul et al., 1997, 2005) at www.ncbi.nlm.nih.gov (June 2010).

3. Results and discussion

3.1. Species determination and quantification of bacteriophages in Norwegian dairies

Bulk starter and whey were sampled on two production days in eight of the largest cheese plants in Norway producing Dutch-type cheese. Altogether, three different commercial starter cultures had been used in the sampled production lots. The samples were analyzed by PCR for the presence of lactococcal phages of the P335, 936 and c2 groups. While 936 and P335 DNA were detected in all, no c2 DNA was found in any samples. The higher susceptibility of these phages to pasteurization (Madera et al., 2004) might contribute to their absence. Moreover, the prolate-headed c2-like phages have been shown to require the host receptor phage infection protein (PIP) for infection in *L. lactis* (Geller, Ivey, Trempy, & Hettinger-Smith, 1993), and strains with mutations in the *pip* gene have been shown to gain complete resistance to prolate-headed phages (Babu, Spence, Monteville, & Geller, 1995). Since pip^- resistant strains can be obtained (Garbutt, Kraus, & Geller, 1997), we assume that the starter culture manufacturers might have selected for this property, making it impossible to propagate c2-like phages in fermentations using these starters. To our knowledge there are no recent reports on c2-like phages being found in the production of Dutch-type cheese. However, phages of the c2 group have been shown to be abundant in dairy environments, most recently by Miklic and Rogelj (2003), Raiski and Belyasova (2009) and Szczepanska et al. (2007). We confirmed the presence of phage belonging to the c2 group in a Spanish Manchego cheese, and an isolated c2-like phage was subsequently used as positive control in species determination analyses in this study.

Bulk starters and wheys from all the cheese plants contained large amounts of 936 phages. Real-time PCR quantification revealed the presence of 936 DNA corresponding to 10^9–10^{10} pfu mL^{-1} in 30 out of 32 samples, the other two contained about 1 log less (data not shown). No correlation was found between phage level and which commercial starter culture had been used.

All dairy samples also contained large amounts of P335 DNA, corresponding to about 10^8 pfu mL^{-1}. Since starter culture strains can be lysogenic for P335 phages, it is likely that P335 DNA is released upon infection by other virulent phages. To test this, the samples were treated with DNase. After this treatment, no P335 DNA was detectable by PCR. The treatment did not affect the quantitative measurements of 936 DNA, showing that unlike 936 the P335 DNA was not packed in viral particles. We thus conclude that phages belonging to the 936 group are the most abundant in Norwegian cheese plants, and that they are present at high levels already at the bulk starter stage.

In one production a failed (slow) fermentation was reported, but neither bulk starter nor whey sampled from this fermentation differed from other productions in levels of 936 DNA.

Unsurprisingly, P335 DNA was detected in all of four different commercial starter cultures. It has been suggested that prophage induction could inhibit milk fermentations (Reyrolle et al., 1982). We could, however, find no evidence for induction of temperate P335 phages in any of the analyzed bulk starter or whey samples produced with these starter cultures. DNA from the c2 and 936 groups of virulent phages were not detected in the commercial starter cultures.

3.2. Bacteriophage diversity assessed by DNA sequencing

To study bacteriophage diversity, a total of 136 bacteriophage isolates were sequenced over a 2.4–3.1 kb genomic region encoding structural proteins (Fig. 1). Brine samples from four of the

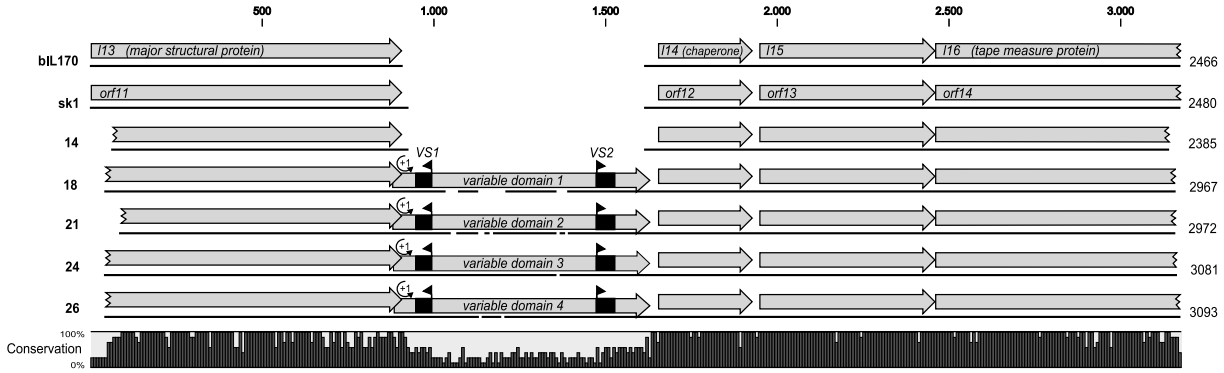

Fig. 1. Alignment of 5 of the 26 unique sequence types (Table 1) and the corresponding genomic regions of lactococcal phages bIL170 and sk1. Phage name or the sequence type number representing each insertion variant is indicated in bold. DNA sequences are indicated by black lines and open reading frames (ORFs) by grey arrows. In bIL170 ORFs *l13* and *l16* putatively encode the major structural protein and tail tape measure protein, respectively, and the gene product of *l14* is likely a tape measure protein chaperone. A protein of unknown function is encoded by *l15*. The high degree of conservation among the sequences is indicated by bars below the alignment. Phages of sequence type 14 contain the sk1-like 17 bp insertion and sequence types 18, 21, 24 and 26 represent phages containing the large insert variants 1–4, respectively. (+1) indicates programmed translational frameshift motifs. Regions encoding variable domain signatures VS1 (triangle pointing left) and VS2 (triangle pointing right) are shown in black. Sequence lengths in base pairs are indicated by the numbered bar above the alignment and at the end of each sequence.

Table 1
Overview of the sequenced bacteriophage isolates.

Sequence type	Insert variant	Number of isolates	Host bacteria used for phage isolation[a]	Source (dairy plant)[b]
1	–	20	Lactococcus lactis ssp. lactis IL1403	D, G, H, J
2	–	15	B-A1, B-A2, B-A11, B-E4, B-F5, B-F10, B-G1, B-H2	D, F
3	–	8	B-A1, B-A11, B-E4, B-F5, B-F10, B-G1, B-H2	B
4	–	6	B-A1, B-A11, B-F5, B-G1	J
5	–	10	B-C5, B-E8	B, D
6	–	3	B-F10, B-G1	J
7	–	1	Lactococcus lactis ssp. lactis IL1403	B
8	–	1	Lactococcus lactis ssp. lactis IL1403	G
9	–	1	B-A2	H
10	–	1	Lactococcus lactis ssp. lactis IL1403	A
11	–	1	Lactococcus lactis ssp. lactis bv. diacetylacis ATCC 15346	A
12	–	1	Lactococcus lactis ssp. lactis bv. diacetylacis ATCC 15346	A
13	Short	1	Lactococcus lactis ssp. lactis IL1403	H
14	Short	1	Lactococcus lactis ssp. lactis IL1403	F
15	Insert variant 1	27	B-A6, B-C9, B-D4, B-D5, B-D7, B-D8, B-G5, B-G9, B-H3, B-H4, L. lactis IL1403	D, F, H
16	Insert variant 1	8	B-A6, B-D5, B-D7, B-D8, B-G9, B-H3	B
17	Insert variant 1	3	Lactococcus lactis ssp. lactis IL1403	G
18	Insert variant 1	3	B-C9, B-D4, B-H4	B
19	Insert variant 1	1	Lactococcus lactis ssp. lactis IL1403	F
20	Insert variant 1	1	Lactococcus lactis ssp. lactis IL1403	H
21	Insert variant 2	7	Lactococcus lactis ssp. lactis IL1403	G
22	Insert variant 2	6	Lactococcus lactis ssp. lactis IL1403	G
23	Insert variant 2	1	Lactococcus lactis ssp. lactis IL1403	D
24	Insert variant 3	1	Lactococcus lactis ssp. lactis IL1403	D
25	Insert variant 4	5	B-C11, B-B1, B-B3	D, F
26	Insert variant 4	3	B-A7, B-D11, B-G8	D, F

[a] Bacterial hosts on which bacteriophages were isolated. Bacteria from array A are given in full name and starter culture isolates by array name (e.g., B) followed by microplate position (e.g., A1).
[b] Dairy plants A, B, D, F, G and H were located in Norway and J located in USA.

largest Norwegian cheese plants were tested with growth inhibition arrays. Brine was sampled since it serves as a reservoir of phages from multiple cheese productions. Seventy seven bacteriophages were isolated by plaque assay from positive wells and subsequently sequenced. Also sequenced were 48 phages isolated from other Norwegian dairies and 11 phages isolated from a US cheese plant producing Dutch-type (Table 1). All isolated phages were determined by PCR to belong to the 936 group, confirming that 936 phages are dominating in the cheese plants.

Twenty six unique sequences were found among the 136 phage isolates. Of these, two were uniquely found in the US cheese plant and 23 only in Norwegian plants. One sequence type was found in three geographically separated Norwegian dairy plants as well as the US plant. In five cases identical sequence types were found in different Norwegian cheese plants (Table 1). No correlation between sequence similarity and the geographical origin of the phages was seen. This could indicate that there is a common source of contamination between the geographically separated cheese plants, but most likely that the sequenced region is too conserved among phages of the 936 group to show any real correlation.

Among the 26 different sequence types, a high degree of sequence identity was seen. Differences were mainly silent or semi-silent point mutations, but about half carried an insertion. Insertion sequences were found between the genes corresponding to *l13* and *l14* in *L. lactis* phage bIL170 (Fig. 1), indicating a hot-spot for insertions. Gene *l13* encodes the major structural protein (MSP) that has been shown to be a major constituent of the capsid (head) structure in bIL170 (Crutz-Le Coq, Cantele, Lanzavecchia, & Marco, 2006) but has also been proposed to be a major tail protein based on its genomic localization (Desiere, Lucchini, Canchaya, Ventura, & Brüssow, 2002). The gene product of *l14* is most likely a chaperone functioning in the tail assembly process (Siponen et al., 2009). Two sequence types had a short (17 bp) insertion with 88% identity to the 17 bp insertion found in lactococcal phage sk1, and 12 were found to carry one of four different large (557–671 bp) insertions (Table 1). All phages carrying a large insertion had a +1

Table 2
Characteristics of gene products predicted on insertion sequences.

Insert sequence variant	ORF size (aa)[a]	Molecular mass (kDa)[b]	Variable domain			
			VS1 sequence[c]	V-domain size (aa)	VS2 sequence[c]	V-domain's closest homolog (Accession no; amino acid identity; aligned region)[d]
1	192	20.96	SADGKDRFMTVYPNLNLL	109	KAEFGPVATPYMPSASEVT	NS
2	208	22.81	SADGKDRFMTVYPNLNLI	124	KWEHGSTATLYMPSLSEVT	Lactococcus phage Tuc2009, minor structural protein 6 (AAK19880; 77/124 [62%]; 441–563)
3	228	25.53	SANGRDRFTTIYPNLNLG	144	KVEEGSTATPWIPSFSEAT	Lactococcus phage 4268, putative tail-host specificity protein (NP_839936; 102/145 [70%]; 857–1001)
4	230	26.16	SKDGTDRFMTVYPNLNIL	147	KLEKGSTATPWMPSFSELKT	NS

[a] Predicted open reading frame (ORF) size in amino acids. ORF start point was set at proline residue encoded at frameshift site.
[b] Molecular mass of the putative gene product of insert sequence ORFs.
[c] Variable domain signature sequences, see Fig. 1 and text.
[d] Closest homolog found using BLASTP. Amino acid identity and region of hit sequences producing alignment are shown. NS: no significant similarity found.

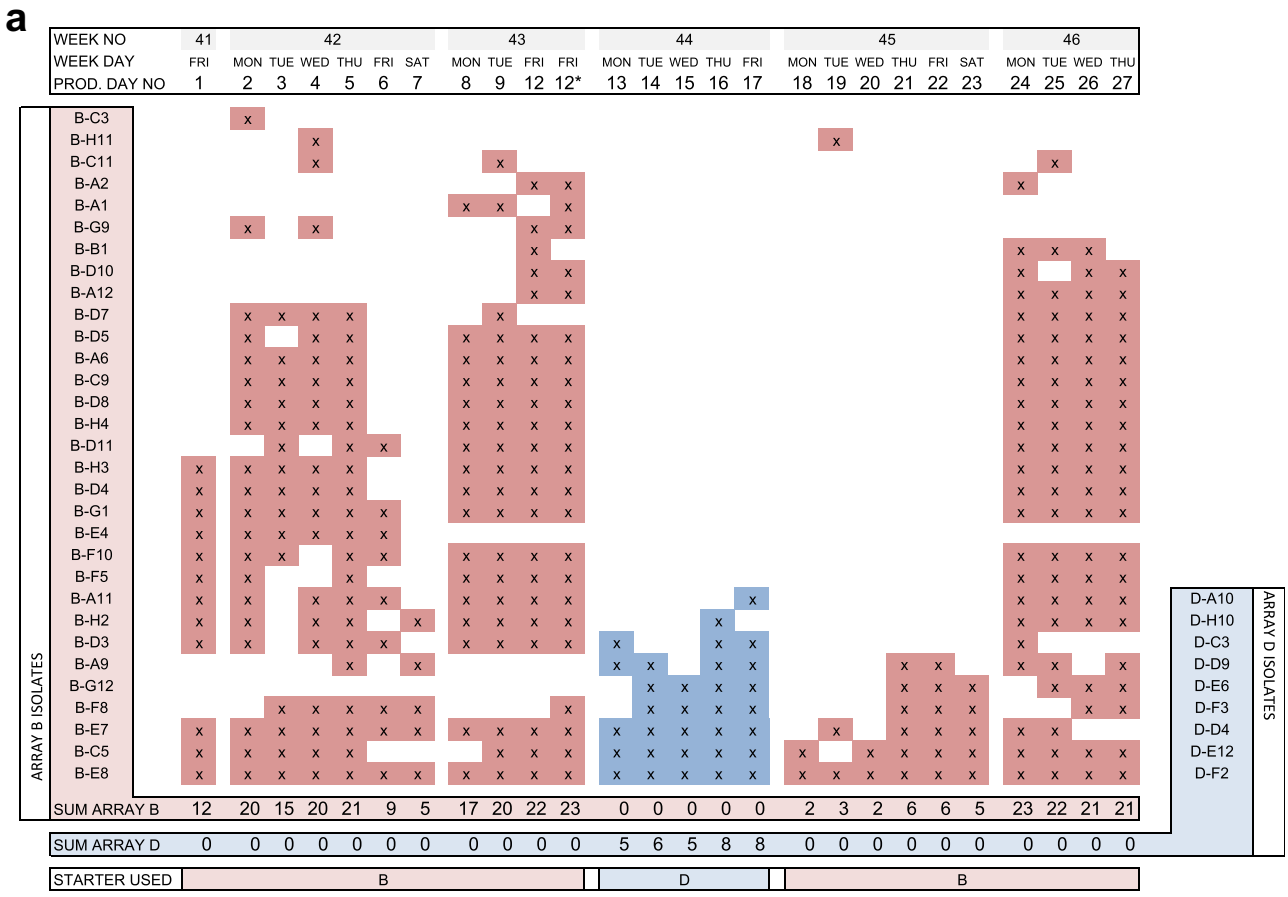

Fig. 2. Panel a: Analysis by growth inhibition arrays B and D, on bulk starters sampled on 25 production days in one cheese plant. All samples were tested against both arrays. Growth inhibition is represented by "x" and background fill indicate whether inhibited isolate was found in array B (■) or D (■). White fields indicate that no inhibition was detected. Isolate names are given in the leftmost column for array B and the rightmost for array D. The actual calendar weeks and week days are indicated, as are production day numbers, assigned to indicate production days when no bulk starter was sampled. All bulk starter samples were taken early morning before cheese production commenced. An additional bulk starter sample was taken on day 12 after a failed fermentation was experienced (12*). The commercial starter culture used on a given production day is shown in the bottom row. The sum of inhibited isolates in plate B (■) and D (■) by a given bulk starter is shown in panel b. Bulk starters were also assayed against array E (see text), and the sum of inhibited isolates was counted for each production day (panel c).

translational frameshift motif, CCC.TAG or CCC.TAA (Auzat, Dröge, Weise, Lurz, & Tavares, 2008) in the 3′ terminus of the putative *msp* gene leading to an extension of *msp* by an open reading frame spanning each of the insertions (Fig. 1). The four different C-terminal extensions of MSP each carried a variable domain flanked by conserved variable domain signature sequences (Crutz-Le Coq, Cesselin, Commissaire, & Anba, 2002). Such variable regions have been found in a variety of phage structural proteins (Crutz-Le Coq et al., 2002) and have in some protein settings been shown to be involved in host recognition (Duplessis & Moineau, 2001; Stuer-Lauridsen, Janzen, Schnabl, & Johansen, 2003). Details on the predicted gene products and variable domains encoded on the different insertions are shown in Table 2. Variable domains encoded on inserts 2 and 3 showed a high degree of sequence identity to variable domains in structural proteins of lactococcal phages Tuc2009 and 4268 respectively. In Tuc2009 the variable domain is located on the collar and whisker structure (Mc Grath et al., 2006) and in 4268 on a putative tail-host specificity protein (Trotter et al., 2006). The role a variable domain fused to the major structural protein might serve remains to be elucidated.

However, phages with different insertion variants were isolated on different array B host bacteria (Table 1). This might suggest a possible function in host specificity.

Several of the sequences differed by point mutations in the putative *msp* gene (supplementary Figs. 1 and 2). This gene is the target for PCR based methods for detection of 936-phages, and knowledge of point mutations commonly occurring will be valuable in improvements of these methods.

3.3. Phage host range diversity

The PCR primers used for phage detection and quantification did not allow for discrimination between different phages of the 936 group, so we devised a biological phage assay to study samples' inhibitory effect on the growth of lactococcal strains. Bacteriophages often show a limited host range, thus growth inhibition assays were used to study the phage biodiversity as measured by the host range of the phages present in a sample. Arrays of indicator bacteria on which a sample's inhibitory effect could be tested were made in microtitre plates. Array A contained 88 lactococcal strains from our laboratory collection. Arrays B, C and D each contained 96 isolates from the commercial starter cultures B, C and D respectively.

The four different arrays showed no or little overlap with respect to phage sensitivity. Samples taken from fermentations using starter culture B ($n = 54$) inhibited several isolates on the B array but none on D and vice versa ($n = 13$). Furthermore, only one of the samples from fermentations using starter culture C ($n = 4$) gave a response on array B. This sample inhibited two isolates on the B array, and was sampled on the first production day after starter cultures had been changed from B to C. We observed no inhibition of array C isolates by phage samples taken from starter B fermentations ($n = 37$). The selectivity of these arrays shows that there is little overlap in bacteriophage sensitivity between the three different starter cultures, and demonstrates the potential of culture rotation in the protection against phage attack. However, *L. lactis* IL1403 present in array A was inhibited by all samples from fermentations with starter cultures B and D, suggesting that there might be some overlap in bacteriophage sensitivity between these two starters. IL1403 was not inhibited by bulk starters or whey produced with starter C.

3.4. Day-to-day fluctuations in bacteriophage diversity and quantity

Changes in bacteriophage diversity over time in a dairy plant was studied by analyzing samples taken daily over a two months period and a second one month period a year later. In the second period, one occasion of fermentation failure (slow acidification) was experienced. A great day-to-day variation in inhibitory spectra was observed (Fig. 2). This finding was similar to that reported by Neve et al. (1995). In both periods starter culture B was the main starter culture in use. Altogether, as many as 59 of the 96 isolates in array B were inhibited on at least one occasion. Fourteen of the isolates were only inhibited by samples from the first period, and 16 were only inhibited by samples from the second. This suggests that a shift in phage host range had occurred between the two periods. Similar shifts have been observed by others (Casey, Morgan, Daly, & Fitzgerald, 1993; Josephsen et al., 1994; Rousseau & Moineau, 2009).

Fig. 2 shows the inhibitory spectra and the number of isolates inhibited by bulk starter samples from the second period. No or only small differences were seen between bulk starter and whey produced on the same day, showing that most phages present in cheese production came from the bulk starter. No clear correlation between bulk starter activity measurements and the inhibitory spectra of bulk starter samples could be found.

In response to the fermentation failure starter culture B was replaced by starter culture D for five production days. This had a dramatic effect on the bacteriophage flora. As seen in Fig. 2, no

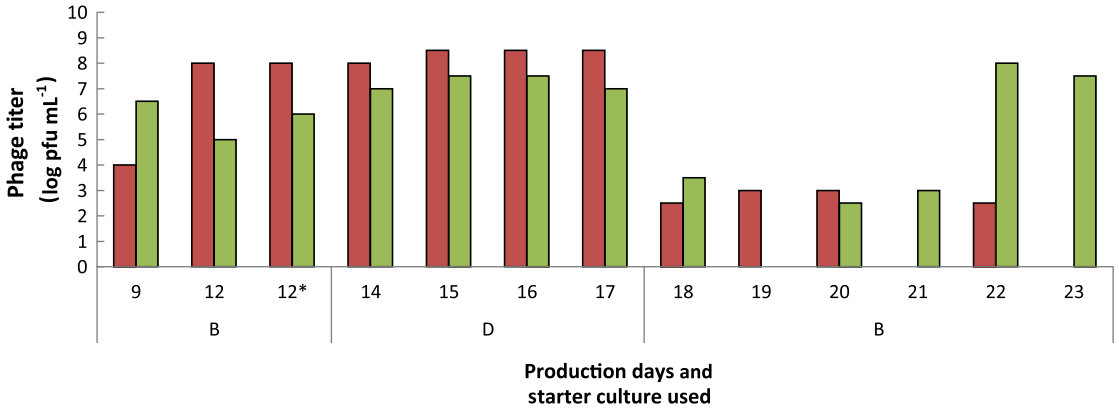

Fig. 3. Plaque quantification of bacteriophages infecting *L. lactis* IL1403. Bulk starter (■) and whey (■) sampled on 12 production days, including one production day when low starter activity was experienced (day 12) and additional bulk starter and whey samples taken from a failed (slow) fermentation (12*). Following fermentation failure, starter culture B was replaced by starter D for five production days. Production days 10 and 11 were not sampled, and production day 13 is not shown since no relevant whey sample was taken.

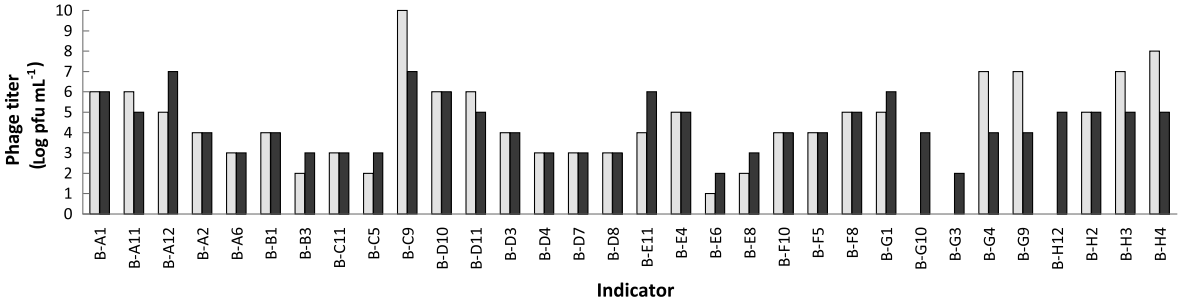

Fig. 4. Plaque quantification with array B indicators. Bacteriophage titres were determined for bulk starter and whey sampled on production day 12. Bulk starters (▒) were sampled early morning before cheese production commenced. Whey (■) was sampled from the first production lot of the day.

phage could be detected by array B after starter cultures had been changed. On the other hand, phages infecting strains of culture D were readily detected from the first day it was used. Moreover, after returning to starter B, phages against this culture rapidly reappeared. The findings clearly demonstrate the potential of starter rotation.

3.5. Bacteriophage analysis results reflected a fermentation failure

Bulk starter and whey sampled from a failed fermentation and a subsequent starter culture rotation were analyzed for bacteriophage quantities by plaque assay. *L. lactis* IL1403 was chosen as indicator since it was sensitive to a large number of different bacteriophages. Results in Fig. 3 show that bacteriophage titres vary greatly between production days. Notably, in fermentations with starter culture B elevated titres were found in bulk starters sampled on the day of the fermentation failure. High titres of bacteriophages against IL1403 were also found when starter culture D was used. Interestingly, normal starter culture activity was reported on these production days.

Bacteriophages in samples taken on production days prior to and after the 5 days of starter D use were quantified using 32

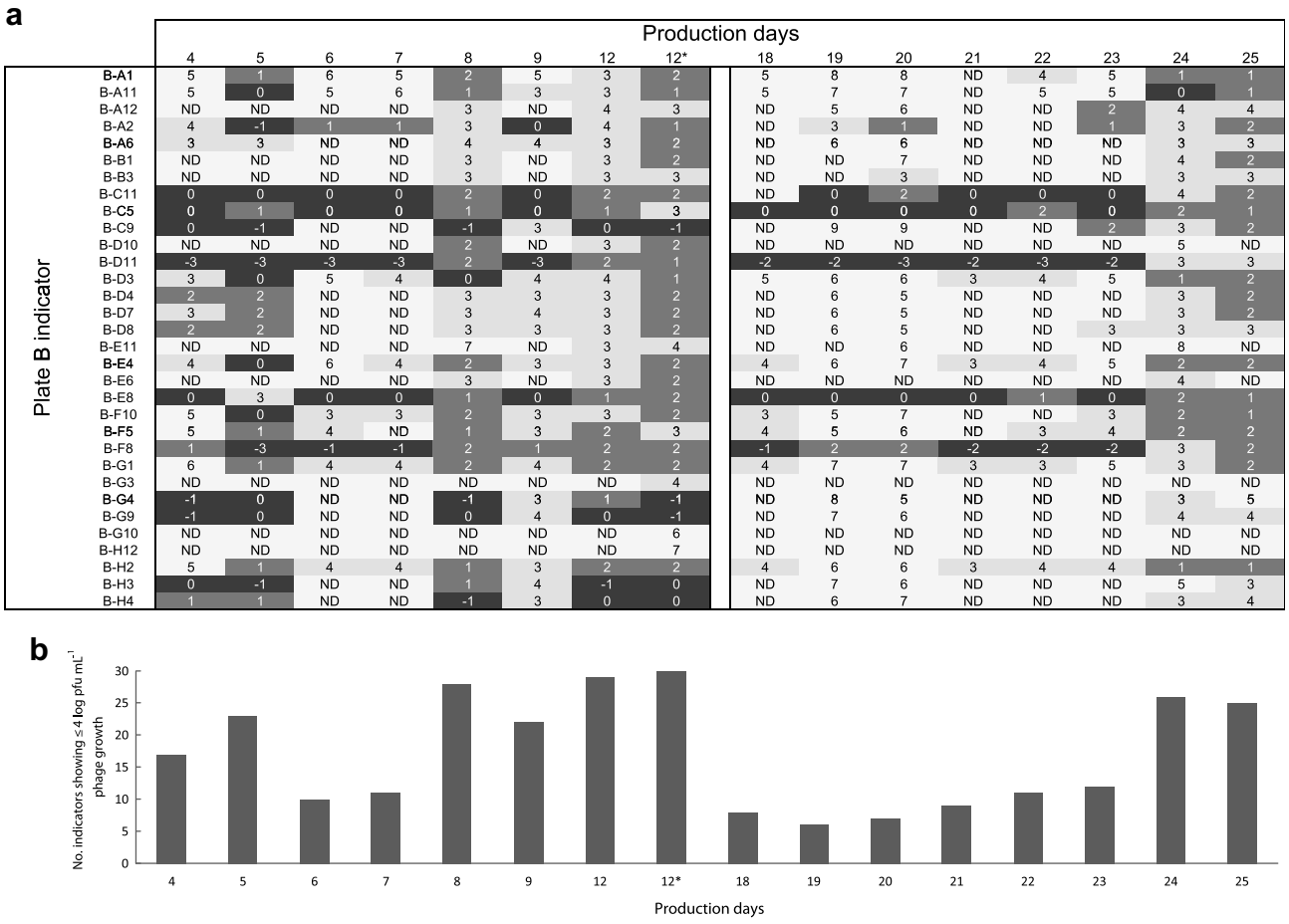

Fig. 5. Bacteriophage growth during cheese production (panel a), assessed by difference in phage titre between bulk starter and whey, and adjusted for 100 fold dilution resulting from the use of 1% inoculum. Bacteriophage titres were determined by plaque assays using array B indicators. Numbers indicate difference in phage titre in log pfu mL^{-1} between whey and bulk starter and "ND" indicates no phage detected. Background fills also indicate phage growth: ND and >4 log pfu mL^{-1} (■), 3–4 log pfu mL^{-1} (▒), 1–2 log pfu mL^{-1} (▓), and no phage growth (■). Bulk starters were sampled before cheese production commenced and whey from the first production of the given production day. Additional samples taken from a failed (slow) fermentation (12*) were also analyzed. All samples analyzed were produced with starter culture B, the gap between production day 12 and 18 represent 5 days of starter D use. Panel b shows the number of indicators showing log 4 or less pfu mL^{-1} bacteriophage growth.

indicators from array B. In Fig. 4, bacteriophage titres in samples from a failed fermentation are presented. For most indicators very little difference in phage titre between bulk starter and whey was observed. This limited growth of bacteriophages during the milk fermentation indicates that host strains were incapacitated, and consequently performed poorly in the cheese production. Fig. 5A presents bacteriophage growth in fermentations sampled on 15 production days, before and after the starter culture rotation. Bacteriophage growth was measured as the difference in phage titre between corresponding bulk starter and whey samples. Bacteria that propagated phages less than 10,000 fold were considered inhibited. Using this criterion we plotted the number of inhibited strains for each of the production days (Fig. 5B). When the fermentation failure was experienced as many as 30 out of 32 strains were inhibited. Our results show that bacteriophage growth analyses can reflect fermentation failure. However, since whey is needed for the determination of bacteriophage growth this assay cannot be used to predict fermentation failure.

3.6. A predictive array

One aim of this study was to find a correlation between bacteriophages and fermentation failure, and thus hopefully making it possible to develop predictive tools. Our results showed correlation between strain inhibition and fermentation failure but did not precisely define what strains are more important for successful fermentation. Such strains could be useful in diagnostic growth inhibition assays as determinants of fermentation failure. We therefore constructed an array (array E) whose indicators were isolated from a successful fermentation rather than directly from a commercial starter. The rationale behind being that it might better represent the strains of most importance for fast acidification. In diversity assays using this array, samples from bulk starters causing slow fermentations scored higher than those from bulk starters used in successful fermentations. As shown in Fig. 2C, 31 isolates were inhibited by the bulk starter of the failed fermentation, the highest number of all samples analyzed. Among the isolates inhibited by the bulk starter of the failed fermentation, four were not inhibited by any other sample analyzed. This suggests that such an array can be used to predict a fermentation failure.

Interestingly, as shown in Fig. 2C, eight of the isolates in this array were inhibited by one or more bulk starter samples produced with starter culture D. This confirms that some overlap in phage sensitivity exists between the two starter cultures B and D.

3.7. Phages in the milk delivery chain

Milk from 112 farm tanks was collected by seven milk trucks into one silo tank at the dairy plant. Samples for bacteriophage analyses were taken from all stages of this milk delivery chain. Bacteriophage concentrations were too low for direct detection by PCR. However, after overnight amplification by starter culture B, the culture in use in the dairy at the time, phages of the 936 group were readily detected. No phages could be detected after amplification with starter culture C, suggesting that phages have spread from the dairy to the raw milk. Phages were detected in all milk trucks, 55 farm tanks and the silo tank. By biodiversity analysis of the positive samples by array B, we found an average of two isolates inhibited by the farm tank samples. 41 strains were inhibited by the silo tank sample, but only six isolates were inhibited by whey from cheese produced with the same milk. Of the six isolates, two were among the 41 inhibited by the silo tank sample. Thus, phages against 39 isolates did not multiply to detectable levels during cheese production even in the presence of suitable host strains. Our results indicate that bacteriophage concentrations in raw milk are too low to have any impact on the milk fermentation. Moreover, considering the thorough sterilization scheme for bulk starter milk, it is unlikely that phage contamination in raw milk is the source for the high bacteriophage levels found in bulk starter samples. This shows that bacteriophages from in-house sources are responsible for contaminating the bulk starter vat.

4. Conclusions

Bacteriophage contamination is common in the dairy industry, but thanks to the use of multi-strain starters successful fermentations can be performed despite high phage levels.

We found that virulent phages of the 936 group contaminate and reach high levels already at the bulk starter stage, and that many hosts are inactivated before they can contribute in the fermentation. Our results strongly indicate that bacteriophages affecting fermentations did not come from the raw milk but more likely from in-house sources of contamination. In bulk starter cultures phages are given ample time to multiply and even a few phages infecting a bulk starter vat can outgrow their hosts before the end of the fermentation, thus equipment sanitation is critical.

Large day-to-day variations in host range were found, indicating contamination from a reservoir of phages with a wide specificity spectrum. Increased host range might result from phages breaking through phage resistance mechanisms, thus some of the variation seen might reflect differences in contamination levels. The potential of starter culture rotation was demonstrated, and despite a small overlap in phage susceptibility between the two starters, an effect was seen on phage diversity in the subsequent fermentations. However, the effect was short lived, emphasizing the ubiquity of phages in the dairy environment and the importance of supplementary phage counter-measures.

A fermentation failure coincided with increased host range of phages in the bulk starter. Identification of the borderline between normal host range fluctuations and that leading to fermentation failure shows promise in the development of a rapid test that can be performed on bulk starters to predict fermentation failure.

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Appendix. Supplementary material

The Supplementary data associated with this article can be found online, at doi:10.1016/j.idairyj.2011.02.010.

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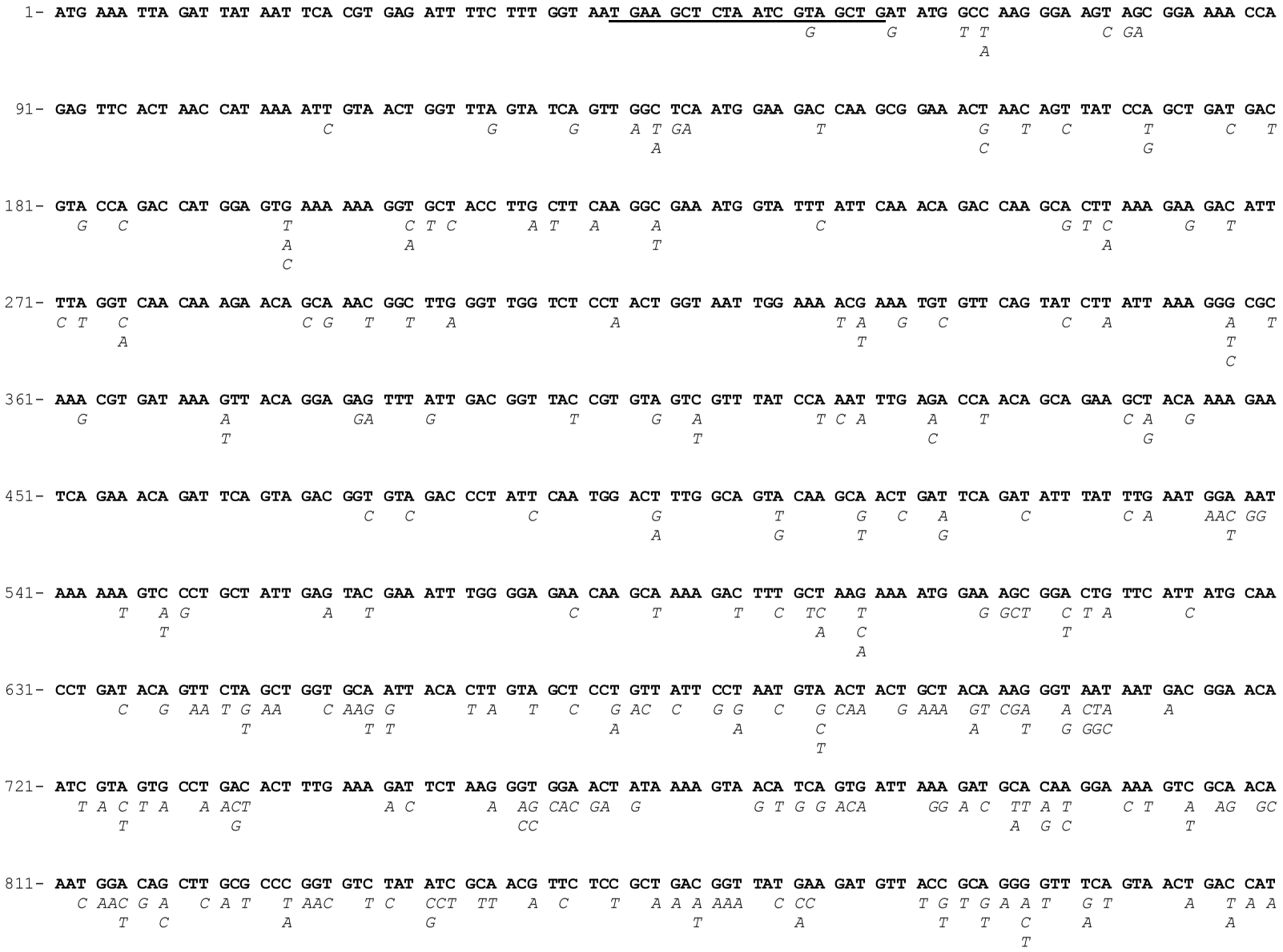

Fig.S1: Overview of variable positions in the MSP gene identified in this study. Primer 936-A is underlined.

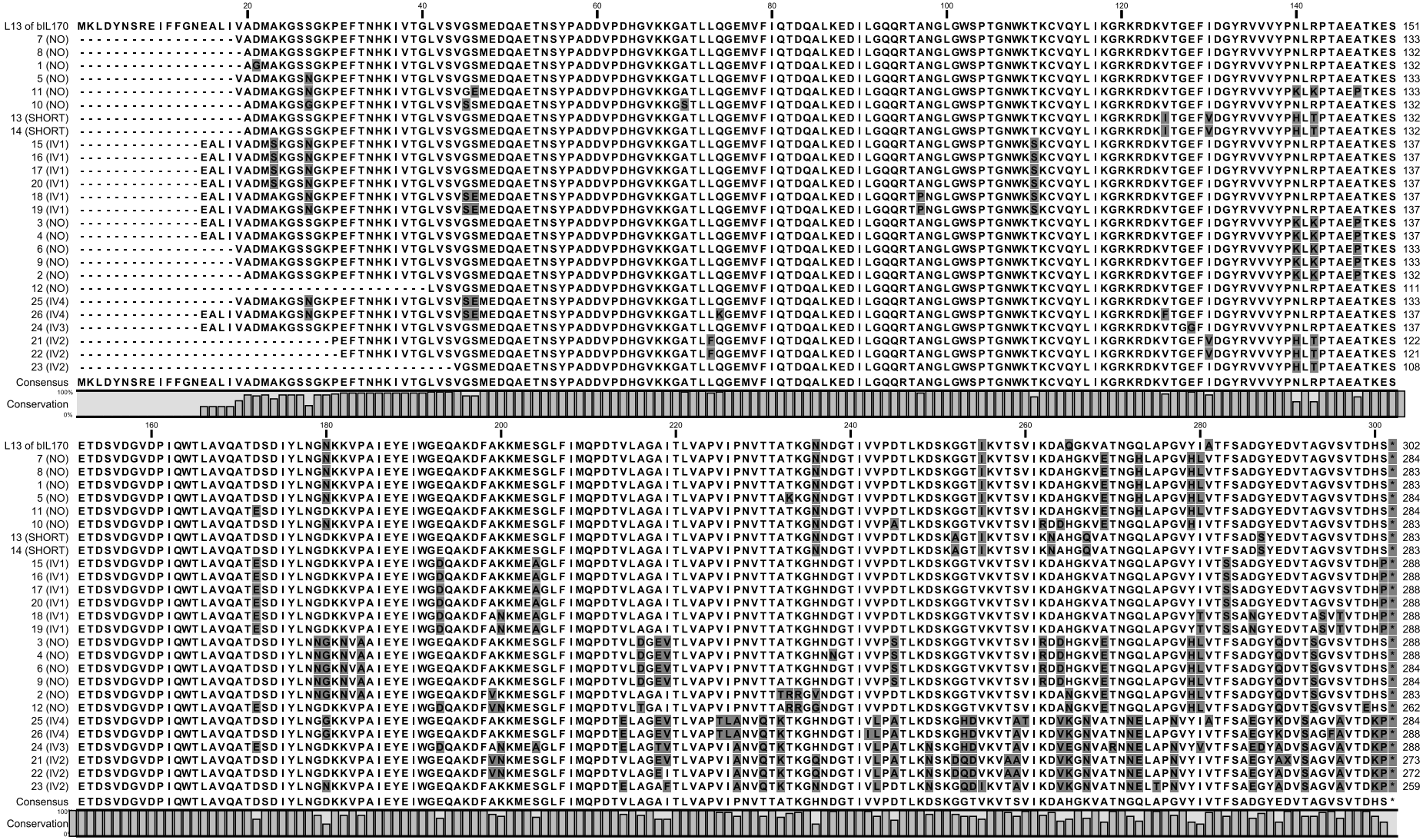

Fig. S2: Amino acid sequence alignment of the putative major structural protein (L13) sequence of *Lactococcus* phage bIL170 (Genbank accession no. AAC27192) and the putative major structural protein sequences dedused from the 26 sequence types identified in this study. Sequence name or sequence type number is indicated to the left of each sequence. Insert types are indicated in parentheses, NO, no insert; SHORT, short insert type; IV1, IV2, IV3 and IV4 indicate insert variant 1 to 4, respectively. Amino acid positions divergent from the consensus sequence are shown on grey background.

Paper II

Characterization of a *Leuconostoc* bacteriophage infecting flavor producers of cheese starter cultures

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Key words: Dairy, fermentation, cheese, lactic acid bacteria, Leuconostoc, bacteriophage, complete genome sequence.

Abstract

Leuconostocs are important flavor producers in fermented milk products such as buttermilk, cultured butter and various cheeses. Bacteriophage Lmd1 infects *Ln. mesenteroides* subsp. *dextranicum* and was isolated from a dairy producing Dutch-type cheese. ϕLmd1 belongs to the *Siphoviridae* family, as do most bacteriophages encountered in dairies and all described phages infecting *Leuconostoc*. It was capable of rapid growth and was unaffected by pasteurization. Interestingly, ϕLmd1 inhibited all *Leuconostoc* isolates of one starter culture and was able to grow on 3 of 4 different starter cultures tested. This identifies ϕLmd1 as a potential threat to flavor producing *Leuconostoc* in dairy fermentation and emphasize the importance of selecting phage unrelated strains for starter cultures. In this, bacteriophage ϕLmd1 could be a valuable selection tool.

Forty open reading frames were predicted in the 26,201 bp genome sequence of ϕLmd1, and the linear genome was found to have cohesive ends. The closest known relative of ϕLmd1 is the *Ln. mesenteroides* phage 1-A4 which was isolated from commercial sauerkraut fermentation. The two phages showed homology in about half of the predicted proteins. Most of these were putatively involved in DNA replication, DNA packaging and virion morphogenesis. Moreover, the gene order in this region was almost completely conserved between the two phages. This indicates a common ancestral origin. The differences between the two phages probably reflect adaptation to different environmental niches.

This study contributes to a better understanding of dairy *Leuconostoc* phages and might help the development of new phage control strategies in the dairy industry.

1 Introduction

Bacteria of the genus *Leuconostoc* (*Ln.*) are incorporated into dairy starter cultures due to their ability to produce important metabolites such as diacetyl and CO_2 from citric acid (6, 10). Diacetyl is the primary source of aroma and flavor compounds in a variety of fermented milk products including buttermilk, butter, quarg and various cheese types (6). *Leuconostocs* are important flavor producers in L-type and DL-type mesophilic starter cultures, in the latter together with *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. The different *Leuconostocs* associated with dairy starters include *Ln. mesenteroides* subsp. *cremoris*, *Ln. mesenteroides* subsp. *dextranicum, Ln. lactis* and *Ln. pseudomesenteroides* (5, 11).

Bacteriophages negatively affect dairy fermentations by inhibiting the growth of key lactic acid bacteria (LAB). Bacteriophages infecting *Lactococcus* have been extensively studied for decades due to their dramatic effect on milk acidification rates (33). Lactococcal phages are ubiquitous in dairy environments (34, 37, 43) and it has been shown that phages resident in the dairy plant are responsible for killing lactococcal starter bacteria early in the fermentation (24). Before phages become dairy residents they are likely to enter dairies through contaminated milk (24, 29) and since natural habitats of *Leuconostoc* include green vegetation and silage (41) a similar route of entry is likely for *Leuconostoc* phages. Atamer and co-workers studied the thermal resistance of 77 *Leuconostoc* phages and found that commonly applied pasteurization conditions were insufficient to ensure complete inactivation of dairy *Leuconostoc* phages (5). Accordingly, *Leuconostoc* phages have been shown to be widely distributed in dairy products (5, 40). Phages infecting dairy *Leuconostoc* have previously been characterized (5, 12) and the genome sequence of a virulent *Ln. mesenteroides* phage and a temperate *Ln. pseudomesenteroides* phage, both isolated from vegetable fermentation have been characterized (21, 28). To our knowledge, there is no publicly available genome sequence of a *Leuconostoc* phage of dairy origin.

In this study we analyzed the complete genomic sequence of a *Leuconostoc* phage isolated from a Norwegian dairy producing Dutch-type cheese, and characterized the phage with respect to its ability to affect dairy fermentation. This study contributes to a better understanding of dairy

Leuconostoc phages and might help the development of new phage control strategies in the dairy industry.

2 Materials and Methods

2.1 Isolation and characterization of Leuconostoc host bacteria.

Forty eight putative *Leuconostocs* were isolated from each of two commercial starter cultures commonly used in the production of Dutch-type cheese. Starter cultures were plated on MRS (Oxoid, Baskingstoke, UK) agar supplemented with 30 µg/ml vancomycin (Sigma-Aldrich, Steinheim, Germany), and incubated overnight (ON) at 30 °C. Random colonies were isolated and plated twice to ensure single strain cultures. Pure cultures were dispersed to microplate wells, added 15 % glycerol and stored at -80 °C until used in a bacteriophage challenge assay. One isolate was characterized using the API 50 CHL kit (BioMérieux, Lyon, France) according to the instructions manual, and 16s rRNA sequence analysis. The 1341-bp 16s rRNA fragment sequenced corresponded to positions 55 to 1387 of the *Escherichia coli* 16s rRNA gene. Identification was done through comparison to available sequences in the NCBI nucleotide collection (nr/nt) using BLASTN 2.2.26+ (47) at www.ncbi.nlm.nih.gov (February 2012).

2.2 Bacteriophage isolation, growth and purification.

A brine sample from a Norwegian dairy producing Dutch-type cheese was dialyzed against phage storage buffer TM (10mM Tris-HCl pH 7.4, 100mM NaCl, 10mM $MgCl_2$, 10mM $CaCl_2$), mixed with 9 volumes MRS supplemented with 5 mM $CaCl_2$ (MRS-C) and used to challenge growth of the 96 bacterial isolates. The growth inhibition array was performed in a microplate as described previously (24). Bacteriophage Lmd1 was isolated from one of the microplate wells displaying growth inhibition. Phage isolation and quantification was done by standard plaque assays performed using MRS-C soft-agar (0.8 % agar) inoculated with approximately 3×10^7 colony forming units/ml and overlaid on MRS-C agar plates. The plates were incubated at 30°C for at least 16 hours before plaque forming units (pfu) were counted or plaques isolated. Phage isolation was repeated three times to ensure single phage isolation.

Bacteriophage amplification was carried out in MRS-C broth by growing host *Leuconostoc* to OD_{600} of about 0.4 followed by infection with ϕLmd1 at a multiplicity of infection (MOI) of approximately 10. Lysis was completed by the addition of 0.5% chloroform and 1 M NaCl. Cell

debris was removed by centrifugation and the clear lysate subjected to filtration (0.45 µm cutoff). Phages were precipitated with polyethylene glycol (PEG) and purified on CsCl gradients as described elsewhere (7). Single step growth curve analysis was performed as described by Hyman and Abedon (20).

2.3 Electron microscopy

Purified phage particles were negatively stained with 2% (w/v) uranyl acetate on a carbon-formvar membrane grid and examined by transmission electron microscopy (TEM). TEM analysis was performed on a FEI Morgagni 268 (FEI Company B.V., Eindhoven, The Netherlands) microscope at an accelerating voltage of 100 kV. Phage dimensions were found by averaging at least 12 measurements.

2.4 DNA purification and sequencing

A PEG precipitate was treated with 1 µg/ml DNase I and 10 µg/ml RNase A for 1 hour at 37 ˚C, followed by 1 hour incubation at 65 ˚C with 25 mM EDTA, 0.5 % sodium dodecyl sulphate (SDS) and 200 µg/ml proteinase K (QIAGEN). After residual PEG was removed by chloroform extraction, standard phenol/chloroform extraction and ethanol precipitation was used to obtain phage DNA. A shotgun library was made in pUC19 (46) after partial digestion with AluI. Insert sizes ranged from 0.3 to 5 kb with an approximate average of 1.6 kb. Clones were sequenced using BigDye 3.1 chemistry (Applied Biosystems, Foster City, CA, USA) and standard M13 primers. Gaps were filled by primer walking using PCR amplified genomic DNA as template.

2.5 Bioinformatic analysis

Sequence assembly, bioinformatic analyzes and genome annotation were done using CLC Main Workbench version 6.5 (CLC bio, Aarhus, Denmark). Open reading frames (ORFs) and ribosomal binding sites (RBS) were identified using the Prodigal (19) online tool (http://prodigal.ornl.org) and homology searches were done using BLASTP and PSI-BLAST build 2.2.26+ (3, 4) at www.ncbi.nlm.nih.gov (February 2012). Conserved domains were found by searching the Conserved Domains Database (30-32) at www.ncbi.nih.gov (February 2012). Genome comparison was carried out using Easyfig software version 1.2.1 (42) with the following cutoff

settings: minimum alignment length = 20, maximum tblastx e-value = 0.001. This corresponded to a minimum sequence identity of 19.23 %.

2.6 Analysis of structural proteins

Purified phage particles were denatured at 100 ˚C for 10 minutes in Laemmli buffer (27) and proteins separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were visualized with Coomassie Brilliant Blue R250 (BIORAD), and visible protein bands excised, trypsin treated and extracted as described elsewhere (39). Extracted peptides were desalted with C_{18} Stage tips (36), eluted with 70 % acetonitrile and mixed with an equal volume of matrix solution (15 mg/ml alpha-cyano-4-hydroxy cinnamic acid in ethanol:acetonitrile 1:1). Peptides were then applied to a matrix-assisted laser desorption ionization (MALDI) target plate (Bruker Daltonik GmbH, Bremen, Germany), and peptide mass finger-printing (PMF) and tandem mass spectrometry (MS/MS) performed on an Ultra Flex MALDI-tandem time of flight (MALDI-TOF/TOF) instrument (Bruker Daltonik). The mass range for MALDI-TOF/MS was 800 to 4,000 Da, with a mass accuracy of 50 ppm. The mass range for MALDI-TOF/MS data acquisition was 800 to 4,000 Da and the spectra were externally calibrated using a peptide calibration mixture (Bruker Daltonik) ranging from 757 to 3147 Da. Protein identification was carried out using Mascot software (Matrix Science Ltd., London, UK) with searches against the non-redundant NCBI database and a database containing all translated ORF sequences (≥ 50 amino acids) from the genome sequences of ϕLmd1.

2.7 Database accession number

The complete genome sequence of *Leuconostoc* phage Lmd1 has been deposited in GenBank under accession number JQ659259.

3 Results

3.1 Host Leuconostoc

To study bacteriophages infecting dairy *Leuconostoc* we isolated 48 vancomycin resistant bacteria from each of two commercial starters commonly used in the production of Dutch-type cheese. The isolates were challenged with a brine sample by use of a growth inhibition assay performed in a microplate. The brine sample was taken from a dairy plant producing Dutch-type cheese and we had previously shown that dairy brine samples were good sources of dairy phage (24). All isolates of one starter culture (A) were inhibited but none of the isolates from the other starter (B) were affected. One isolate from starter culture A (isolate A1) was chosen to serve as a host for bacteriophage isolation from the brine sample. Isolate A1 was identified as *Ln. mesenteroides* subsp. *dextranicum* by its 16s ribosomal RNA gene sequence, its sugar fermentation pattern and its ability to grow in the presence of 6.5% NaCl. The partial 16s rRNA gene sequence of isolate A1 was found to be 100.0 % identical to that of *Leuconostocs* belonging to ribospecies CHCC 2114 (35). When compared to the type strains closely related to this ribospecies, the 16s rRNA sequence of isolate A1 showed 99.5 % identity to the 16s rRNA genes of the type strains of *Ln. mesenteroides* subsp. *mesenteroides* (NCFB 523; Genbank accession AB023244) and *Ln. mesenteroides* subsp. *dextranicum* (NCFB 529; AB023242), and 99.6 % identity to *Ln. pseudomesenteroides* (NRIC 1777; AB023237). The sugar fermentation pattern of isolate A1 was assayed using the API50 CHL test, and acid production was observed during growth on the following carbohydrates: D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, methyl-αD-glucopyranoside, N-acetylglucosamine, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, sucrose, D-trehalose, D-raffinose, starch, gentibiose and D-turanose. No growth was observed on the other carbohydrates included in the API50 CHL test. These results gave a 99.4 % identity hit (at www.apiweb.biomerieux.com) to *Ln. mesenteroides* subspecies *dextranicum*, however, the sugar fermentation pattern could also be consistent with that of *Ln. pseudomesenteroides* (6). The ability to grow in the presence of 6.5 % NaCl has previously been used to distinguish between *Ln. mesenteroides* and *Ln. pseudomesenteroides* (16, 17). *Leuconostoc* isolate A1 was able to grow in MRS supplemented with 6.5 % a trait seen in *Ln.*

mesenteroides but not in *Ln. pseudomesenteroides*. In the following the strain name *Ln. mesenteroides* subsp. *dextranicum* A1 will be shortened to *Ln. mesenteroides* A1.

One virulent bacteriophage isolated on host *Ln. mesenteroides* A1 was named ϕLmd1 and selected for further study.

3.2 Morphology of φLmd1

The bacteriophage Lmd1 produced large clear plaques on bacterial lawns containing *Ln. mesenteroides* A1. Plaques were measured to about 1.4 mm in diameter and 3.5 mm halos of partial lysis appeared around the clear plaques after continued incubation for about 48 hours (Fig. 1A).

Transmission electron microscopy showed that ϕLmd1 is a tailed phage of the *Siphoviridae* family (1). It has a capsid diameter of 41 nm and a tail measuring 115 by 10 nm, typical for the B1 morphotype (2). The tail consists of 30 or 31 tail segments and a distinct baseplate could be observed at the tail tip (Fig. 1B and C).

3.3 Phage virulence, growth on commercial starter cultures and thermal resistance

Single-step growth curve analysis showed that ϕLmd1 had an average burst-size of 51 when assayed in MRS-C at 30°C. Lysis had not started 20 minutes after adsorption but was completed before 30 minutes had passed (data not shown). We did not observe growth of any phage resistant mutants of *Ln. mesenteroides* A1 during routine amplification of ϕLmd1 in broth media or on plates.

Interestingly, ϕLmd1 inhibited all *Leuconostocs* isolated from starter culture A but none of the isolates that originated from starter B. To test the ability of ϕLmd1 to multiply on different starter cultures commonly used in industrial dairy fermentation, ϕLmd1 was suspended in MRS-C and inoculated with four different starters, approximately 10^7 cells/ml. Phage titers were assayed before and after 16 hours incubation at 25°C. As shown in Fig. 2, ϕLmd1 was unable to proliferate on starter B strains but grew well on starters A, C and D.

To study the thermal resistance of ϕLmd1, 3 × 10^9 pfu/ml phage particles were suspend in whole milk or TM buffer and incubated at 73°C for 2 minutes or 96°C for 30 minutes. Temperatures and incubation times were chosen to resemble commonly employed pasteurization and bulk starter sterilization schemes, respectively. No reduction in titer was observed after incubation at 73°C, but 30 minutes incubation at 96°C resulted in phage titers dropping below our detection limit of 200 pfu/ml.

These findings shows that ϕLmd1 can survive pasteurization, is capable of rapid growth and can multiply on 3 of the 4 different commercial starter cultures tested in this study. Based on these characteristics and the observation that ϕLmd1 inhibited all *Leuconostoc* isolates of one starter culture, ϕLmd1 has the potential to seriously affect flavor producing *Leuconostoc* in dairy fermentation.

3.4 The genome of φLmd1

The sequence of the 26,201 base pair linear genome of *Leuconostoc* phage Lmd1 was found by a combination of shotgun sequencing and primer walking with an average 6.1 fold sequence coverage. The G+C content of the ϕLmd1 genome was 36.4%, very close to the G+C content of *Ln. mesenteroides* subsp. *mesenteroides* ATCC 8293. The presence of cohesive (cos) genome ends was examined by analyzing the effect of heating on EcoRI digests of ϕLmd1 genomic DNA (8). One of the two digests was heated to 77°C for 10 minutes prior to visualization in an agarose gel (Fig. 3). The three recognition sites for EcoRI are plotted on the ϕLmd1 genome map in Fig. 4. EcoRI digestion of a circularized genome would produce DNA fragments of 20476, 5725 and 679 bp. As shown in Fig. 3, two additional bands of about 2.0 kb and 3.5 kb were visible. The lane with DNA that had been denatured showed higher intensity bands at 2.0 and 3.5 kb compared to the unheated sample that showed a higher intensity band at about 5.7 kb. This is indicative for the presence of cohesive (cos) ends in the ϕLmd1 genome and that the cos-site is located near position 22,900 or 24,400. Position 24,400 lies within a large non-coding region of the ϕLmd1 genome and is most probably the location of the cos-site (Fig. 4).

A putative origin of replication (ori) was found upstream of the predicted *orf 1*. This region comprises an A-T rich region and multiple repeats and hairpin structures typical of phage replication origins (44).

Forty open reading frames (ORF) were predicted. Starting with the ORF immediately downstream of ori, ORFs were given numbers consecutively (Table 1 and Fig. 4). Half of the ORFs were located on one strand and the other half on the complementary strand. The predicted coding sequence of ϕLmd1 constituted 91.7 % of the genomic sequence. As shown in Table 1, 36 ORFs initiated translation with the ATG start codon and 4 with TTG. Putative ribosomal binding sites (RBS) similar to the consensus sequence (AGGAGG) of Shine-Dalgarno elements were found upstream of 36 of the 40 ORFs.

Based on PSI-BLAST analysis and searches against the Conserved Domains Database, putative functions were assigned to 24 ORFs by homology to previously characterized proteins. Eight ORFs were similar to uncharacterized database entries (conserved hypotheticals), one, ORF 21, was found by tBLASTx to be similar to a possible ORF in a non-coding region of *Ln. mesenteroides* phage 1-A4 (28), and the remaining 7 ORFs gave no significant hits to known sequences (Table 1).

To identify ORFs coding for structural proteins, a purified phage sample was separated by SDS-PAGE and proteins visualized with Coomassie stain. Visible protein bands were excised and identified by mass spectrometry (Fig. 5). Eight proteins were identified as structural proteins and they were encoded by *orf9* and *orf14* through *orf20*. The molecular masses deduced from these ORFs were in line with the masses estimated from SDS-PAGE. The only exception to this was ORF17, discussed below.

Similar to most characterized bacteriophage genomes, the genome of ϕLmd1 is organized in functional modules. Four modules are clearly identifiable: the DNA replication module, DNA packaging module and the head and tail morphogenesis modules (Fig. 4).

The modular organization of ϕLmd1 is very similar to that of *Ln. mesenteroides* phage 1-A4, which was isolated from industrial vegetable fermentation (28). As shown in table 1 and figure

6, twenty-four ORFs of ϕLmd1 show homology to ORFs of ϕ1-A4. Eighteen of these are located on the same strand, in the predicted functional modules for DNA replication, packaging and virion structure. The predicted gene order in this region is nearly completely conserved between the two phages. Among the 20 predicted ORFs on the negative strand, only 6 show homology to ϕ1-A4 ORFs. *Orf23, orf37, orf38* and *orf40* have similar genomic locations as their homologs in ϕ1-A4, but for *orf22* and *orf36* a gene relocation and an insertion event, respectively, seems to have taken place in the ϕ1-A4 genome compared to ϕLmd1 (Fig.6).

An alignment of the complete genome sequences of ϕLmd1 and ϕ1-A4 revealed generally low sequence similarity at the DNA level, even within *orfs* encoding homologous proteins (not shown). The great number of acquired point mutations suggests that there is a large evolutionary distance between the two phages.

DNA replication module

The putative DNA replication module found downstream of the predicted origin of replication consisted of *orf1* through *orf6*. Five of the ORFs had predicted functions indicating involvement in DNA replication (Table 1) and the sixth was assigned to the replication module based on its genomic location. ORF1 resembles the DNA helicase of a *Lactobacillus plantarum* prophage but was also partly similar to the VirE family of virulence associated proteins (Pfam05272). The predicted gene product of *orf2* has an N-terminal DNA primase/polymerase domain (Pfam09250) and is highly similar to the putative DNA primase/polymerase of ϕ1-A4. ORF3 is likely a homing endonuclease, similar to HNH_3 (Pfam13392) and a putative HNH endonuclease of *Lactobacillus* phage A2 (Table 1). ORFs 4, 5 and 6 are highly similar to ORFs 5, 6 and 7 of ϕ1-A4 (Table 1 and Fig. 6). The gene product of *orf4* is probably a type B DNA polymerase. The six conserved motifs found in type B polymerases, ExoI, ExoII, ExoIII and motifs A, B and C (44) are all present (data not shown). Type B polymerases are only rarely found in phages belonging to the *Siphoviridae* family. ORF6 can be a tRNA 3' endonuclease involved in preparing tRNAs for the addition of a CCA tail based on its similarity to the beta-lactamase family of metal-dependent hydrolases (COG1235) and RNaseZ (TIGR02651).

No significant similarity in sequence or gene order was found to any one of the well characterized phage replication modules described by Weigel and Seitz (44). Experimental data would be necessary to elucidate the mechanism of replication for ϕLmd1.

DNA packaging module

During phage assembly, terminase proteins function as DNA packaging machines when newly synthesized phage DNA is packaged into empty viral capsids (9). ORF8 shows significant homology to the phage terminase family of proteins (Pfam03354) and is 56% identical to the putative terminase large subunit of ϕ1-A4. ORF7 was assigned the putative function of terminase small subunit based on its genomic location and 41 % identity to the putative terminase small subunit, ORF8, of ϕ1-A4 (Table 1).

Head and tail morphogenesis modules

The head and tail morphogenesis modules probably encompass 12 ORFs, *orf9* through *orf20* (Fig. 4). As shown in Table 1 and Fig. 6 the structural modules of ϕLmd1 and ϕ1-A4 are highly similar in sequence, and, with the exception of *orf20*, identical in gene order.

The head morphogenesis module consists most likely of a putative portal protein, ORF9, a putative phage prohead protease, ORF10, and a putative major capsid protein, ORF11. All three ORFs are highly similar to their counterparts, ORFs 11 through 13, in ϕ1-A4 (Table 1). The putative head morphogenesis proteins of ϕLmd1 and ϕ1-A4 resemble those of several lactococcal phages and they weakly resemble the well described portal, prohead protease and capsid proteins of *Escherichia coli* phage HK97 (22). It is possible that the capsid formation and maturation follows a mechanism similar to that of phage HK97 (45).

Of the ORFs in the putative head morphogenesis module, only the putative portal protein (ORF9) was identified by mass spectrometric analysis as a structural component of the ϕLmd1 virion (Fig. 5). This is in agreement with similar experiments performed on ϕ1-A4 (28).

Downstream of the putative head morphogenesis module is the putative tail morphogenesis module. This region encompasses 8 ORFs, *orf13* through *orf20*, encoding a putative tail protein and 7 experimentally verified structural proteins, including the putative major tail protein, tape

measure protein, baseplate protein and receptor binding protein (Table 1). ORF13 is similar to the putative phage tail protein, ORF15, of ϕ1-A4 but no significant similarity was found to other available sequences. Experimental data is required to determine the function of this protein. *Orf14*, *orf15* and *orf18* encode structural proteins that share a high degree of identity to hypothetical proteins of ϕ1-A4 (Table 1) but no significant similarities were found to other database entries. ORF16 shares 63% identity to the major tail protein of ϕ1-A4 and was the most abundant protein observed in the protein gel (Fig. 5). This protein is most probably the major tail protein. Downstream of the major tail protein gene is the putative tape measure protein, encoded by *orf17*. ORF17 is likely a tape measure protein since it contains a conserved tape measure protein domain (TIGR02675) and produced significant hits to tape measure proteins of several different LAB phages (not shown). Interestingly, the calculated 83.5 kilodalton (kDa) molecular mass of the ϕLmd1 tape measure protein did not match the ~60 kDa molecular mass estimated by SDS-PAGE. It is possible that the tape measure protein of ϕLmd1 is subject to post-translational cleavage prior to phage tail assembly. The gene products of *orf19* and *orf20* share a high degree of similarity to the putative baseplate and putative receptor binding proteins of ϕ1-A4, respectively. However, no significant similarities were found to other database entries. The putative functions assigned to ORFs 19 and 20 are based on genomic location but further study would be necessary to verify this.

ORFs found on the negative strand

Of the 20 ORFs found on the negative strand seven ORFs showed no similarity to database entries, and we were only able to assign putative functions to six ORFs. Of these, two ORFs are probably involved in host lysis: ORF23 is likely a holin based on its significant similarity to the phage holin family 4 (Pfam05105, not shown). ORF35 harbor an amidase 2 domain (smart00644) and share 83 % identity with an N-acetylmuramoyl-L-alanine amidase of *Leuconostoc* sp. C2 (Table 1). Holins and lysins are often found clustered together to form lysis cassettes (26). Interestingly, the putative holin and lysin encoding genes of ϕLmd1 are separated by 11 ORFs of unknown function (Fig. 4). An ORF upstream of the putative lysin gene (*orf36*) might encode a second holin to form a typical lysis cassette. The deduced gene product of *orf36* resembles a putative holin (ORF36) of ϕ1-A4 but no other significant database hits

could corroborate this functional assignment. ORF37 contains a ribbon-helix-helix domain of the CopG family (18) and is thus likely a transcriptional regulator. Its genomic location upstream of the putative lysis gene might indicate a function in regulating host cell lysis.

Genomic regions comprising many relatively small ORFs with no assigned function are commonly found in phage genomes. The gene products encoded in such regions are often assumed to be involved in transcription regulation, DNA modification or host control since they are frequently encoded on early transcripts. The ϕLmd1 genomic region comprising *orf21, orf22 and orf24* through *orf34* resembles such regions. Seven of the 11 predicted gene products of *orf24* through *orf34* did not show any sequence similarity to database entries. ORF26 showed sequence similarity to a predicted protein of *Paenibacillus lactis* 154, encoded in a region also encoding putative transcription regulators and membrane associated proteins. ORFs 27, 28 and 30 showed sequence similarity to predicted prophage proteins of *Weissella paramesenteroides*, *Leuconostoc gelidum* and one *Leuconostoc* sp. (Table 1). All of these bacteria have been associated with plants and raw milk (6, 23, 38), the common habitats likely promoting genetic exchange.

Three ORFs are located between the predicted cos-site and the origin of replication (Fig. 4). All show homology to different endonucleases. ORF38 and ORF39 resemble homing endonucleases of *Leuconostoc* phage 1-A4 and *Enterococcus* phage EFRM31, respectively (Table 1). ORF40 resembles endodeoxyribonucleases of the RusA family (Pfam05866, not shown). The RusA protein of *Escherichia coli* is an endonuclease that can resolve Holliday intermediates and phage-encoded RusA-like endonucleases are thought to be involved in triggering replication restart in the case of replication fork stalling (44). ORF40 is thus possibly involved in DNA replication.

4 Discussion

In this study we have isolated and characterized a bacteriophage, ϕLmd1, which infects *Leuconostoc mesenteroides* subsp. *dextranicum* A1. The study was performed in order to gain knowledge on phages infecting dairy *Leuconostoc*, important flavor producers in a variety of fermented milk products.

The host bacterium, *Ln. mesenteroides* subsp. *dextranicum* A1, was isolated from a commercially available mixed mesophilic DL starter culture commonly employed in the industrial production of cultured butter and various cheese types. The host bacterium of ϕLmd1 was identified based on 16s rRNA gene sequencing, API50 CHL tests and the ability to grow in the presence of high salt concentrations. We were unable to determine the taxonomy of isolate A1 to a species level based on 16s rRNA gene sequencing. The sugar fermentation pattern of the isolate was clearly different from *Ln. mesenteroides* subsp. *cremoris* and *Leuconostoc mesenteroides* subsp. *mesenteroides* was excluded since isolate A1 did not ferment arbutin (6). The sugar fermentation of isolate A1 was in best accordance with fermentation patterns of *Ln. mesenteroides* subsp. *dextranicum* and *Ln. pseudomesenteroides*, however, the species *Ln. pseudomesenteroides* was excluded since isolate A1 was able to grow well in the presence of 6.5 % NaCl (16, 17). Interestingly, the 16s rRNA sequence of *Ln. mesenteroides* A1 is 100.0 % identical to that of *Leuconostoc* strains belonging to ribospecies CHCC2114. Strains of this ribospecies have repeatedly been isolated from fermented dairy products and have been assigned to both *Ln. mesenteroides* and *Ln. pseudomesenteroides* species (35). The taxonomic status for the CHCC2114 ribospecies was not resolved, and the authors suggested an investigation of the phylogenetic relationship between the two closely related species *Ln. mesenteroides* and *Ln. pseudomesenteroides* (35).

Bacteriophage Lmd1 was isolated from brine used in the production of Dutch-type cheese in a Norwegian dairy. ϕLmd1 belongs to the *Siphoviridae* family of tailed phages and has a B1 morphotype (Fig 1). All 76 *Leuconostoc* phages that had been described by the year 2000 were of the B1 morphotype, and this is also the most frequently encountered morphotype of dairy phages infecting *Lactococcus lactis* (2). In single step growth curve experiments, phage Lmd1

had an average burst size of 51, and lysis was completed by 30 minutes after adsorption (performed in MRS-C at 30°C). This shows that ϕLmd1 is capable of rapid growth, suggesting that only minute contamination of fermentation vessels can potentially affect flavor producing *Leuconostocs* sensitive to ϕLmd1. Many cheese manufacturers prepare bulk starter cultures which are used to inoculate cheese production vats. Bulk starters are often preferred for sensory reasons and because they give an increased initial fermentation speed, however, bulk starters are also especially vulnerable to phage attack (24) since phages are given ample time to multiply during both bulk starter production and the subsequent fermentation in cheese production vats. Thermal inactivation studies on ϕLmd1 revealed that the phage is unaffected by pasteurization but its titer was reduced by more than 7-log when exposed to a thermal inactivation scheme resembling commonly employed bulk starter vat sterilization schemes. It has been shown that other dairy *Leuconostoc* phages are rapidly inactivated at temperatures above 80°C and that 1.2 minutes holding time at 90°C was sufficient for a 9-log reduction in titer of the heat resistant Leuconostoc phage P793 (5). Since pasteurization does not affect ϕLmd1, there is no barrier for the bacteriophage to enter cheese fermentation vats, however, infection of bulk starter vats would require contamination during or after bulk starter milk cooling.

Surprisingly, ϕLmd1 inhibited the growth of all 48 *Leuconostoc* isolates from one starter culture. One important point of using a mixed starter culture is to reduce the impact of contaminating phages (34), and starter culture producers put great effort to incorporate phage unrelated strains into starter cultures. We were previously unable to isolate bacteriophages able to infect all *Lactococcus lactis* strains of a starter culture, even from samples of a failed fermentation (24). The finding that one phage was able to inhibit all isolated Leuconostocs of a starter culture can be explained by a large host range of ϕLmd1, or possibly, by a low number of phage unrelated Leuconostoc strains in the starter. In face of new *Leuconostoc* bacteriophages, like ϕLmd1, there is likely a need for an increased focus on selecting new phage-insensitive *Leuconostoc* strains for L- and DL-type starter cultures. Bacteriophage Lmd1 could be a valuable selection tool for identifying phage-insensitive strains.

To reduce phage impact on dairy fermentation, many dairies practice rotation of different phage-unrelated starter cultures (34). The observation that bacteriophage Lmd1 was able to multiply on 3 out of the 4 starter cultures examined in this study emphasize the importance of assaying for *Leuconostoc* phages during selection of starter cultures for rotation schemes.

Starter activity measurements can be suitable to identify phage attacks affecting important acid producers like *Lactocuccus lactis* but not flavor producing *Leuconostocs*. A growth inhibition assay containing key *Leuconostoc* strains from each starter culture could be a useful tool in identifying variable performance of flavor producing *Leuconostoc* strains due to phage attack, and could be a useful tool in advising starter culture rotation routines.

The genome of ϕLmd1 is closely related to the previously characterized genome of *Ln. mesenteroides* phage 1-A4, isolated from vegetable fermentation. The two phages are closely related both with respect to sequence similarity and genome organization (Table 1 and Fig. 6). Lu and co-workers used a functional distribution analysis to show the functional distance between ϕ1-A4 and 111 phages infecting lactobacilli, lactococci, streptococci, enterococci, listeriaea, bacilli, clostridia and others (28). They showed that *Leuconostoc* phage 1-A4 cluster most closely with several lactococcal phages including Q54-like, c2-like and 936-like phages (14), but they suggested that ϕ1-A4 should form a separate functional cluster based on the relatively large distance between it and its closest relatives. This is in agreement with the relatively low number of significant BLAST hits we found to phage sequences other than ϕ1-A4. The extensive similarities between ϕLmd1 and ϕ1-A4 are clearly indicative of a common evolutionary origin of these phages. It is possible that a common ancestor of the two phages found its way into dairy- and vegetable fermentations via the natural habitat of *Leuconostoc*, green plants and silage (6, 41). The two phages, ϕLmd1 and ϕ1-A4, shared homologous genes and nearly identical gene order in the putative modules for DNA replication, DNA packaging and virion morphogenesis, however, almost half of the predicted proteins in ϕLmd1 did not show any similarity to ϕ1-A4 ORFs (Fig 6.). The dissimilar ORFs were mostly found on the negative strand in both phages, in modules possibly involved in transcription regulation or host interaction. This putative

functional assignment is in agreement with the presence homologs of conserved *Leuconostoc* and *Weissella* prophage genes in this region.

PCR-based methods for detection, identification and quantification of lactococcal bacteriophages have been very useful in the study of dairy phages infecting *Lactococcus lactis* (13-15, 25). In an attempt to design PCR primers specific for *Ln. mesenteroides* bacteriophages, the complete genome sequences of ϕLmd1 and ϕ1-A4 were aligned (not shown). Due to low genetic conservation at the DNA level no primer pair specific for both phages could be designed. However, the genetic region corresponding to the major tail protein gene was the most conserved region, and will probably be the target of PCR primers specific to dairy *Ln. mesenteroides* phages when more sequences become available.

5 Conclusions

To our knowledge this work represents the first characterization of a publicly available genome from a bacteriophage infecting dairy *Leuconostoc*. Bacteriophage Lmd1 infects *Leuconostoc mesenteroides* subsp. *dextranicum* A1, isolated from a commercial mesophilic DL starter culture commonly employed in the production of cultured butter and various cheeses. Bacteriophage Lmd1 was capable of rapid growth and was unaffected by pasteurization. Moreover, ϕLmd1 inhibited all *Leuconostoc* isolates of one starter culture and was able to grow on 3 out of 4 starter cultures examined in this study. This emphasize the importance of selecting phage unrelated *Leuconostoc* strains for starter cultures and the importance of better monitoring of *Leuconostoc* phages in dairy environments. The new information gained from the genome sequence of a dairy *Leuconostoc* phage may deepen our understanding of phage genetics, and may aid the development of phage control technologies in dairy fermentations.

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8 Figure legends

Figure 1

A: Plaque morphology of ϕLmd1. Halos surrounding the clear plaques formed only after 48 hours incubation. Scale bar indicates 1 cm. B and C: Transmission electron micrographs of ϕLmd1 particles. Scale bars indicate 200 nm and 100 nm in B and C, respectively.

Figure 2

ϕLmd1 titers before (light grey) and after (dark grey) 16 hours incubation with *Ln. mesenteroides* A1 or commercial starter cultures A, B, C or D.

Figure 3

Analysis of ϕLmd1 genome ends. EcoRI restriction enzyme digested genomic DNA was analyzed by agarose gel electrophoresis with (+) or without (-) melting of cohesive genome ends prior to electrophoretic separation. Estimiated DNA fragment sizes are indicated on the right. Lane M contains 1 kb ladder (Invitrogen).

Figure 4

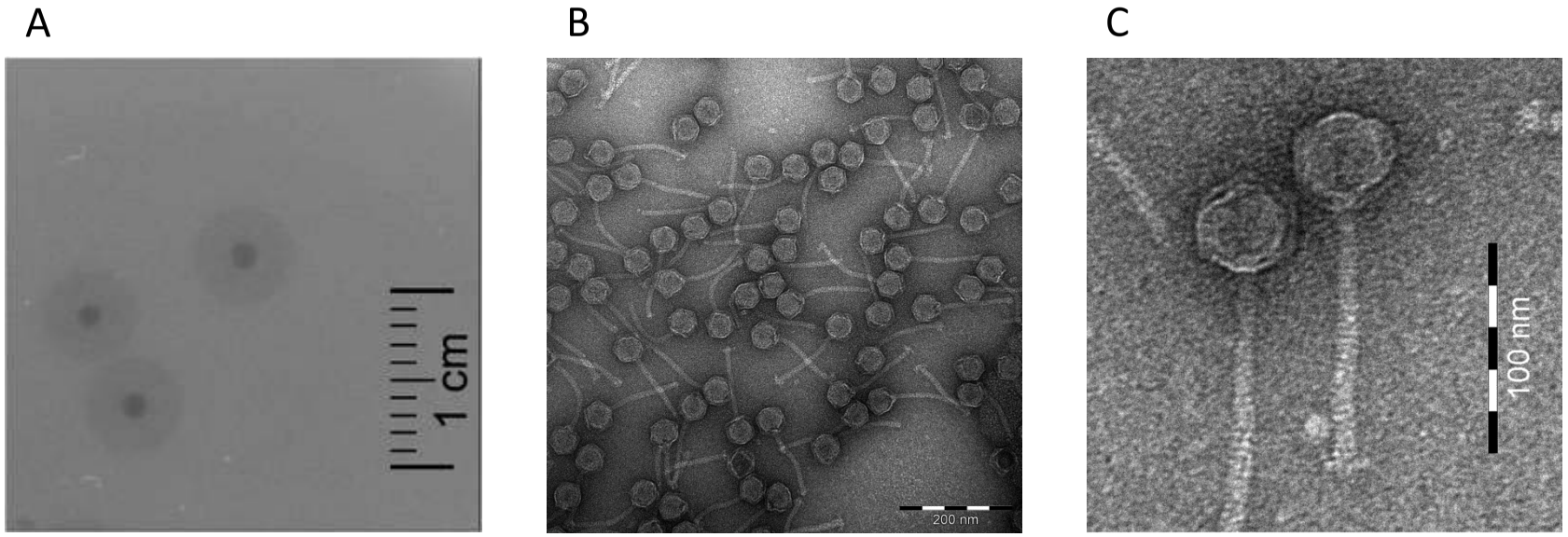
Genome map of ϕLmd1. Positions of the predicted open reading frames are indicated by arrows. Putative functions and functional modules are indicated above. Structural proteins identified by mass spectrometry in this study are indicated by grey arrows. The putative origin of replication is indicated by a black square, the three EcoRI recognition sites by crosses and the estimated cos-site position by an open circle. The scale bar marks genome positions at 2000 bp intervals.

Figure 5

SDS-PAGE analysis and identification of ϕLmd1 structural proteins. Lane M shows the protein ladder with molecular masses in kilodaltons (kDa) marked on the right. Lane ϕ contains purified phage particles. Protein identities determined by MS, calculated molecular masses in kDa and putative functions are shown on the left.

Figure 6

Genome comparison between *Leuconostoc mesenteroides* phages Lmd1 and 1-A4 (Genbank accession GQ451696). ORFs are indicated by numbered arrows. Grey connecting lines between ORFs indicate identities. Light grey indicate 20% identities and black lines 100%, according to the greyscale bar on the right. For detailed BLASTP scores between ORFs see Table 1. The locations of putative origins of replication (ori) and cos-sites (cos) are indicated. The scale bar below indicates 5000 bp.



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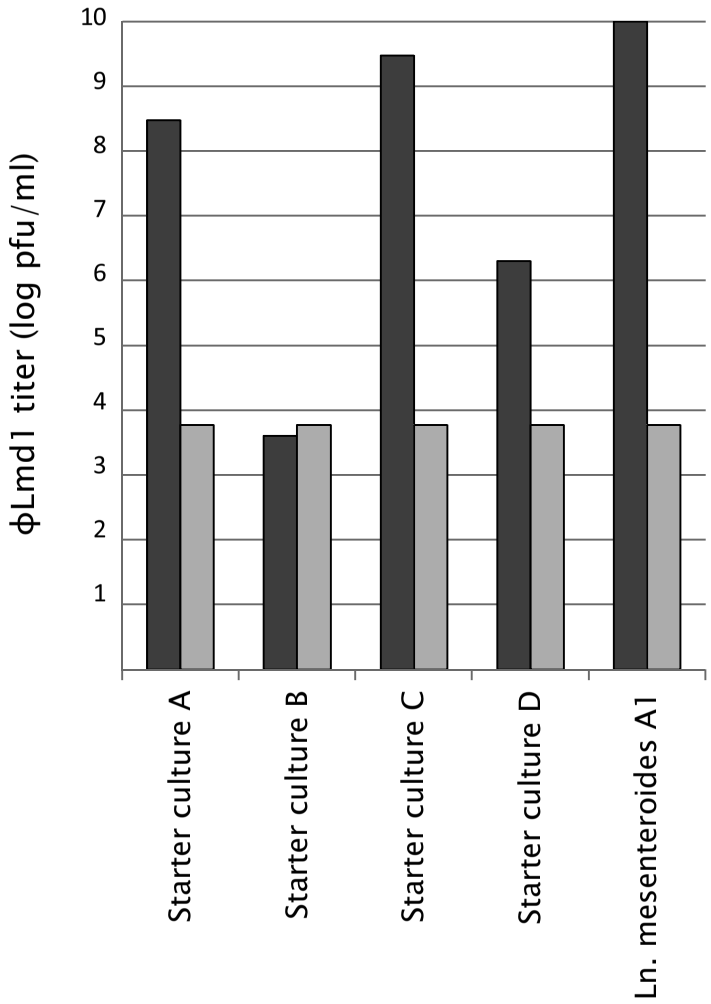

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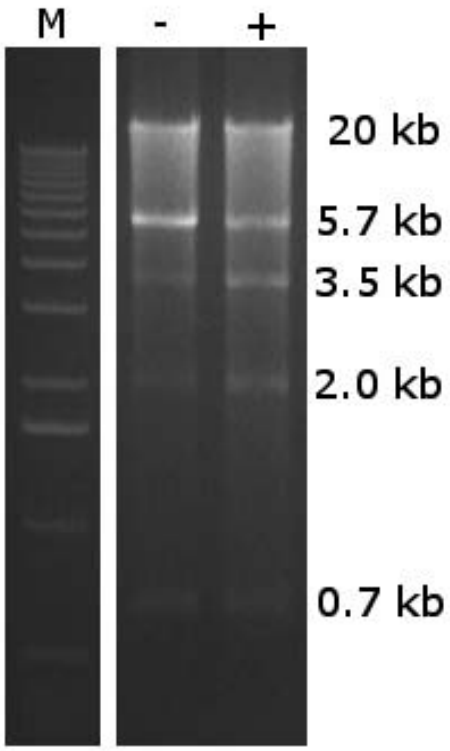

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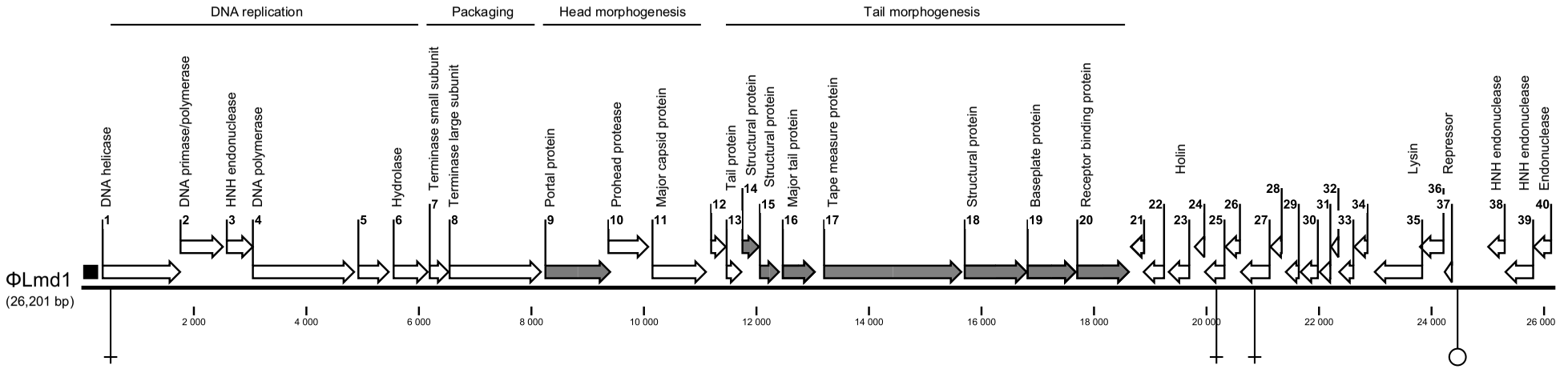

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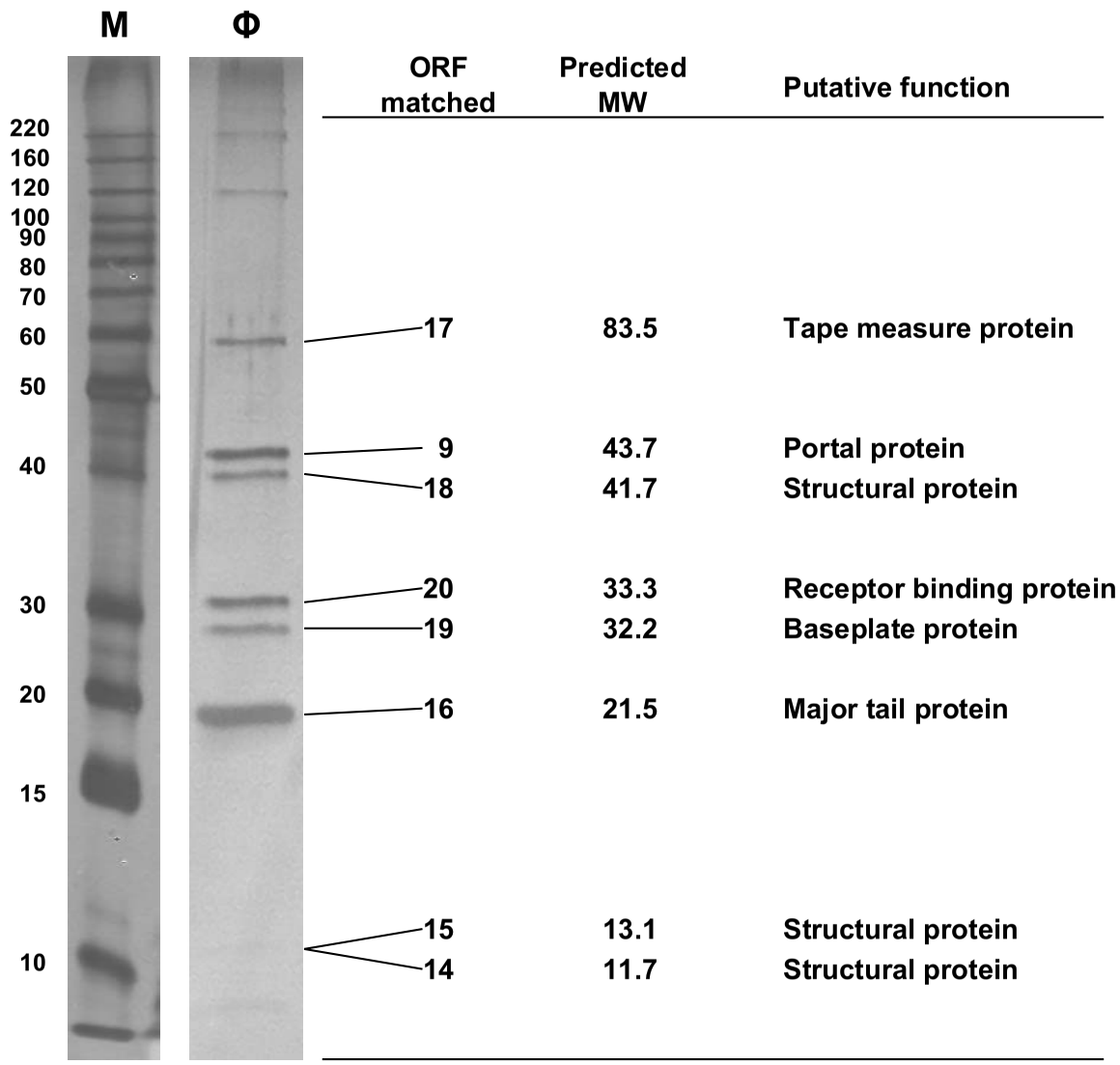
Table 1: Open reading frames of ϕLmd1 examined in this study.

ORF	Putative ribosomal binding site and start codon sequence [A]	Position [B] Start .. End	Size (aa)	Mol. wt. (kDa)	pI	Putative function [C]	Closest homolog (Extent, % identities, % positives, % gaps)	Size (aa)	Reference
1	TAAAGGAGGCCTAACATG	370 .. 1764	464	52.7	5.55	DNA helicase	*Lactobacillus plantarum* , WCFS1, Lp3 protein 8 (442/464, 21, 36, 11)	464	NP_785924
2	ACGGAGGTGCTTTTTATG	1754 .. 2524	256	29.8	7.01	DNA primase/polymerase	*Leuconostoc* phage 1-A4, LM1A4_003 (250/256, 41, 58, 6)	251	ADD71726
3	CCAAGAGGTGATGATATG	2577 .. 3056	159	18.4	9.88	HNH endodeoxyribonuclease	*Lactobacillus* phage A2, ORF57 (139/159, 40, 58, 0)	207	NP_680539
4	CGGGAGGATATACTTTTG	3040 .. 4857	605	68.6	6.17	Possible DNA polymerase	*Leuconostoc* phage 1-A4, LM1A4_005 (605/605, 52, 68, 1)	611	ADD71728
5	CAAGGAAGTGTAACAATG	4915 .. 5472	185	21.0	8.53	Cons.	*Leuconostoc* phage 1-A4, LM1A4_006 (188/185, 46, 68, 4)	190	ADD71729
6	TAAGAGAAGATAATCATG	5542 .. 6165	207	24.2	6.40	Hydrolase (tRNA 3' endonuclease)	*Leuconostoc* phage 1-A4, LM1A4_007 (209/207, 62, 77, 1)	210	ADD71730
7	GAGGTAATAACCAATATG	6184 .. 6534	116	13.4	4.95	Terminase small subunit	*Leuconostoc* phage 1-A4, LM1A4_008 (98/116, 41, 70, 0)	108	ADD71731
8	TACGGAGAATTGAGTATG	6537 .. 8177	546	62.9	5.50	Terminase large subunit	*Leuconostoc* phage 1-A4, LM1A4_010 (534/546, 56, 73, 1)	548	ADD71733
9	TTGAAAGGGCATGCTATG	8240 .. 9409	389	43.7	5.16	Portal protein *	*Leuconostoc* phage 1-A4, LM1A4_011 (357/389, 52, 70, 4)	373	ADD71734
10	CAGGGAGCACGGCTAATG	9360 .. 10085	241	26.0	4.88	Phage prohead protease	*Leuconostoc* phage 1-A4, LM1A4_012 (185/241, 57, 72, 4)	239	ADD71735
11	GAGGAAAATATTATAATG	10143 .. 11111	322	34.6	5.85	Major capsid protein	*Leuconostoc* phage 1-A4, LM1A4_013 (303/322, 49, 64, 3)	321	ADD71736
12	AAAGGAAACCGACTATTATG	11186 .. 11464	92	11.0	4.49	Cons.	*Leuconostoc* phage 1-A4, LM1A4_014 (86/92, 34, 65, 0)	90	ADD71737
13	TAGGTGGTGGCAAGAATG	11461 .. 11742	93	10.5	9.95	Phage tail protein	*Leuconostoc* phage 1-A4, LM1A4_015 (70/93, 39, 51, 1)	92	ADD71738
14	AGGAGGCAATCGCTAATG	11742 .. 12053	103	11.7	4.83	Structural protein *	*Leuconostoc* phage 1-A4, LM1A4_016 (105/103, 43, 66, 2)	105	ADD71739
15	AAGATGTAGTGGTGCTCTGATG	12053 .. 12409	118	13.1	10.48	Structural protein *	*Leuconostoc* phage 1-A4, LM1A4_017 (114/118, 40, 54, 7)	109	ADD71740
16	ACGTGAGGATAATAAAAACATG	12460 .. 13044	194	21.5	5.10	Major tail protein *	*Leuconostoc* phage 1-A4, LM1A4_018 (189/194, 63, 74, 1)	193	ADD71741
17	AAAAGGAGCTTTTAAATG	13194 .. 15650	818	83.5	10.36	Tape measure protein *	*Leuconostoc* phage 1-A4, LM1A4_019 (859/818, 34, 52, 13)	889	ADD71742
18	TGATATAATCGTAGTATG	15694 .. 16809	371	41.7	4.93	Structural protein *	*Leuconostoc* phage 1-A4, LM1A4_020 (404/371, 28, 49, 11)	398	ADD71743
19	ATTTGGAGACTAGAGATG	16812 .. 17681	289	32.2	5.11	Baseplate protein *	*Leuconostoc* phage 1-A4, LM1A4_021 (334/289, 33, 48, 14)	332	ADD71744
20	AAGAAAGGTAATAATATG	17695 .. 18627	310	33.3	8.79	Reseptor binding tail protein *	*Leuconostoc* phage 1-A4, LM1A4_023 (142/310, 39, 61, 5)	255	ADD71746
21	ATTGTTTAATCAATTTCTTG *(18648 .. 18899)*		83	9.4	9.83	Cons.	*Leuconostoc* phage 1-A4, position (19145..19360) (41/83, 54, 78, 0)	72	GQ451696
22	AAAGGACGAACCAACATG *(18874 .. 19254)*		126	14.6	4.78	Cons.	*Leuconostoc* phage 1-A4, LM1A4_022 (114/126, 49, 67, 2)	133	ADD71745
23	AATGGAAACGTAATAATG *(19319 .. 19702)*		127	14.2	6.09	Holin	*Leuconostoc* phage 1-A4, LM1A4_024 (109/127, 43, 62, 0)	123	ADD71747
24	TAGGAGATAAAAATAATG *(19782 .. 19970)*		62	7.0	7.09		No significant similarity		
25	AAAGGGTGAATTTTAATG *(19963 .. 20328)*		121	13.2	4.47		No significant similarity		
26	AAAGGAGAACAACTAATG *(20328 .. 20603)*		91	10.7	9.46	Cons.	*Paenibacillus lactis* 154, predicted protein (87/91, 41, 57, 0)	106	ZP_09002987
27	TAAGGAGCCAGAGAGATG *(20603 .. 21130)*		175	20.4	5.19	Cons.	*Weissella paramesenteroides* ATCC33313, predicted protein (68/175, 53, 71, 1)	141	ZP_04782166
28	AGGAGATGATATATTTTG *(21127 .. 21345)*		72	8.1	5.52	Cons.	*Leuconostoc* sp. C2, LGMK_06970 (60/72, 47, 73, 0)	61	YP_004706067
29	TTAAAGGAAAATAGAATG *(21401 .. 21649)*		82	9.7	4.66		No significant similarity		
30	TGATGGAGATATATGATG *(21665 .. 21988)*		107	12.6	4.63	Cons.	*Leuconostoc gelidum* KCTC 3527, predicted protein (79/107, 41, 59, 11)	112	ZP_08479517
31	GAGAGGATTTTCAAAAAATG *(22001 .. 22210)*		69	8.2	9.42		No significant similarity		
32	ACGAGGTAAATTGGTATG *(22207 .. 22353)*		48	6.1	11.41		No significant similarity		
33	AAAGGCGCAATTATAATG *(22350 .. 22619)*		89	10.7	9.97		No significant similarity		
34	AAAGGAGGTCACAAAAGATG *(22619 .. 22870)*		83	9.7	9.47		No significant similarity		
35	AAGAGGCAGAGGCAGTTG *(22982 .. 23842)*		286	32.1	6.83	Lysin	*Leuconostoc* sp. C2, LGMK_06825 (180/286, 83, 89, 0)	319	YP_004706038
36	GAAGAGGATTAACACATG *(23784 .. 24221)*		145	15.5	7.19	Cons.	*Leuconostoc citreum* KM20, LCK_01036 (132/145, 48, 65, 0)	140	YP_001728307
37	AAAGGAACGATAAGACTATG *(24225 .. 24371)*		48	5.6	5.47	Repressor	*Clostridium scindens* ATCC 35704, CLOSCI_00042 (45/48, 42, 64, 0)	59	ZP_02429839
38	ATTGTGTTCAAAGAATTATG *(24998 .. 25309)*		103	12.4	8.88	HNH endonuclease	*Leuconostoc* phage 1-A4, LM1A4_047 (90/103, 42, 58, 0)	100	ADD71770
39	AAGGGGGCTAAAAACAAAAATG *(25306 .. 25815)*		169	18.9	10.08	HNH endonuclease	*Enterococcus* phage EFRM31, gp11 (166/169, 45, 61, 5)	173	YP_004306639
40	GGAATGAGGTTGCACAAATG *(25808 .. 26134)*		108	12.7	9.03	Endodeoxyribonuclease	*Leuconostoc* phage 1-A4, LM1A4_050 (106/108, 69, 80, 1)	124	ADD71773

[A] *Predicted ribosomal binding site and start codons are underlined.*

[B] *ORF positions on complement strand are shown in parentheses*

[C] *Putative function based on BLASTP and PSI-BLAST results. Structural proteins identified in this study are indicated with an asterix.*



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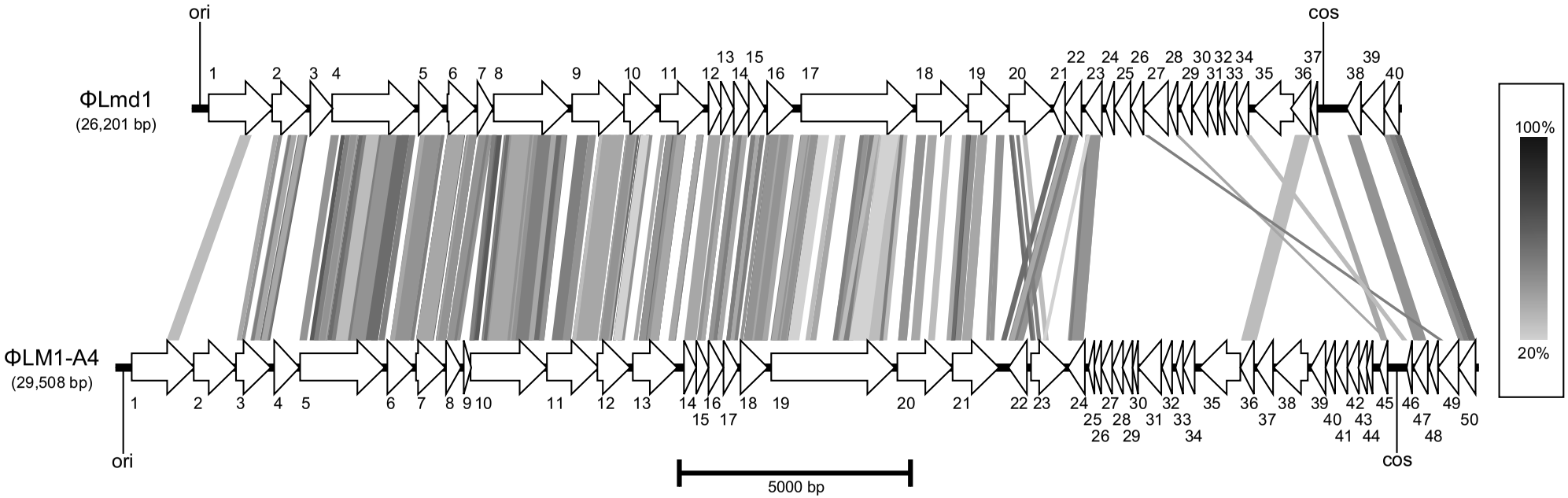

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Paper III

A novel bacteriophage of the *Podoviridae* family infecting *Weissella cibaria* isolated from kimchi

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ABSTRACT

The first complete genome sequence of a phage infecting *Weissella cibaria* (*Weissella kimchii*) is presented. The bacteriophage phiYS61 was isolated from kimchi, a Korean fermented vegetable dish. Bacteriophages are recognized as a serious problem in industrial fermentations, however, phiYS61 differed from many virulent phages associated with food fermentations since it was difficult to propagate and was very susceptible to resistance development. Sequence analysis revealed that phiYS61 resembles *Podoviridae* of the subfamily *Picovirinae*. Within subfamily *Picovirinae*, the phi29-like phages have been extensively studied, and their terminal protein-primed DNA replication is well characterized. Our data strongly suggest that phiYS61 also replicates by a protein-primed mechanism. Weissella phage phiYS61 is, however, markedly different from the *Picovirinae* with respect to genome size and morphology. *Picovirinae* are characterized by small (approximately 20 kb) genomes which were contrasted by the 33,594 bp genome of phiYS61. Based on electron microscopy analysis, phiYS61 was classified as a *Podoviridae* of morphotype C2, similar to the phi29-like phages, but its capsid dimensions were significantly larger than those reported for these phages. The novelty of phiYS61 was also emphasized by the low number of ORFs showing significant similarity to database sequences. We propose that the bacteriophage phiYS61 should represent a new subfamily within the family *Podoviridae*.

1 INTRODUCTION

Kimchi, a traditional Korean dish, is manufactured by fermentation of vegetables such as Chinese cabbage and radish. Hundreds of kimchi varieties are produced by the addition of different seasonings, such as scallions, powdered chili peppers, garlic, ginger and fermented seafood. Lactic acid produced during fermentation contributes to preservation and gives kimchi its characteristic sour taste. Proper ripening and preservation is ensured by 2-5 % (wt/vol) salt content and anaerobic fermentation at low temperatures. Traditionally, kimchi is prepared by spontaneous fermentation by lactic acid bacteria (LAB) indigenous to the vegetable ingredients. However, starter cultures have been developed in order to better control the fermentation and thus improve the safety and shelf-life of kimchi (16, 17, 20, 23), or its potential health benefits (37).

The microbial composition of kimchi fermentations has been investigated by culture dependent (6, 18, 19, 33, 36, 38, 40, 57) and culture independent methods (30, 34, 39, 50, 53). Several LAB species have been identified as likely contributors in kimchi fermentations, including the *Leuconostoc* species *Leuc. mesentereoides, Leuc. kimchii, Leuc. citreum, Leuc. gasicomitatum* and *Leuc. gelidum*, the *Lactobacillus* species *Lb. brevis, Lb. curvatus, Lb. plantarum* and *Lb. sakei, Lactococcus lactis, Pediococcus pentosaceus*, and the *Weissella* species *W. confusa, W. kimchii* and *W. koreensis*. The species *Weissella kimchii*, first described in 2002 (19), was reclassified as *Weissella cibaria* in 2004 (9, 22).

Several studies have been carried out to investigate the microbial population dynamics in kimchi fermentations (6, 15, 18, 30, 36, 50, 52). Reportedly, successions of various LAB species and their metabolic activities are responsible for the quality and safety of these products. The kimchi fermentation process is characterized by an initial heterofermentative phase followed by a homofermentative phase. Which bacterial species dominates the different stages of kimchi fermentation varies with the ingredients used and fermentation conditions such as temperature, salinity etc. Generally, *Weissella* species are abundant in the late fermentation and can continue to grow during storage at low temperatures (-1°C). *Weissella* species have thus been associated with the excessive acidic taste of over-ripened kimchi products.

Metagenomic analysis of kimchi microbial dynamics revealed high abundance of phage DNA, indicating that bacteriophages affect kimchi fermentation and might be an important determinant of kimchi microbial dynamics (30). Bacteriophage involvement in bacterial community succession has also been reported in sauerkraut fermentation (41). Phages are apparently responsible for the variability observed in such vegetable fermentations (8). Bacteriophages have long been recognized as a problem in industrial fermentations, and various countermeasures are employed for their control (49). Repeated

use of standardized starter cultures fixes the phage sensitivity of the bacterial population throughout the period one starter is used. This will lead to an increased vulnerability to phage induced fermentation failure, and to the possible establishment of an in-house flora of detrimental bacteriophages (49). More knowledge on bacteriophages affecting industrial fermentations will be valuable for the development of improved bacteriophage counter-measures.

Recently, a bacteriophage infecting a *Weissella cibaria* starter culture strain used in the fermentation of Thai Nham sausage was reported (54). Bacteriophages likely infecting the genus *Weissella* was also reported in a sauerkraut fermentation, but the bacterial isolate was not conclusively identified (41). Here we report on a novel *Weissella cibaria* bacteriophage, phiYS61, isolated from kimchi fermentation, and present the first complete genome sequence of a phage infecting the genus *Weissella*.

2 MATERIALS AND METHODS

2.1 Bacteriophage isolation, growth and purification

Bacteriophage phiYS61 was isolated from a commercial Chinese cabbage kimchi purchased at a Korean hypermarket one week after manufacture. For bacteriophage isolation and determination of phage titers, log-phase host strain *Weissella cibaria* YS61 cells were inoculated (≈3×10^7 colony forming units/ml) in MRS (Oxoid, Basingstoke, Hampshire, United Kingdom) soft agar supplemented with 5 mM $CaCl_2$ (MRS-C) and cast on MRS-C agar slants. Phage suspensions were spotted on top and plaques isolated and/or counted after overnight incubation at 30 ˚C. Bacteriophage amplification was carried out at 30 ˚C in MRS-C. Cells were grown to OD_{600} = 0.3-0.4 before infected with 1/20 volume of filtered (0.45 µm) phage lysate (MOI approx. 10) and incubated until lysis occurred (approximately 2 hours). Lysis was completed by the addition of 0.5 % chloroform and 1 M NaCl. The mix was incubated on ice for one hour before chloroform and cell debris was removed by centrifugation. Phage particles were precipitated with polyethylene glycol (PEG) and purified on Caesium Chloride (CsCl) gradients as described elsewhere (12), except that all centrifugation steps before ultracentrifugation in CsCl gradients were carried out at 5000 × *g*, 4 ˚C. Purified phage particles were dialyzed against TM buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$) and stored at 4 °C. Single step growth curve analysis was performed essentially as described elsewhere (29).

2.2 DNA purification

Phage precipitated with PEG was treated with 1 µg/ml DNase I and 10 µg/ml RNase A for 1 hour at 37 ˚C, and then incubated for 1 hour at 65 ˚C with 25 mM EDTA, 0.5 % sodium dodecyl sulphate (SDS) and 200 µg/ml proteinase K (QIAGEN). After removal of residual PEG by chloroform extraction, standard phenol/chloroform extraction and ethanol precipitation was used to obtain phage DNA.

2.3 Sequencing and sequence analysis

A shotgun library was made in pUC19 (61) after partial digestion with Sau3A. Insert sizes ranged from 0.3 to 5 kb with an approximate average of 1.6 kb. Clones were sequenced using BigDye 3.1 chemistry (Applied Biosystems, Foster City, CA, USA) and standard M13 primers. Gaps were filled by primer walking on PCR amplified genomic DNA. Sequence assembly, bioinformatic analyzes and genome annotation were done using CLC Main Workbench version 6.1.1 (CLC bio, Aarhus, Denmark). Open reading frames

(ORFs) and ribosomal binding sites (RBS) were identified using the Prodigal (28) online tool (http://prodigal.ornl.org) and homology searches were done using BLASTP and PSI-BLAST build 2.2.25+ (3, 4) at www.ncbi.nlm.nih.gov (September 2011). Conserved domains were found by searching the Conserved Domains Database (43-45) at www.ncbi.nih.gov (September 2011). Promoters were predicted by manual inspection of intergenic regions and by use of the Prokaryote Promoter Prediction tool (http://bioinformatics.biol.rug.nl/websoftware/ppp)(62). Putative transcription terminators were found through manual inspection of RNA secondary structures predicted using the CLC software.

2.4 Analysis of structural proteins

Purified phage particles were denatured at 100 ˚C for 10 minutes in Laemmli buffer (35) and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stained with Coomassie Brilliant Blue R250 (BIORAD). Visible protein bands were excised, trypsin treated and extracted as previously described (56). Extracted peptides were desalted with C_{18} Stage tips (55). The peptides were eluted with 70 % acetonitrile before being mixed with an equal volume of matrix solution (15 mg/ml alpha-cyano-4-hydroxy cinnamic acid in ethanol:acetonitril 1:1), and applied to a matrix-assisted laser desorption ionization (MALDI) target plate (Bruker Daltonik GmbH, Bremen, Germany). Peptide mass finger-printing (PMF) and tandem mass spectrometry (MS/MS) were performed on an Ultra Flex MALDI-tandem time of flight (MALDI-TOF/TOF) instrument (Bruker Daltonik). The mass range for MALDI-TOF/MS was 800 to 4,000 Da, with a mass accuracy of 50 ppm. The mass range for MALDI-TOF/MS data acquisition was 800 to 4,000 Da and the spectra were externally calibrated using a peptide calibration mixture (Bruker Daltonik) ranging from 757 to 3147 Da. Protein identification was carried out using Mascot software (Matrix Science Ltd., London, UK) with searches against the NCBI database and a database containing all predicted ORF sequences (≥ 50 amino acids) from the genome sequences of phiYS61 and one member of *Siphoviridae* infecting *Leuconostoc spp* (unpublished data).

2.5 Electron microscopy

Purified phage samples were negatively stained with 2% (w/v) phosphotungstic acid (pH 7.2) on a carbon-formvar membrane grid and examined by transmission electron microscopy (TEM). TEM analysis was performed on an EF-TEM Leo 912AB (Carl Zeiss Inc., Germany) at an accelerating voltage of 120 kV. Electron micrographs were taken at 200,000 × magnification at the Korea Basic Science Institute in Chuneheon. The phage sizes were determined from the average of five independent measurements.

2.6 Transcription analysis

The host bacterium was grown to OD_{600} = 0.3 in 50 ml MRS-C, infected with 1-2 MOI of phages, incubated on ice for 30 minutes and then rapidly brought to 30 ˚C in a water bath. After 10 and 36 minutes, 10 ml samples were taken, cells washed twice in ice cold TE (pH 7.4) and quickly frozen in ethanol and ice. Cell disruption in a FP120 FastPrep bead beater (Bio101/Savant) and total RNA isolation by use of the RNeasy Mini kit (QIAGEN) were performed as described elsewhere (59). To avoid residual DNA carryover an additional digest with RNase-Free DNase I (QIAGEN) in RDD buffer (QIAGEN) was done at 37 ˚C for 30 minutes. DNase was removed by phenol:chloroform extraction and RNA precipitated with ethanol at -20 ˚C over-night. The RNA was washed once in 70 % (v/v) ethanol in DEPC water, once in 96 % ethanol, dried for 5 minutes at 45 ˚C in a SPD 2010 SpeedVac concentrator (Savant) and dissolved in RNase-free water. RNA concentrations were measured on a ND-1000 spectrophotometer (NanoDrop Technologies) and RNA integrity assessed using the RNA 600 Nano LabChip kit and an Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's instructions. cDNA was synthesized by use of random hexamer primers and the SuperScript™ III Reverse Trascriptase kit (Invitrogen) as instructed by the manufacturer. Transcript boundaries were analyzed by PCR using the primers listed in table S1, and with genomic DNA as positive control.

2.9 Nucleotide sequence accession number.

The genome sequence of phage phiYS61 has been deposited in the GenBank database under accession number JQ341413.

3 RESULTS

Bacteriophage phiYS61 infecting *Weissella cibaria* YS61 was isolated from a one week old vegetable kimchi purchased at a Korean hypermarket. To better understand bacteriophages infecting the genus *Weissella* and possibly gain insight on the dynamic microbial populations of kimchi fermentation the virulence, morphology and genome of phiYS61 was explored.

3.1 Virulence and resistance development

The infection characteristics of phiYS61 were assessed by single step growth analysis. After a latent period of about 48 minutes the lytic life circle ended with the release of approximately 50 progeny phages. When spotted on host strain YS61 in MRS-C soft-agar, phiYS61 produced small plaques (approximately 0.5 mm in diameter) with slightly diffuse edges. Growth of phage resistant bacteria was observed during phage amplification. To investigate this, YS61 and mixtures of YS61 and phiYS61 (MOI=0.01) were inoculated on MRS-C plates. Resistant colonies appeared at a rate of about 10^{-4} compared to the colony counts of uninfected YS61. Twelve phage insensitive colonies (YS61-R1 to R12) were isolated, cast in soft-agar and challenged with 10-fold dilution series of phiYS61. Compared to YS61, two of the isolates showed similar phage sensitivity in this assay, however, plaques produced on these isolates differed from those on YS16 being diffuse and hard to identify. Seven isolates displayed similar diffuse plaque morphologies and showed 2-3 log reduction in plaque counts compared to the YS61 strain. One isolate (YS61-R10) showed a 3 log reduction in plaque counts but with clear plaque morphology, similar to those displayed by the wild-type YS61. Two isolates (YS61-R2 and R3) showed complete resistance to phiYS61 infection. All but two isolates retained their reduced phage sensitivity after repeated subculturing without phage challenge. YS61-R10 reverted to wild-type phage sensitive phenotype while the more persistent YS61-R3 isolate displayed only slight growth inhibition when challenged with undiluted phage suspension (2×10^8 pfu/ml).

3.2 Morphology

Electron microscopy (Fig. 1) showed that phage phiYS61 has a moderately elongated capsid (85 by 36 nm) and a short non-contractile tail. This identified phiYS61 as a member of *Podoviridae* of the C2 morphotype (1). The distinct baseplate (28 nm wide) has 6 appendages (6 nm long) and a central spike. The distance from the head-tail interface to the spike tip was measured to 29 nm.

3.3 The genome of phiYS61

The complete genome sequence of phiYS61 was obtained through a combination of shotgun sequencing and primer walking with an average 6.3 fold sequencing coverage. The linear double-stranded DNA genome was 33,594 bp long, with a DNA base composition of 43.9 % G+C. A total of 48 open reading frames (ORFs) were predicted (Table 1 and Fig. 2). Forty one of the ORFs had a putative ribosomal binding site (RBS) upstream of the predicted start codon, and one ORF's translation was probably initiated by a frameshift event (described below). The predicted ORFs comprised 86 % of the genome. Also present were 25-bp inverted terminal repeats, which are characteristic for the *Picovirinae* subfamily that include the phi29-like phages (42).

Transcription

To identify transcript borders, RNA was purified from infected YS61 cells and analyzed by reverse transcription PCR amplification of intergenic regions. Four early and five late transcripts were found. Transcripts are mapped on the genome in Fig. 2. Our results indicated that early transcription progresses inward from the ends of the linear genome, transcripts E1, E2 and E3 from the right end and transcript E4 from the left. Predicted genes on late transcripts were found on the positive strand downstream of E4 with the exception of *orf21*, transcript L3, which was found on the negative strand.

Putative promoters, resembling the consensus *Escherichia coli* σ^{70} promoters were identified upstream of all transcript regions. Upstream of early transcripts E1 and E2 were also found promoter signals resembling recognition sites for the lactococcal two-component regulator LlrB (51). Moreover, putative promoters upstream of E1 were found in a tandem repeat region comprised of two repeat units, 1 (66 bp) and 2 (35 bp) repeated in the order 1-2-1-2-1-2-1. Tandemly repeated promoters have been shown to result in strong promoter activity in the *Ralstonia solanacearum* phage ϕRSB1 (32).

As shown in Fig. 2B, putative Rho-independent terminators were detected in seven of the eight intergenic regions found by PCR to exert transcription termination. A putative mechanism for transcription termination in the *orf46* (E2) - *orf45* (E3) intergenic region was not identified.

Several putative promoters and terminators identified outside the confirmed start-stop regions are probably involved in modulating transcription activity and consequently downstream gene expression.

3.4 Functional assignment of ORFs.

BLAST searches revealed that 18 of the 48 ORFs were similar to other known sequences whereas 30 ORFs produced no significant hits in database searches. Putative function was assigned to 16 ORFs by sequence comparison or MS identification of structural proteins (Table 1). Based on putative function, ORFs could be grouped in functional modules. Predicted module borders matched well with transcript borders identified by PCR (Fig. 2).

ORFs on early transcripts E1, E2 and E3

Twenty open reading frames, *orf28* to *orf47*, were predicted on early transcripts E1 to E3. BLAST searches revealed similarity to known sequences for 5 of these ORFs (Table 1). ORF41 showed weak resemblance to a *Lactobacillus* transcriptional regulator (YP_004286453). A putative function was assigned to the *orf42* gene product by its significant similarity to proteins of the HNH family of homing endonucleases commonly found in phage genomes.

DNA replication

Four open reading frames (*orf 1* to *orf 4*) were predicted in the genomic region corresponding to early transcript E4. BLAST searches revealed that *orf4* putatively encoded a DNA polymerase (DNAP) type B, similar to that of *Streptococcus* phage Cp-1 (47) and the phi29-like phages (48). We identified the highly conserved DNAP motifs ExoI, ExoII, ExoIII, CT, motif-1, 2a, 2b and 3, and found insertion sequences similar to TPR-1 and TPR-2 conserved among protein-primed DNA polymerases (31, 48). Sequence comparison of ORF4 and the 36 seed sequences of the Pfam DNAP family B (PF03175) showed that ORF4 grouped phylogenetically with the phage encoded protein primed DNA polymerases (Fig. 3). In addition to the polymerase, three proteins are essential to DNA replication in the phi29-like phages (10). These are the terminal DNA-bound protein (TP; gp3 in phi29) a single strand DNA binding protein (SSB; gp5 in phi29) and a double strand DNA binding protein (DSB; gp6 in phi29), and are encoded by genes immediately upstream of the DNAP encoding gene. Based on their genomic localization, *orfs 1*, *2* and *3* possibly encode proteins with similar involvement in DNA replication. The high pI values of the predicted ORF 1-3 proteins might indicate interactions with negatively charged molecules such as DNA, but BLAST results did not corroborate this. The presence of a covalently bound terminal protein was, however, indicated by the observation that Proteinase K treatment was essential for purification of phiYS61 DNA. An alignment of the deduced 261 amino acid (aa) sequence of phiYS61 ORF3 to the terminal proteins (167-266 aa) of *Bacillus* phages phi29, B103, GA-1 and Nf, *Enterobacteria* phages PRD1 and L17,

Lactococcus phage asccphi28 and *Streptococcus* phage Cp-1 revealed conservation of functional amino acids (31) and predicted secondary structures, despite the low overall sequence similarity (Fig. S1). These data and the presence of inverted terminal repeats in the phiYS61 genome strongly suggest that phiYS61 DNA replication occurs by a protein-primed mechanism, and that *orf3* and *orf4* encode the terminal protein and the protein primed DNA polymerase respectively.

DNA packaging

A strong indication of homology was found between the predicted *orf5* encoded protein and the DNA encapsidation protein of Bacillus phage B103 and other phi29-like phages. The phi29-like DNA encapsidation protein is a DNA packaging ATPase that together with packaging RNA (pRNA) and the head tail connector protein form a packaging motor (58). Strong similarity was also observed between the putative head tail connector of phiYS61 and those of the phi29-like phages. Comparison of phi29 and Cp-1 pRNA structures (5, 46) to computer predicted phiYS61 RNA secondary structures revealed no significant pRNA prediction.

Structural proteins

Structural proteins were analyzed by SDS-PAGE and five protein bands were identified by mass spectrometry analysis (Fig. 4). Three distinct SDS-PAGE bands were identified as variants of ORF12. Of these, the lower two bands were identified as ORF12A and ORF12B. The predicted masses of ORF12A and ORF12B, 45.4 and 53.9 kDa, respectively, were consistent with the observed protein migration during SDS-PAGE. The third ORF12 variant with an observed mass of about 56 kDa could possibly result from an additional frameshift event or from post-translational modification. We were unable to identify the additional peptides observed in this band. BLAST searches revealed that ORF12A is homologous to the major capsid protein of *Enterococcus* phage EF62phi. ORF12B contains a bacterial group 2 immunoglobulin-like domain (Big_2, Pfam accession PF02368) and is more similar to the major capsid protein of the phi29-like phages. Immunoglobulin-like (Ig-like) domains are frequently found on the surface of tailed dsDNA phages and have been proposed to interact with carbohydrates on the cell surface and thereby facilitate phage adsorption (24). Moreover, Ig-like domains are commonly added to phage structural proteins by programmed ribosomal frameshifts (25). We identified two possible slippery sequences in the *orf12A*-*orf12B* overlapping region: CAG.GGG.TAA (position 9609-9618, +1 frame codons marked with dots and the +3 frame alternative codon underlined) in which a $tRNA^{Gly}$ could slip back one

base, and GAC.CCG.TCC (position 9598-9606) where a $tRNA^{Thr}$ (3'-UGG-5') could bind the proline CCG codon thereby making the third codon base available for pairing with the following $tRNA^{Val}$ anticodon (7). Our MS data did not distinguish between the two frameshift positions.

The gene products of *orf9* and *orf26* were also identified by MS as components of the phiYS61 virion. Based on its genomic location we suspect that *orf9* encodes a capsid protein, however, no sequence similarity was found to known proteins. *Orf26* is probably a tail protein based on its location on transcript L5 downstream of the genes encoding the putative tail, connector, lower collar and tail fiber proteins (*orfs 21*, *22*, *23* and *24*, respectively).

The predicted *orf20* encoded protein shares homology and essential catalytic residues with the tail associated cell wall degrading enzyme, gp13, of Bacillus phage B103 and the phi29-like phages. Gp13 is most likely involved in the penetration of the phage tail through the peptidoglycan layer (60). Gp13 of phi29 have also been shown to be essential for phage tail assembly interacting with gp9 (tail protein) after gp11 (lower collar) has assembled onto the phage capsid (26, 27).

Host cell lysis

We identified a putative muramidase (ORF18) with 50 % amino acid identity to lytA of prophages integrated in *Weissella paramesenteroides* ATCC33313 and several *Leuconostoc* sp. A putative holin (ORF27) was identified by its sequence similarity to the holin (gp24) of Leuconostoc phage 1-A4. Significant sequence similarity was also found to the Pfam holin_4 family (PF05105).

4 DISCUSSION

The bacteriophage phiYS61 infecting *Weissella cibaria* YS61 was isolated from Korean kimchi. Here we report the characterization of phiYS61 and present the first complete genome sequence of a phage infecting the genus *Weissella*.

The host strain was originally classified as *Weissella kimchii* (19), however the two species *W. kimchii* and *W. cibaria* (9) are very closely related and have been proposed to be one species named *Weissella cibaria* (22). The G+C content of the 33.594 bp phiYS61 genome was 43.9 %, which is more similar to the reported 43.9 to 44.9 % G+C of *Weissella cibaria* (9) than the 48.2 % reported for the *Weissella kimchii* strain CHJ3 (19). Most of the predicted phiYS61 ORFs with significant similarity to known sequences showed homology to proteins of phages and bacteria commonly associated with soil, vegetables and vegetable fermentation (Table 1). This is in agreement with the extensive horizontal gene transfer observed between bacteriophages in closely related environments (14, 21).

Bacteria of the genus *Weissella* are commonly encountered in vegetable fermentations and bacteriophages infecting them could be responsible for variability in fermentation progress and product quality. Better understanding of bacteriophages in industrial fermentations is of great importance for advising phage counter-measures. We showed that phage resistant YS61 mutants readily appear during infection experiments with phiYS61, and it was necessary to filter out resistant bacteria during serial propagation of phiYS61. A kimchi fermentation is a highly competitive environment (30), and phage resistance is often associated with reduced fitness (11). Thus, the readily appearing phiYS61 resistant mutants might not be able to prevail in actual kimchi fermentation.

Another aspect of kimchi fermentation where bacteriophage knowledge could be useful is in the prevention of over-ripening. *Weissella* sp. have been associated with excess acid formation and reduced shelf-life of kimchi. Bacteriophages or phage lysins could possibly be used to significantly reduce the growth of *Weissella* and other unwanted bacteria during kimchi storage. This could also be implemented for other fermented products, for instance in Korean rice wine where *Weissella cibaria* is a likely cause of quality deterioration (S. K. Yum, Seoul Takju Manufacturers' Assossiation, personal communication). The putative lysin identified in this study showed homology to lytA of a *Weissella paramesenteroides* prophage (Table 1). It is possible that the phiYS61 lysin could be applied to increase shelf life of kimchi and other fermented vegetable products.

Morphologically, phage phiYS61 resembled the recently reported *Weissela cibaria* phage phi22 isolated from a fermented pork sausage (54). The C2 morphotype of *Podoviridae* is relatively rare and had by the year 2000 only been reported for 39 bacteriophages, whereas the C1 *Podoviridae* morphotype had been

reported 631 times (2). The observed capsid dimensions (85 by 36 nm) were smaller than the 92 by 50 nm capsid reported for *Podoviridae* phi22 infecting *Weissella cibaria* N22 (54). Genome size is often proportional to capsid dimensions, however, the genome of phiyS61 (34.5 kb) is larger than the genome size (29 kb) reported for phi22 (54).

Predicted proteins encoded by *orfs* in the putative structural modules of the phiYS61 genome resembled structural proteins of phages of the *Podoviridae* subfamily *Picovirinae*, as well as the predicted enterococcal phage EF62phi (13) (Table 1). EF62phi is a phage-like extrachromosomal linear genetic element identified during *Enterococcus faecalis* EF62 genome sequencing. *Enterococcus* species have been found along with *Weissella* in vegetable fermentation (30) and genetic exchange between these species are thus likely. The morphology of the EF62phi-like enterococcal phages is unknown but the phi29-like *Picovirinae* belong to the C2 morphotype, however, with smaller capsid dimensions and different baseplate structures than phiYS61 (48).

With respect to DNA replication and packaging, phiYS61 resembles the *Picovirinae* with protein-primed DNA replication. Although we were unable to purify TP-DNA from phiYS61, our data strongly indicate that phiYS61 replicates by a protein primed DNA polymerase (ORF4) and that the terminal protein is encoded by *orf3*. This was supported by the presence of inverted terminal repeats in the phiYS61 genome, a trait that is characteristic for phages with protein-primed replication within the order *Caudovirales* (42). To our knowledge, this is the largest *Podoviridae* genome to replicate by this mechanism. The novelty of phiYS61 was also emphasized by distinct regions of dissimilarity when DNA polymerase sequences were aligned.

The characterization and genome sequence analysis of phiYS61 has revealed a novel *Podoviridae* with a clear relationship to the *Picovirinae*. phiYS61 differs, however, in typical *Picovirinae* traits such as genome and capsid size. We propose that the bacteriophage phiYS61 should represent a new subfamily within the family *Podoviridae*.

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7 Figure legends

Figure 1:

Transmission electron micrograph of a phiYS61 particle taken at 200,000 × magnification.

Figure 2:

Graphic representation of the linear phiYS61 dsDNA genome. A: Relative position and direction of each ORF is indicated by a white arrow. Putative functions are indicated in grey letters: *tp*, terminal protein; *dnap*, DNA polymerase; *enc*, encapsidation; *nmn*, NMN transporter; *sp*, structural protein; *mcp*, major capsid protein; *lys*, lysin; *taa*, tail assembly; *tap*, tail protein; *ucc*, upper collar connector; *lcp*, lower collar protein; *taf*, tail fiber; *hol*, holin; *hnh*, HNH endonuclease. The 25 bp inverted terminal repeats (ITR) are also indicated. Scale bars mark genome positions at 5000 bp intervals. B: Early (light grey bars) and late (dark grey bars) are shown at their relative positions on the upper or lower strand. Predicted terminators (o) and promoters (→) located between transcripts are indicated on tall stems, or on short stems when found within transcript regions.

Figure 3:

Neighbor-Joining tree based on amino acid sequence alignment of phiYS61 ORF4, *Lactococcus* phage asccphi28 ORF7, *Bacillus* phage phi29 gp2 and the 36 seed sequences of the Pfam Type B DNA polymerase family (PF03175). The alignment was produced in CLC after alignment fixpoints had been introduced at the positions of conserved motifs ExoI, ExoII, ExoIII, CT, motif-1, 2a and 3 (48). Bootstrap values calculated from 100 replicates are indicated by each node.

Figure 4:

SDS-PAGE analysis of phiYS61 structural proteins. Lane M: molecular weight marker with molecular masses in kilodaltons indicated on right side. Lane ϕ: phiYS61 proteins visualized with Coomassie stain. Protein identities determined by MS analysis are shown on left side with deduced molecular masses shown in parentheses. No molecular mass could be predicted for the protein band marked ORF12 since we were unable to identify the mechanism by which a protein of this size is produced from the coding sequence of *orf12A* or *orf12B*.

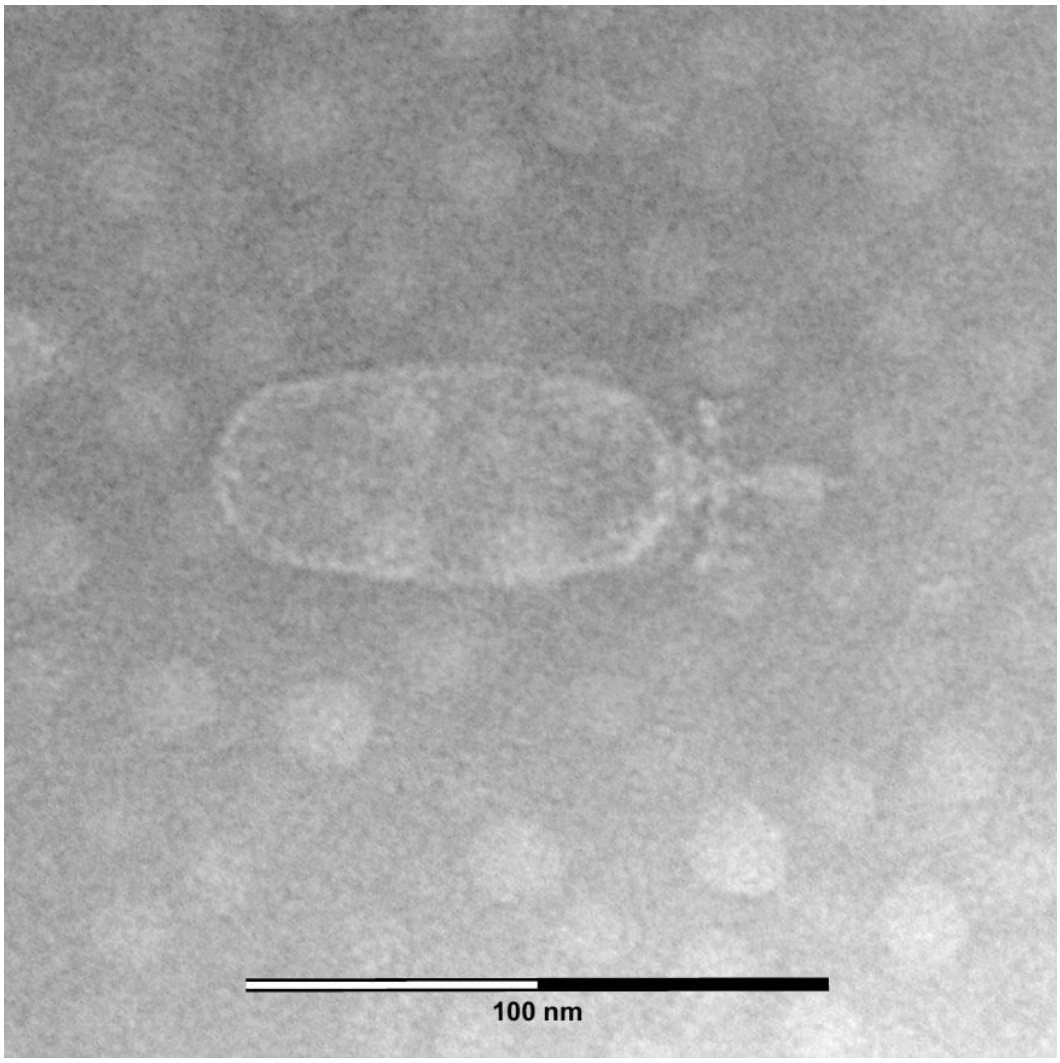
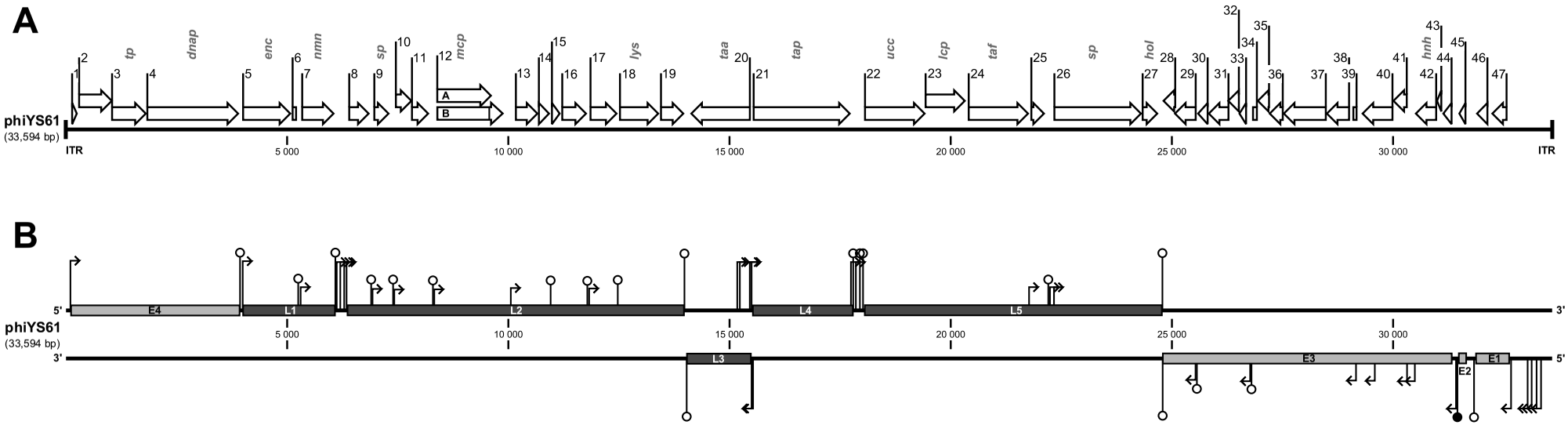


Figure 1: Transmission electron micrograph of a phiYS61 particle taken at 200,000 × magnification.



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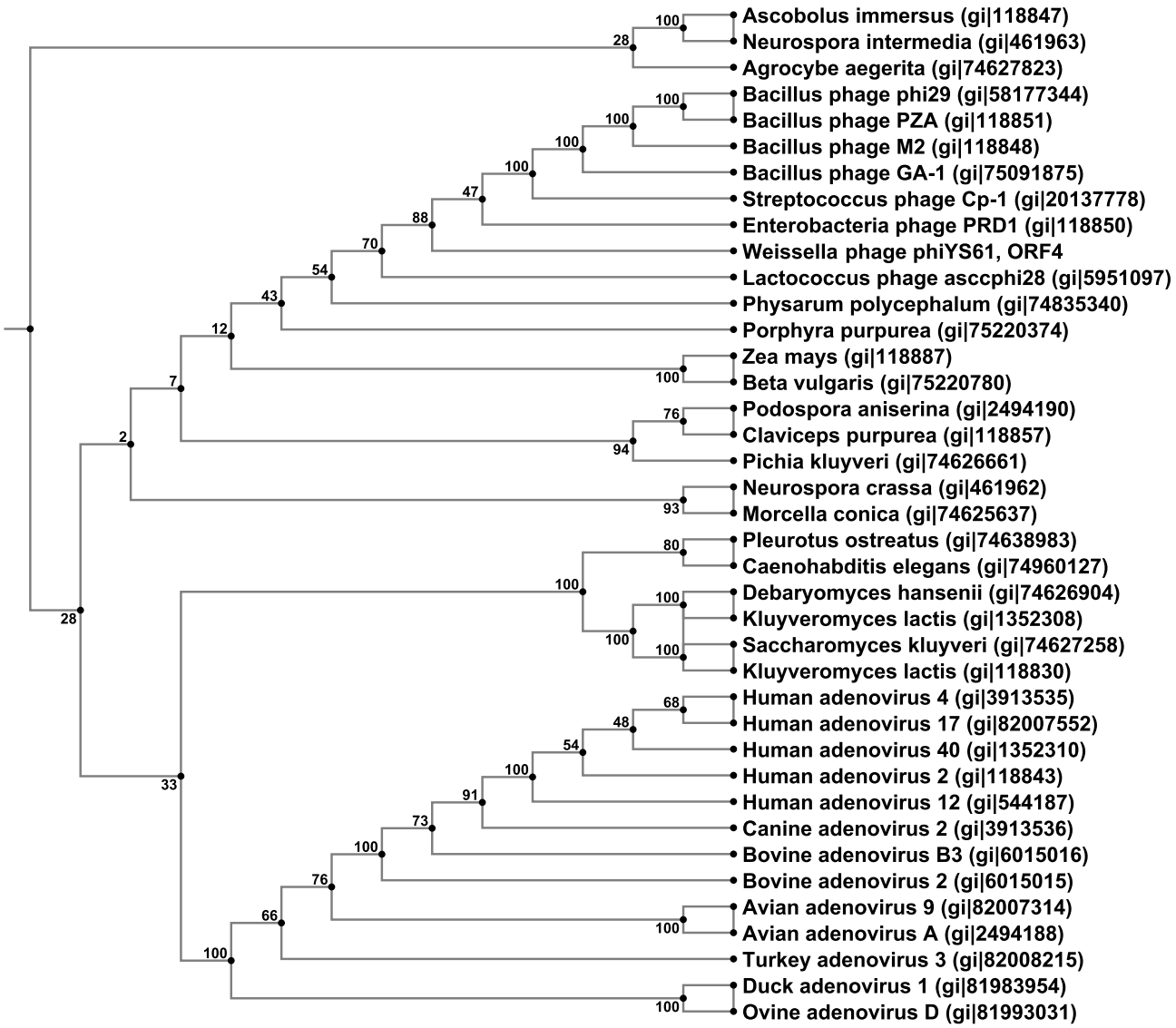
ORF	Ribosomal binding site and start codon sequence [a]	Position [b] Start	Stop	Predicted protein Size (aa)	Mol. wt. (kDa)	pI	Putative function [c]	Closest homolog (Extent, % identities, % positives, % gaps)	Size (aa)	Reference
1	AGGAGGTAAAAATTTATG	129	257	42	5.0	9.35		No significant similarity		
2	AGGAGGGTAATTGAATATG	287	1048	253	29.5	9.00		No significant similarity		
3	AAGGAGAAACAAAATG	1032	1817	261	30.0	10.06	Terminal protein	No significant similarity		This study
4	AGGAGAAACAAATG	1829	3904	691	79.6	5.04	DNA polymerase (PHA02563, 1e-09)	Streptococcus phage Cp-1, p05 (523/568, 18, 32, 14)	568	NP_044817
5	GAAAGGTTTATAAACCATG	3994	5082	362	42.2	8.88	DNA encapsidation protein (PHA00149, 2e-51)	Bacillus phage B103, gene 16 (303/321, 33, 53, 11)	321	NP_690650
6	AGGGTGGCATTGACTTGTG	5113	5214	33	3.8	6.74		No significant similarity		
7	AGGAGGATAACAATG	5330	6061	243	27.3	8.75	NMN transporter (PF04973, 1e-18)	Lactococcus phage KSY1, gp052 (229/249, 53, 73, 0)	249	YP_001469050
8	GAAAGGACGGTCAATTATG	6393	6854	153	16.5	4.79		No significant similarity		
9	**AGGAGTCTAATAATG**	**6960**	**7304**	**114**	**12.0**	**3.74**	**Structural protein**	**No significant similarity**		**This study**
10	AGGAGAAACACAATG	7447	7821	124	14.4	4.81		No significant similarity		
11	GGAGGAATTGAATAACAATG	7811	8200	129	14.8	4.18		No significant similarity		
12A	**AGGAGAAATATTATG**	**8383**	**9618**	**411**	**45.4**	**5.02**	**Major capsid protein (PHA00144, 1e-37)**	**Enterococcus phage EF62phi, ORF 40 (421/469, 31, 52, 5)**	**469**	**This study and ADX81364**
12B	**AGGAGAAATATTATG**	**8383**	**9881**	**500**	**53.9**	**5.31**	**Major capsid protein with bacterial Ig-like domain (PF02368, 1e-6)**	**Bacillus phage phi29, major head protein (483/448, 25, 42, 17)**	**448**	**This study and YP_002004536**
13	TGGAACATCAAGCACTTATG	10160	10669	169	18.2	5.42		No significant similarity		
14	AGGGGCACAGCATG	10680	10931	83	10.0	6.58		No significant similarity		
15	AGGAGGAATTATG	10975	11172	65	7.7	4.47		No significant similarity		
16	AGTAGAAAGGAGATG	11208	11768	186	21.1	9.95		No significant similarity		
17	AGGAGGCCATACAAAATG	11847	12458	203	21.1	6.76		No significant similarity		
18	GAAAGGAAATAGATTAAATG	12513	13400	295	32.7	6.40	Lysin, N-acetyl-L-alanine amidase (PF01510, 7e-4)	Weissella paramesenteroides ATCC 33313, lytA (270/305, 50, 61, 12)	305	ZP_04782183
19	AGGAGAACAAACATG	13440	13967	175	17.1	9.48		No significant similarity		
20	GGAGGAAAATAATTTTG	*(14126*	*15472)*	448	49.4	4.75	Tail lysin	Bacillus phage B103, gene 13 (303/365, 28, 44, 13)	365	NP_690647
21	AGGAGAAATTCAAATG	15535	17730	731	82.1	5.84	Tail protein (PHA00380, 3e-16)	Bacillus phage PZA, GP 9 (731/599, 23, 39, 24)	599	P07534
22	ATTTTGCAAGCAAAATTATG	18052	19422	456	49.8	4.69	Upper collar connector (PHA00147, 9e-27)	Enterococcus faecalis T2, EFBG_02899 (328/333, 33, 53, 12)	333	ZP_05423975
23	AGGAGGAGAAATGTAATG	19422	20330	302	34.5	4.46	Lower collar protein (PHA01077, 6e-13)	Enterococcus phage EF62phi, ORF 33 (245/255, 32, 51, 12)	255	ADX81357
24	AAACCGCAAATAATTCAATG	20396	21760	454	47.5	4.96	Tail fiber protein (PF01391, 9e-9)	Mycobacterium phage Bxz2, gp4 (108/344, 62, 72, 10)	344	NP_817595
25	GCATTATTTAGCGCCTGATG	21812	22120	102	11.8	6.40		No significant similarity		
26	**GCGGTATACAGCACATG**	**22333**	**24312**	**659**	**74.6**	**5.01**	**Structural protein**	**Escherichia coli MS 60-1, HMPREF9533_04376 (419/712, 24, 41, 6)**	**712**	**This study and EGB80821**
27	AGGAGAGAAAATAGTG	24328	24681	117	13.0	5.65	Phage holin 4 (PF05105, 8e-11)	Leuconostoc phage 1-A4, LM1A4_024 (106/123, 33, 55, 0)	123	ADD71747
28	AGGAGGCAGAGCATG	*(24800*	*25087)*	95	11.0	6.72		No significant similarity		
29	AGAGGGTCTTTTTTG	*(25059*	*25550)*	163	18.8	5.10		No significant similarity		
30	AGGAGACTAAGGAATG	*(25581*	*25820)*	79	9.2	4.78		Lactobacillus acidophilus 30SC, LAC30SC_06460 (66/67, 36, 50, 0)	67	YP_004292355
31	AGGAGGGTTTTAAATTATG	*(25824*	*26291)*	155	17.3	5.81		No significant similarity		
32	TTGCTTTATCGTGCGAGGTG	*(26263*	*26526)*	87	10.2	8.77		No significant similarity		
33	AGGAGGCGGCTTATG	*(26516*	*26689)*	57	6.5	4.11		No significant similarity		
34	AGGAGGCAGGCGTTTAATG	*(26823*	*26933)*	36	4.0	5.99		No significant similarity		
35	AGGGGAAAGATAATG	*(26933*	*27208)*	91	10.3	4.11		No significant similarity		
36	GGAGGACAAGTAACATG	*(27201*	*27518)*	105	12.0	4.28		No significant similarity		
37	AGGAGGACAAGTAACATG	*(27520*	*28491)*	323	36.4	6.36		No significant similarity		
38	GAAAGGATTTTATAATG	*(28493*	*29017)*	174	19.9	5.30		Listeria phage B025, ORF 62 (169/212, 24, 38, 25)	212	YP_001468701
39	AGGAGGCTTGACATG	*(29091*	*29186)*	31	3.6	5.89		No significant similarity		
40	GAAAGGAAGCATAATG	*(29300*	*29998)*	232	24.3	4.63		No significant similarity		
41	AGGAGGATATAACATG	*(29998*	*30321)*	107	11.5	9.00		Lactobacillus acidophilus 30SC, LAC30SC_00610 (98/227, 29, 41, 1)	227	YP_004286453
42	GAGAGGAAATGCAATG	*(30504*	*30986)*	160	18.8	10.04	HNH endonuclease	Enterococcus phage EFRM31, gp11 (120/173, 38, 60, 4)	173	YP_004306639
43	GGAACGCTATG	*(30983*	*31102)*	39	4.8	9.58		No significant similarity		
44	GGGGTGGAAATTATG	*(31125*	*31334)*	69	8.1	4.23		No significant similarity		
45	GAGGTGGAAATTATG	*(31487*	*31648)*	53	6.2	6.71		No significant similarity		
46	GAGGTATGGAAATATG	*(31887*	*32144)*	85	9.7	4.71		Lactobacillus phage Lb338-1, ORF 13 (57/102, 35, 65, 4)	102	YP_002790692
47	GAGGAAATTATCATG	*(32233*	*32577)*	114	13.4	3.79		No significant similarity		

[a] *Predicted ribosomal binding sites and start codons are underlined. Putative translation initiation by frameshifting indicated with asterix.*

[b] *ORF positions on complement strand are shown in parentheses.*

[c] *Best hits in the Conserved Domains Database accession numbers and E-values are shown in parentheses. PHA: CDD accession; PF: Pfam accession. Structural proteins identified by MS analysis are shown in bold letters.*



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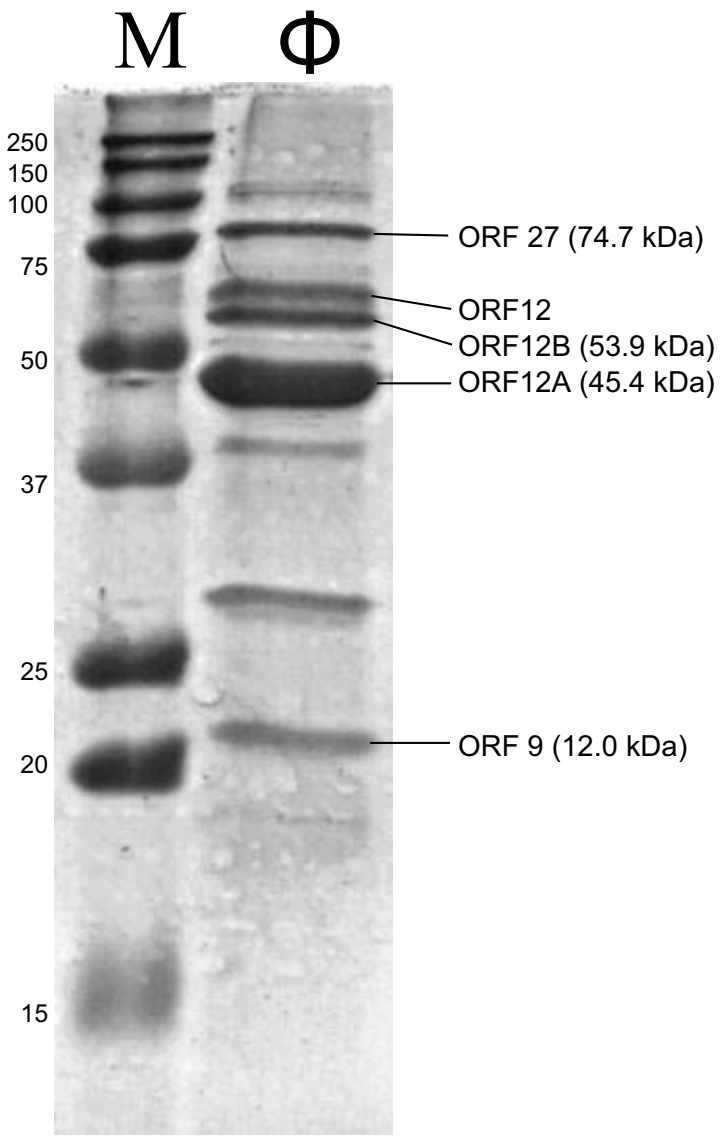

Figure 4: SDS-PAGE analysis of phiYS61 structural proteins. Lane M: molecular weight marker with molecular masses in kilodaltons indicated on right side. Lane Φ: phiYS61 proteins visualized with Coomassie stain. Protein identities determined by MS analysis are shown on left side with deduced molecular masses shown in parentheses. No molecular mass could be predicted for the protein band marked ORF12 since we were unable to identify the mechanism by which a protein of this size is produced from the coding sequence of *orf12A* or *orf12B*.

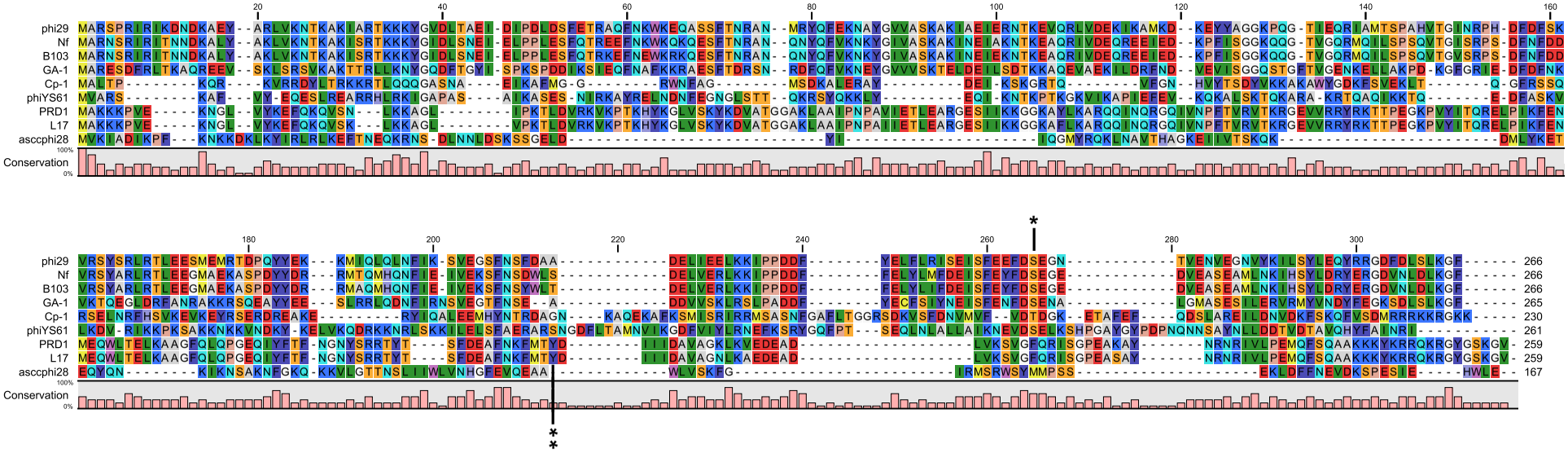

Figure S1: Alignment of phiYS61 ORF3 and terminal protein sequences from *Bacillus* phages Nf (accession: ACH57070), phi29 (YP_002004530), B103 (NP_690636), GA-1 (NP_073686), *Streptococcus* phage Cp-1 (NP_044816), *Enterobacteria* phages PRD1 (AAA32449) and L17 (AAX45555), and *Lactococcus* phage asccphi28 (YP_001687519). Priming site residues Ser-232 and Thr-189 of phi29 and Cp-1, respectively (*), and Tyr-190 of PRD1 (**) form a phospho-diester bond to 5'-dNMP during replication initiation.

TABLE S1. PCR primers used in transcript boundary analysis.

#	Sequence (5'-3')	Position [A]
1	TCAGAAGCCCAGAAGAAA	135..152
2	GAGCTGGTAACGGAATTG	(300..317)
3	AATCTATAAACGGGGGTC	(693..710)
4	GACCCCCGTTTATAGATT	693..710
5	TATTTGACTCACTGGCCT	(1129..1146)
6	AGCAAGACCGCAAAAAGAA	1474..1492
7	GTCTGATAAGTCATTCATTCCC	(1999..2020)
8	ATGTTAAGCCGTGGAACCAG	2211..2230
9	GTCTCTAATTCTCCCTCCC	(2985..3003)
10	CATGGTTTGAAGCTAGAGAACG	3503..3524
11	TTCTCGAACTTACCCTCGTC	(3707..3726)
12	CGAATATAGGCTGAGTGT	(4033..4050)
13	CAACCCTTATTTTGAAGCC	4569..4587
14	CTCCTTCCAAACACCGTA	(4834..4851)
15	ACGAGTCCAAAAGCACAA	4945..4962
16	TTCCACTCCCACAACTTT	5594..5611
17	TGACATTAACCCTTGTAACGTCC	(5883..5905)
18	CGGCTTATTCTTCTCACA	5995..6012
19	AACACCAGAAGACGCCGA	6536..6553
20	TTCCCCAGTAGCACCGTCA	(6650..6668)
21	ACGAAGAGAAGAAGACAGA	6964..6982
22	CATCTTCTGTCATCGTGT	(7252..7269)
23	GGTGGCTTTTCTTTTGCT	7395..7412
24	AGCACAAAAGAAAAGACCC	(8300..8318)
25	AGCCTCAATCGCAAACCCA	8598..8616
26	CCGTGTGATAGATAGCCTT	(8758..8776)
27	GCAATAAGTCAACCCCAGAA	10419..10438
28	TGGGGCGGTTGATGTTGTG	(10552..10570)
29	ACCGGATTGCTTGATTGA	(12309..12326)
30	GACGCTTATTACACACAC	12669..12686
31	TAGGAAGGCAATAGGGTCAA	(12985..13004)
32	GGCTTGCTTGTTGATTTGCT	(13675..13694)
33	CAGAACAAAAGGCAAGCA	(14329..14346)
34	TGAGAAAGGCCAACGGAA	14915..14932
35	TTCAAATGGCAGAACGAC	15530..15547
36	CTGAATCGGTCTTGTTTGT	(16042..16060)
37	CCGCAAGGGCAAATAATAA	17075..17093
38	TGGAACTGAGGATTTGAAC	17529..17547
39	AAAGACGACAACAACGGA	18330..18347
40	CAACCAGCGAGTATTTAC	(18466..18483)
41	GGGGTTGACAAGAAAGAG	19111..19128
42	CACCGCCTGGATAAATAGAA	(19487..19506)
43	GGTAACTTGTGGTATGCT	21491..21508
44	TTGGGTTTGAGTTGGGTAT	(21700..21718)
45	CGAAACCAGCCCAAAATA	(22892..22909)
46	CGTATTTTGGGCTGGTTT	22890..22907
47	CCCGGTTGATTTTGAGTA	23046..23063
48	CAAAATAACCCGACGACA	(23822..23839)
49	TTTCACTCCATTCGTTCC	(24682..24699)
50	GGCTCATTGTACTTGACCCT	(24998..25017)
51	TCGCTCTGCTTCATCAATCTC	25101..25121
52	GTTAAACGCAATGCAACTTGG	(25699..25719)
53	AGTTGCATTGCGTTTAACAG	25702..25721
54	GGAACCTATCACTCAACT	(26396..26413)
55	AAATATCCAGGACGAGCA	26453..26470
56	TTGACAAAGAGCAATTCCAG	(27083..27102)
57	AAGCCACTCTCGTTCATTAGTC	27185..27206
58	GGCTTTGATGTTCCTTGT	27856..27873
59	TGTGTAGCCTTTCGACTT	28009..28026
60	CGAGTACACAACGAGAAG	(28982..28999)
61	CAGGGTGTTCTGATTTGT	29323..29340
62	GTCAAGCTCAGACAAGCA	(29781..29798)
63	CCAGCACCTATCAATACA	29967..29984
64	GGTGTATTGATAGGTGCTG	(29968..29986)
65	GAGAATGGTGTTAAAAGGG	(30567..30585)
66	CCCTTTTAACACCATTCTC	30567..30585
67	TGAGGGTGACAGTATTGA	(31228..31245)
68	TGTTCTTACCGCCTTCTG	31549..31566
69	ACTTAGTTGGTGGCTTTG	(31692..31709)
70	CTTCCTCTTCCATTTCTTC	32301..32319
71	GCGAAAAGCAGAAGGATA	(32387..32404)
72	CGCTTCAGTACACCATATT	(32946..32964)

[A] *Positions on negative strand marked by parentheses.*