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Identification of the Receptor-Binding Protein in Lytic *Leuconostoc pseudomesenteroides* Bacteriophages

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Two phages, P793 and ΦLN04, sharing 80.1% nucleotide sequence identity but having different strains of *Leuconostoc pseudomesenteroides* as hosts, were selected for identification of the host determinant gene. Construction of chimeric phages leading to the expected switch in host range identified the host determinant genes as $ORF21_{P793}$/$ORF23_{ΦLN04}$. The genes are located in the tail structural module and have low sequence similarity at the distal end.

Bacteriophages cause large problems in the dairy industry (1, 2). The results of phage infection are changes of product quality, fermentation delays, or failures (3). Phages attacking *Leuconostoc* spp. are responsible for quality-related failures, i.e., changes in the concentration of aromatic compounds or in CO_2 production (4).

The phage infection process begins with the adsorption of the phage to the host cell. This specific interaction occurs between the phage receptor binding protein (RBP) and the bacterial receptor, which is present on the cell surface. Various RBPs have been studied in phages attacking Gram-negative bacteria, especially in phages of *Escherichia coli* (5, 6). However, a small number of RBPs in phages of lactic acid bacteria (LAB) have been identified. Duplessis and Moineau in 2001 were the first to report on the identification of the RBP gene of a *Streptococcus thermophilus* phage (7). They constructed a chimeric phage with altered host range and concluded that variable region 2 (VR2), located in the C-terminal part of the RBP, is responsible for host specificity (7). However, further investigation revealed that more than one gene may be responsible for the host range interaction in *S. thermophilus* phages (8). A similar approach was used to identify the RBPs of the three major phage families of *Lactococcus lactis* (9–11). More recently, the RBP structures of phages p2, bIL170, TP901-1, and Tuc2009 have been determined (12–16).

In 2010, the first full genomic sequence of a lytic bacteriophage attacking *L. mesenteroides* was reported (17). In 2012, Kleppen et al. reported the first full genomic sequence of a dairy *Leuconostoc* phage (18). An additional nine complete genomic sequences of *Leuconostoc* phages virulent to either *L. mesenteroides* or *L. pseudomesenteroides* were recently determined in our laboratory (W. Kot, L. H. Hansen, H. Neve, K. Hammer, S. Jacobsen, P. D. Pedersen, S. J. Sørensen, K. J. Heller, and F. K. Vogensen, unpublished data). The 11 described lytic *Leuconostoc* phages all belong to the *Siphoviridae* family (19), with genome sizes varying from 25 to 30 kbp. Based on sequence similarities, *Leuconostoc* phages can be gathered into two groups, which correlate with the species of the host. Genomic comparison of the sequenced phages revealed high levels of conservation in the structural module within phages attacking the same species. However, two different, nonoverlapping

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TABLE 1 Strains and plasmids used in the study

Bacterial strain, phage, or plasmid	Relevant feature	Source and/or reference
E. coli strains		
TOP10 (DH10B)	Transformation strain	Invitrogen, USA
Leuconostoc pseudomesenteroides strains		
LN02	*L. pseudomesenteroides*, host for ΦLN04	Kot et al., unpublished
BM2	*L. pseudomesenteroides*, host for P793	20, MRI[a]
Phages		
ΦLN04	*L. pseudomesenteroides* phage, virulent to strain LN02	
P793	*L. pseudomesenteroides* phage, virulent to strain BM2	20, MRI
ΦLNR1– ΦLNR4	*L. pseudomesenteroides* phages, with altered host range	This study
Plasmids		
pGhost8	Shuttle vector, tetracycline resistance, 4.3 kbp	21
pWPK1	pGhost8 with cloned region 19096 to 20725 from ΦLN04, 6.1 kbp	This study

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TABLE 2 Primers used in this study

No.[a]	Primer sequence (5′ to 3′)		Relevant feature(s)
	Forward	Reverse	
1	GACGTTGTAAAACGACGGCCAG	CGTTAAATCGACTGGCGAAA	Targeting pGhost8, flanking MCS
2	GGTGGGACTAAAAAATACA	CTCACAGGTCAAAAGTAA	Amplifies regions 19096 to 20725 in ΦLN04 and 18031 to 19792 in P793
3	GCAATCGCCTATTAAAC	CCAGAGGGTGTATTATT	Amplifies region 17985 to 19828 in P793

[a] Primer set number.

host ranges could be observed for phages lytic to *L. pseudomesenteroides*.

Bacteria, bacteriophages, and growth conditions. The bacterial strains and phages used in this study are listed in Table 1. *Escherichia coli* strains were grown at 28°C in Luria-Bertani medium with 10 μg/ml of tetracycline (Oxoid, Basingstoke, United Kingdom). *Leuconostoc* strains were grown at 28°C on MRS agar or in MRS broth (Difco, Sparks, MD), and 3 μg/ml of tetracycline was added when appropriate. Phages were propagated on their hosts, as shown in Table 1, in MRS supplemented with 10 mM $CaCl_2$ (MRS-Ca). Phage host range and phage titers were determined on MRS-Ca top agarose (MRS-Ca broth, 0.4% agarose) as described before (20). Phages were further analyzed by electron microscopy as described earlier (11).

DNA techniques. Primers used in this study are listed in Table 2. Primers were obtained from IDT (Munich, Germany). The pGhost8 plasmid (21) was digested and simultaneously dephosphorylated using FastDigest SmaI enzyme and FastAP thermosensitive alkaline phosphatase (Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol. Ligation of linearized plasmids and PCR product was done using T4 ligase (NEB, Ipswich, MA) according to the manufacturer's protocol. The ligation product was cloned in *E. coli* One Shot TOP10 (Invitrogen, Carlsbad, CA). Plasmid DNA from *E. coli* strains was isolated with a PureLink HiPure plasmid filter maxiprep kit (Invitrogen) according to the manufacturer's recommendations.

Electrocompetent cells of *Leuconostoc* were prepared similarly to the method of Wyckoff et al. (22) with MRS used instead of MRS-V8 and with triple wash steps.

Cells were transformed with 2 μg of plasmid DNA with a single discharge (25 μF capacitor, 400 Ω resistance, 10 kV/cm). Instantly 950 μl of MRS broth was added to the cells. The suspension was incubated for expression of tetracycline resistance at 28°C for 90 min and afterwards plated on MRS plates containing 3 μg/ml of tetracycline. Plates were incubated at 28°C for 48 h, by which time transformants had appeared. Transformants possessing plasmid pWPK1 were verified by colony PCR followed by sequencing.

Generation of recombinant phages. Strain BM2(pWPK1) was grown to an optical density at 600 nm (OD_{600}) of 0.4 and infected with phage P793 at a low multiplicity of infection (MOI) (approximately 0.01). After 16 h of incubation, the lysate was filtered using a 0.45-μm-pore-size syringe filter and 150 μl of the filtrate was added to MRS-Ca top agarose along with 100 μl of the selection strain LN02.

Adsorption assay. The bacterial strains were grown in 10 ml of MRS-Ca broth to an OD_{600} of 0.4 to 0.6. Phage solution was added to a final concentration of approximately 10^5 PFU per ml. The mixture was incubated for 10 min at 28°C to allow phage adsorption. The solution was then centrifuged for 5 min at 8,000 × *g*. The phage concentration in the supernatant was determined using a plaque assay, and the titer was compared to the titer of a cell-free phage solution. The adsorption test was performed in triplicate for each of the phages.

RBP exchange. Phages ΦLN04 and P793 were chosen for determination of the RBP gene because they have nonoverlapping, stable host ranges and both attack strains of *L. pseudomesenteroides*. Different baseplate structures could be distinguished on electron micrographs of the two phages (Fig. 1). Data obtained from the comparative genomics study suggested that the tail structural module in the ΦLN04 genome encompasses open reading frames (ORFs) 16 to 23. The structural modules are highly similar in both organization and nucleotide sequence. However, the two phages revealed different host ranges, and their RBP genes were therefore expected to have low or no identity. ORF23 from phage ΦLN04 fulfills the requirements of both being located within the structural module and having only partial similarity to the corresponding gene in phage P793 (Fig. 2). BLASTP analysis of gpORF23 from ΦLN04 performed on the NCBI nonredundant protein database revealed similarities to the putative RBP of two other *Leuconostoc* phages: ΦLmd1 (18) and Φ1A4 (17). The homologous gene in phage P793 was determined to be $ORF21_{P793}$. Proteins $gpORF23_{\Phi LN04}$ and $gpORF21_{P793}$ share only a limited similarity that is localized to the N-terminal part of the proteins. BLAST comparison showed 80% similarity of the amino acid sequences when the first 30% of the N-terminal parts of the proteins were compared. Then similarity decreased to 64%

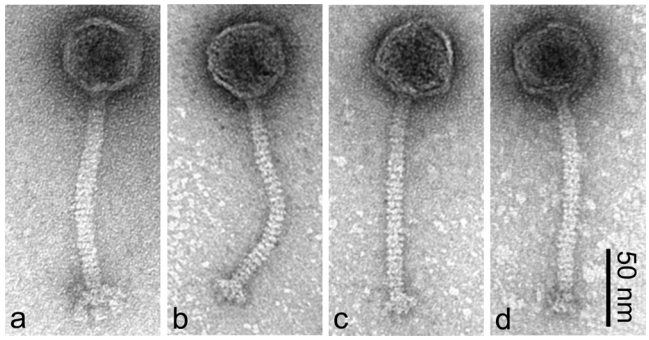

FIG 1 Transmission electron micrographs of *L. pseudomesenteroides* wild-type phages P793 (a) and ΦLN04 (d), with different host ranges, and of the two recombinant phages ΦLNR02 (b) and ΦLNR03 (c). Negative staining was done with 2% (wt/vol) uranyl acetate.

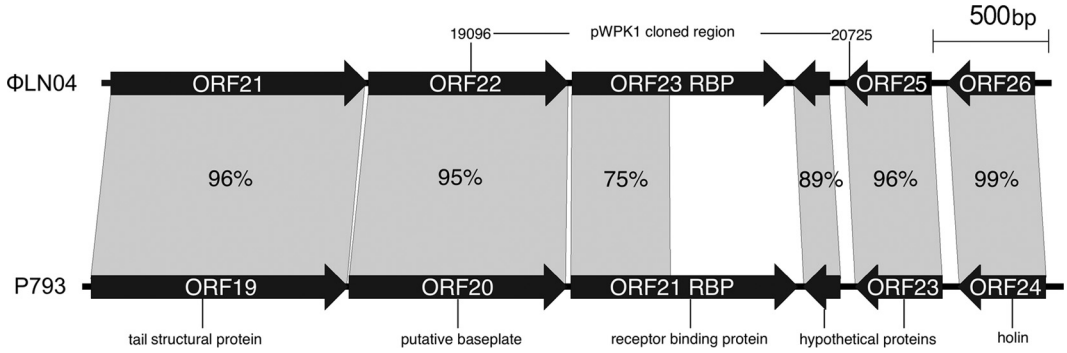

FIG 2 Genetic overview of the RBP region of *L. pseudomesenteroides* phages P793 and ΦLN04, with different host ranges. The figure illustrates the nucleotide similarities between the two phages as calculated by the BLASTN algorithm. The distal parts of the RBP genes did not show any significant similarities to each other. The hypothetical functions of genes are indicated for phage P793. The region cloned into plasmid pWPK1 is indicated on top of the figure.

when 65% of the N-terminal parts were compared. The remaining 35% of the proteins localized on the C-terminal part did not shown any significant similarities to each other. Genes that neighbor the RBP gene show high similarity along the whole length.

A plasmid containing pGhost8 with cloned region 19096 to 20725 from ΦLN04 was constructed and designated pWPK1. The pWPK1 plasmid was cloned to the *E. coli* strain DH10B/TOP10 and purified. The transformation efficiency of *L. pseudomesenteroides* strain BM2(pWPK1) was 8.8×10^2 transformants per μg of DNA. The average time before colonies appeared was 36 h. Because no spontaneous antibiotic-resistant mutants were seen on the control plates, all colonies were assumed to carry the pWPK1 plasmid. When strain BM2(pWPK1) was infected with phage P793, we were able to isolate phages which were lytic to strain LN02. Phages with altered host range occurred at a frequency 10^{-4}. Plaques that formed on strain LN02 were characterized by unchanged morphology compared to the plaques formed by *L. pseudomesenteroides* phages on their own hosts. Single plaques could be picked up and readily propagated both on a soft agar medium and in a planktonic culture of strain LN02. Four generated host range mutants were selected for further examination and were designated accordingly ΦLNR1 to ΦLNR4. Sequence comparison between recombinant phages P793 and ΦLN04 revealed that recombination occurred in the regions 18150 to 18537 and 19557 to 19811 in P793. All recombinant phages had the sequence of the putative receptor with 100% similarity to $ORF23_{\Phi LN04}$ (Fig. 3).

The levels of adsorption of phages ΦLN04 and P793 to their own hosts were very high (i.e., 98.3% ± 2% and 99.6% ± 0.5%, respectively). When phages were tested with the strain that they could not infect, the adsorption levels were 14.3% ± 9.6% and 14.2% ± 9.2%, respectively. The recombinant phage ΦLNR2 adsorbed efficiently (99.9% ± 0.1%) to strain LN02 but poorly (16.6% ± 17.9%) to strain BM2.

Furthermore, the baseplate of the tail of the recombinant phage ΦLNR2 was analogous to the baseplate of phage ΦLN04 and lost the characteristic "fluffy" appendices present in the baseplate of phage P793 (Fig. 1).

Exchange of $ORF21_{P793}$ with $ORF23_{\Phi LN04}$ resulted in alternation of the host range of the phage, adsorption pattern, and morphological details at the end of the tail tip; thus, it is concluded that $ORF21_{P793}$ and $ORF23_{\Phi LN04}$ are the RBPs of the *L. pseudomesenteroides* phages.

Nucleotide sequence accession numbers. The nucleotide sequences of phages P793 and ΦLN04 can be found in GenBank under accession numbers KC013021 and KC013023, respectively.

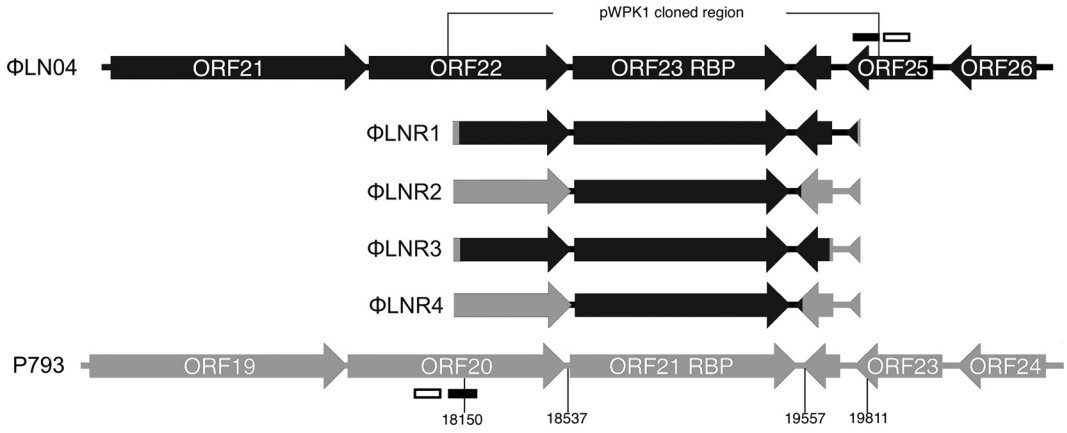

FIG 3 Comparison of recombinant *L. pseudomesenteroides* phages and of original phages ΦLN04 and P793. Black indicates 100% similarity to the ΦLN04 sequence. Gray indicates 100% similarity to the P793 phage genome. The positions of the primers used for cloning are indicated with small black boxes. The region cloned into plasmid pWPK1 is indicated on top of the figure. The position of primers used for clone verification is indicated with a small empty box. The nucleotide positions of phage P793 are indicated at the bottom.

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REFERENCES

1. **Moineau S, Tremblay D, Labrie S.** 2002. Phages of lactic acid bacteria: from genomics to industrial applications. ASM News **68**:388–393.
2. **Brüssow H.** 2001. Phages of dairy bacteria. Annu. Rev. Microbiol. **55**:283–303.
3. **Kleppen HP, Bang T, Nes IF, Holo H.** 2011. Bacteriophages in milk fermentations: diversity fluctuations of normal and failed fermentations. Int. Dairy J. **21**:592–600.
4. **Hemme D, Foucaud-Scheunemann C.** 2004. *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. Int. Dairy J. **14**:467–494.
5. **Randall-Hazelbauer L, Schwartz M.** 1973. Isolation of the bacteriophage lambda receptor from *Escherichia coli*. J. Bacteriol. **116**:1436–1446.
6. **Wang J, Hofnung M, Charbit A.** 2000. The C-terminal portion of the tail fiber protein of bacteriophage lambda is responsible for binding to LamB, its receptor at the surface of *Escherichia coli* K-12. J. Bacteriol. **182**:508–512.
7. **Duplessis M, Moineau S.** 2001. Identification of a genetic determinant responsible for host specificity in *Streptococcus thermophilus* bacteriophages. Mol. Microbiol. **41**:325–336.
8. **Duplessis M, Lévesque CM, Moineau S.** 2006. Characterization of *Streptococcus thermophilus* host range phage mutants. Appl. Environ. Microbiol. **72**:3036–3041.
9. **Stuer-Lauridsen B, Janzen T, Schnabl J, Johansen E.** 2003. Identification of the host determinant of two prolate-headed phages infecting *Lactococcus lactis*. Virology **309**:10–17.
10. **Dupont K, Vogensen FK, Neve H, Bresciani J, Josephsen J.** 2004. Identification of the receptor-binding protein in 936-species lactococcal bacteriophages. Appl. Environ. Microbiol. **70**:5818–5824.
11. **Vegge CS, Vogensen FK, Mc Grath S, Neve H, van Sinderen D, Brøndsted L.** 2006. Identification of the lower baseplate protein as the antireceptor of the temperate lactococcal bacteriophages TP901-1 and Tuc2009. J. Bacteriol. **188**:55–63.
12. **Spinelli S, Desmyter A, Verrips CT, de Haard HJW, Moineau S, Cambillau C.** 2006. Lactococcal bacteriophage p2 receptor-binding protein structure suggests a common ancestor gene with bacterial and mammalian viruses. Nat. Struct. Mol. Biol. **13**:85–89.
13. **Tremblay DM, Tegoni M, Spinelli S, Campanacci V, Blangy S, Huyghe C, Desmyter A, Labrie S, Moineau S, Cambillau C.** 2006. Receptor-binding protein of *Lactococcus lactis* phages: identification and characterization of the saccharide receptor-binding site. J. Bacteriol. **188**:2400–2410.
14. **Sciara G, Bebeacua C, Bron P, Tremblay D, Ortiz-Lombardia M, Lichière J, van Heel M, Campanacci V, Moineau S, Cambillau C.** 2010. Structure of lactococcal phage p2 baseplate and its mechanism of activation. Proc. Natl. Acad. Sci. U. S. A. **107**:6852–6857.
15. **Campanacci V, Veesler D, Lichière J, Blangy S, Sciara G, Moineau S, van Sinderen D, Bron P, Cambillau C.** 2010. Solution and electron microscopy characterization of lactococcal phage baseplates expressed in *Escherichia coli*. J. Struct. Biol. **172**:75–84.
16. **Siponen M, Spinelli S, Blangy S, Moineau S, Cambillau C, Campanacci V.** 2009. Crystal structure of a chimeric receptor binding protein constructed from two lactococcal phages. J. Bacteriol. **191**:3220–3225.
17. **Lu Z, Altermann E, Breidt F, Kozyavkin S.** 2010. Sequence analysis of *Leuconostoc mesenteroides* bacteriophage 1-A4 isolated from an industrial vegetable fermentation. Appl. Environ. Microbiol. **76**:1955–1966.
18. **Kleppen HP, Nes IF, Holo H.** 2012. Characterization of a *Leuconostoc* bacteriophage infecting flavor producers of cheese starter cultures. Appl. Environ. Microbiol. **78**:6769–6772.
19. **Ackermann HW.** 2011. Bacteriophage taxonomy. Microbiol. Aust. **32**:90–94.
20. **Atamer Z, Ali Y, Neve H, Heller KJ, Hinrichs J.** 2011. Thermal resistance of bacteriophages attacking flavour-producing dairy *Leuconostoc* starter cultures. Int. Dairy J. **21**:327–334.
21. **Maguin E, Prevost H, Ehrlich SD, Gruss A.** 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. J. Bacteriol. **178**:931–935.
22. **Wyckoff HA, Sandine WE, Kondo JK.** 1991. Transformation of dairy *Leuconostoc* using plasmid vectors from *Bacillus*, *Escherichia*, and *Lactococcus* hosts. J. Dairy Sci. **74**:1454–1460.