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 ORF21P793/ORF23ΦLN04. 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 CO₂ 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 thermophillus* 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. thermophillus* 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

### TABLE 1 Strains and plasmids used in the study

<table>
<thead>
<tr>
<th>Bacterial strain, phage, or plasmid</th>
<th>Relevant feature</th>
<th>Source and/or reference</th>
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<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
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</tr>
<tr>
<td>TOP10 (DH10B)</td>
<td>Transformation strain</td>
<td>Invitrogen, USA</td>
</tr>
<tr>
<td><strong>Leuconostoc pseudomesenteroides strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN02</td>
<td><em>L. pseudomesenteroides</em>, host for ΦLN04</td>
<td>Kot et al., unpublished</td>
</tr>
<tr>
<td>BM2</td>
<td><em>L. pseudomesenteroides</em>, host for P793</td>
<td>20, MRI*</td>
</tr>
<tr>
<td><strong>Phages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΦLN04</td>
<td><em>L. pseudomesenteroides</em> phage, virulent to strain LN02</td>
<td>20, MRI</td>
</tr>
<tr>
<td>P793</td>
<td><em>L. pseudomesenteroides</em> phage, virulent to strain BM2</td>
<td>20, MRI</td>
</tr>
<tr>
<td>ΦLN1–ΦLN4</td>
<td><em>L. pseudomesenteroides</em> phages, with altered host range</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<tr>
<td>pGhost8</td>
<td>Shuttle vector, tetracycline resistance, 4.3 kbp</td>
<td>21</td>
</tr>
<tr>
<td>pWPK1</td>
<td>pGhost8 with cloned region 19096 to 20725 from ΦLN04, 6.1 kbp</td>
<td>This study</td>
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*MRI, Max Rubner-Institut, Kiel, Germany.*
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 CaCl₂ (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 ligated into vector with Taq DNA ligase (NEB, Ipswich, MA) according to the manufacturer’s protocol. The ligation product was cloned in One Shot E. coli electrocompetent cells (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations.

Electrocompeent 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 were verified by colony PCR followed by sequencing.

**Generation of recombinant phages.** Strain BM2(pWPK1) was grown to an optical density at 600 nm (OD₆₀₀) 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 OD₆₀₀ of 0.4 to 0.6. Phage solution was added to a final concentration of approximately 10⁵ 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 ORF21P793. Proteins gpORF23ΦLN04 and gpORF21P793 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%.

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**Note:** The table below provides information on the primers used in this study.

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Relevant feature(s)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>GACGTTGTAAAAACGACGCGCAG</td>
<td>Targeting pGhost8, flanking MCS</td>
</tr>
<tr>
<td>2</td>
<td>GGTGGGACTAAAAATATACA</td>
<td>Amplifies regions 19096 to 20725 in ΦLN04 and 18031 to 19792 in P793</td>
</tr>
<tr>
<td>3</td>
<td>GCAATCGGCCTATTTAAC</td>
<td>Amplifies region 17985 to 19828 in P793</td>
</tr>
</tbody>
</table>

*Primer set number.*
when 65% of the N-terminal parts were compared. The remaining 35% of the proteins localized on the C-terminal part did not show 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 \( \Phi LN04 \) 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 \( \times 10^{10} \) transformants per \( g \) 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 \( \Phi LN01 \) to \( \Phi LN04 \). Sequence comparison between recombinant phages P793 and \( \Phi LN04 \) 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 \( \Phi LN04 \) 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 \( \Phi LN02 \) 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 \( \Phi LN02 \) was analogous to the baseplate of phage \( \Phi LN04 \) 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 alteration 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 \( \Phi LN04 \) can be found in GenBank under accession numbers KC013021 and KC013023, respectively.

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**FIG 2** Genetic overview of the RBP region of \( L. pseudomesenteroides \) phages P793 and \( \Phi LN04 \), 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.

**FIG 3** Comparison of recombinant \( L. pseudomesenteroides \) phages and of original phages \( \Phi LN04 \) and P793. Black indicates 100% similarity to the \( \Phi LN04 \) 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.
ACKNOWLEDGMENTS

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REFERENCES