Receptor-Binding Protein of *Lactococcus lactis* Phages: Identification and Characterization of the Saccharide Receptor-Binding Site

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Phage p2, a member of the lactococcal 936 phage species, infects *Lactococcus lactis* strains by binding initially to specific carbohydrate receptors using its receptor-binding protein (RBP). The structures of p2 RBP, a homotrimeric protein composed of three domains, and of its complex with a neutralizing llama VH domain (VHH5) have been determined (S. Spinelli, A. Desmyter, C. T. Verrips, H. J. de Haard, S. Moineau, and C. Cambillau, Nat. Struct. Mol. Biol. 13:85–89, 2006). Here, we show that VHH5 was able to neutralize 12 of 50 lactococcal phages belonging to the 936 species. Moreover, escape phage mutants no longer neutralized by VHH5 were isolated from 11 of these phages. All of the mutations (but one) cluster in the RBP/VHH5 interaction surface that delineates the receptor-binding area. A glycerol molecule, observed in the 1.7-Å resolution structure of RBP, was found to bind tightly ($K_d = 0.26 \mu\text{M}$) in a crevice located in this area. Other saccharides bind RBP with comparable high affinity. These data prove the saccharidic nature of the bacterial receptor recognized by phage p2 and identify the position of its binding site in the RBP head domain.

Bacteriophage infection is a major problem impairing any industrial fermentation that relies on bacteria to transform a substrate into fermented products or specific molecules. Several strains of the gram-positive bacterium *Lactococcus lactis* are used quite extensively worldwide for the manufacture of fermented milk products. For decades, the dairy industry has been dealing with phage infections of their *L. lactis* strains because virulent phages are ubiquitous within their environment (28).

Phages of *L. lactis* are classified within several groups (21), although those commonly found in dairy plants belong to three different species, i.e., 936, P335, and c2 (18, 27, 29). From a taxonomical standpoint, these phages are all members of the *Siphoviridae* family (order *Caudovirales*) that also includes the coliphage lambda. Siphophages are characterized by a double-stranded DNA genome and a long noncontractile tail. The phages belonging to the species 936 and P335 have a small isometric capsid, while those from the c2 species have a prolate capsid.

The bacteriophage infection process starts by the specific recognition between the phage receptor-binding protein (RBP) located at the tip of the tail and the receptor distributed over the host cell surface (14, 35). Bacterial receptors have been well studied in gram-negative bacteria, particularly in

Escherichia coli, while similar information lags behind in gram-positive bacteria (12). It has been previously shown that phages infecting some *L. lactis* strains adsorb initially to the cell wall surface and likely to various carbohydrates containing rhamnose, glucose, or galactose (for a review, see Forde and Fitzgerald [15]). For many phages, this binding step is reversible, and phages of the c2 species, for example, require a second irreversible binding step to a predicted membrane-attached protein (PIP) of 901 amino acids. However, phages of the species 936 and P335 do not use PIP as a secondary receptor (14). A better understanding at a molecular level of these phage-host interactions in gram-positive bacteria should lead to better tools for the control of phage infection in various biotechnological processes as well as reveal insights into the molecular basis of host specificity.

A novel antiphage strategy was recently designed based on the use of a phage-neutralizing heavy chain antibody fragment (VHH5) obtained from llama (31). VHH5 produced at large scale in the food microorganism *Saccharomyces cerevisiae* prevented the infection of an *L. lactis* strain by phage p2 (936 species) during the manufacture of cheese (23). VHH5 recognized the RBP of phage p2, a 30-kDa protein (ORF18) located at the distal part of the phage tail, with a high affinity ($K_d = 1.4 \text{ nM}$) (8).

We previously determined the crystal structure of the p2 RBP trimer at 2.3-Å resolution and that of its complex with three molecules of VHH5 at 2.7-Å resolution (34). The RBP ternary complex is formed of three subunits, each assembling three symmetry-related domains: the shoulders, the interlaced neck, and the heads, from N to C terminus (Fig. 1A). The shoulder domains, encompassing residues 1 to 141, have a β -sandwich fold formed of two β -sheets of four antiparallel

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FIG. 1. Views of the receptor-binding protein from phage p2 alone or in complex with VHH5. (A) View of the RBP trimer in ribbon representation (34). The threefold axis is vertical, within the paper plane. Monomers A, B, and C are colored pink, blue, and yellow, respectively. The names of the domains (head, neck, and shoulders) are explained in the text. (B) Ribbon representation of the RBP/VHH5 complex. The head domains are the only parts of RBP observed. The shoulders and neck domain (in gray) are taken from the native structure and depicted here for consistency with panel A. The head monomers are colored in pink, dark blue, and yellow and the VHH5s in red, marine blue, and orange.

β -strands each. The neck (residues 142 to 163) folds as a triple-stranded interlaced β -helix that forms an equilateral triangular prism around the threefold axis (Fig. 1A). The receptor recognition domain (residues 164 to 264), designated as the head, is a β -barrel formed of seven antiparallel β -strands. Each domain is parallel to the threefold axis and interacts with the two others, forming a very compact structure (Fig. 1A). In the structure of the head trimer complexed with VHH5, each of the three VHH5 domains binds the RBP head at the top half of the trimer and forms a regular trimeric complex, sharing the same threefold axis as the isolated RBP (Fig. 1B).

Here, we further investigated the molecular interactions between various RBPs of *Lactococcus lactis* phages and VHH5. First, the broadness of the neutralization conferred by VHH5 was tested against 50 distinct lactococcal phages of the 936 species. Then, phage mutants escaping the VHH5 neutralization were isolated, and their mutated RBPs were analyzed by surface plasmon resonance (SPR). We extended the resolution of the p2 RBP structure to 1.7-Å resolution and localized glycerol molecules in the RBP head, within the VHH5 binding area. We performed fluorescence quenching experiments with saccharides to demonstrate the existence of a high-affinity saccharide binding site overlapping with the structural glycerol attachment site.

MATERIALS AND METHODS

Bacterial strains, phages, and host range. The phages and hosts used in this study are listed in Table 1. *L. lactis* strains were grown at 30°C in M17 broth (Quélab) supplemented with 0.5% glucose (GM17). For phage propagation, GM17 was supplemented with 10 mM CaCl_2 as described previously (27). The neutralization assays were performed in BCP broth (2% tryptone, 0.5% yeast

extract, 0.5% glucose, 0.4% NaCl, 0.15% Na-acetate, 40 mg of bromocresol purple per liter [2]).

Native and mutant RBPs, VHH5, and head domain production. Phage p2 wild-type RBP and llama VHH5 were cloned, expressed, and purified as described previously (8, 23). Single-amino-acid mutations of RBP were generated using a QuickChange site-directed mutagenesis kit (Stratagene). Briefly, the pET28a/ORF18 (8, 23) was entirely amplified by *Pfu*Turbo DNA polymerase using two complementary mutated primers: the Thr 165 codon was mutated to encode Met using the primers T165M-F (5'-TCGATGTTCCAGTTCAAATGT TGACAGTTGAAGCTGG-3') and T165M-R (5'-CCAGCTTCAACTGTCAA CATTGGAACGTGAACATCGA-3'); Trp 201 was mutated to encode Arg using the primers W201R-F (5'-GTGTCAAATATACAAAAAGCCGGAATATGT CTGGAACATGGG-3') and W201R-R (5'-CCCAGTTCAGACATATTC GGCC TTTTGTATATTGACAC-3'); Val 217 was mutated to encode Ile using the primers V217I-F (5'-GACCATTTCTGTCAGCTGCTATTCAAAGTCTTGT TGGTCATTTTGG-3') and V217I-R (5'-CAAAATGACCAACAAGACTTTGAA TAGCAGCTGGACGAAATGGTC-3'); and Asn 236 was mutated to encode Lys using the primers N236K-F (5'-GATACCTCTTCCATATTGATATAAAGCCA AATGGTAGTATTACTTGGT-3') and N236K-R (5'-ACCAAGTAATACTAC CATTGGCTTTATATCAATATGGAAGAAGATATC-3') (bold nucleotides mark mutational changes). Parental plasmid was digested by DpnI, and the vector containing the mutation was transformed in supercompetent XL1-Blue cells. Transformants were selected onto LB ampicillin (100 $\mu\text{g}/\text{ml}$) agar plates. Mutations were verified by automated DNA sequencing (Genome Express, France). The DNA fragments encoding the phage p2 RBP head segment (residues 164 to 264) were amplified by PCR from the pET28a/ORF18 plasmid (8, 23) using the following primers: forward primer, 5'-ACAAGTTTGTACAAAAGCAGGCTagaaggagata gaaccatgaagtgcaaacgttgacagtggaagctgg-3' (attB1 sequence in uppercase, initial atg codon in bold, and the ribosome binding site and the first part of the open reading frame in lowercase); reverse primer, 5'-ACCACCTTTGTACAAGAAAGCTGGGT cTAGTGATGGTGATGGTGATGtttaatgaagtaactccgttacc-3' (attB2 sequence in uppercase, stop codon in bold, six-His tag in italic, and the last part of the open reading frame in lowercase). The PCR product was subcloned in the pDest14 vector by recombination (36) using Gateway technology (Invitrogen). Expression of mutant proteins and the RBP head was carried out using the BL21(DE3) and Rosetta(DE3)pLysS *E. coli* strains in Terrific broth (Gibco) and 2YT supplemented with antibiotics, respectively. When the optical density at 600 nm

TABLE 1. Bacteriophages used in this study

Phage	Host	Origin	GenBank accession number of the RBP gene	Reference or source
Phages neutralized by VHH5				
HD8	SMQ-423	Canada	AY576315	22
HD9	SMQ-425	Canada	AY576280	1
HD10	SMQ-424	Canada	AY576281	1
HD11	SMQ-426	Canada	AY576289	1
HD12	SMQ-426	Canada	AY576288	1
HD20	SMQ-423	Canada	AY576303	22
HD21	SMQ-424	Canada	AY576302	1
HD22	SMQ-425	Canada	AY576325	1
jj50	LM0230	Denmark	AY576279	19
p2	LM0230	United States	AY576278	25
Q50	SMQ-198	United States	AY576299	29
sk1	LM0230	United States	AF011378	5
Phages not neutralized				
712	LM0230	Ireland	AY576282	32
1727	SMQ-453	France	AY576311	Danisco, France
bIL170	IL1403	France	AF009630	7
P008	IL1403	Germany	AF539449	24
Q23	SMQ-194	United States	AY576314	29
Q34	SMQ-217	United States	AY576313	29
Q37	SMQ-217	United States	AY576312	29
Q39	SMQ-217	United States	AY576307	29
Q40	SMQ-217	United States	AY576306	29
Q41	SMQ-217	United States	AY576305	29
Q43	SMQ-217	United States	AY576310	29
Q46	SMQ-217	United States	AY576309	29
Q51	SMQ-198	United States	AY576298	29
Q62	SMQ-573	United States	AY576297	10
Q63	SMQ-573	United States	AY576287	10
Q64	SMQ-573	United States	AY576286	10
Q65	SMQ-420	United States	AY576320	10
Q66	SMQ-420	United States	AY576319	10
Q67	SMQ-420	United States	AY576318	10
Q68	SMQ-420	United States	AY576317	10
SL2	SMQ-402	Canada	AY576293	22
SL3	SMQ-403	Canada	AY576285	22
SL4	SMQ-404	Canada	AY576284	22
SL5	SMQ-405	Canada	AY576283	1
SL6	SMQ-406	Canada	AY576322	1
SL9	SMQ-408	Canada	AY576321	22
SL10	SMQ-408	Canada	AY576296	22
SL11	SMQ-408	Canada	AY576295	1
SL12	SMQ-409	Canada	AY576294	22
HD3	SMQ-392	Canada	AY576324	1
HD5	SMQ-413	Canada	AY576323	1
HD6	SMQ-413	Canada	AY576316	1
HD14	SMQ-422	Canada	AY576301	22
HD15	SMQ-427	Canada	AY576300	1
HD16	SMQ-428	Canada	AY576292	22
HD17	SMQ-428	Canada	AY576291	22
HD18	SMQ-428	Canada	AY576290	22
HD19	SMQ-428	Canada	AY576304	22

reached 0.5, expression was induced with 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside), and the temperature was decreased to 15°C and 17°C for mutants and head, respectively. All proteins produced were soluble and were purified using a two-step procedure on a Ni column, taking advantage of the presence of a six-His tag at the C terminus, followed by gel filtration on a Superdex 200 column in 1.8 mM KH₂PO₄, 10.1 mM Na₂HPO₄, pH 7.2, 2.7 mM KCl, 137 mM NaCl. For each protein, productions yielded between 20 and 50 mg of pure protein per liter of culture.

Neutralization assay. The efficacy of the VHH5 antibody in neutralizing various lactococcal phages was determined by a lactic acid production assay. Briefly, 10⁴ phages and 1 μ g/ml of VHH5 antibody were added to 3 ml of BCP medium supplemented with 10 mM CaCl₂. The mixture was incubated for 1 h at 30°C. Then, the appropriate *L. lactis* host was inoculated at 1% and incubated for an

additional 5 h at 30°C. This neutralization assay also included a control that consisted of the BCP medium inoculated with only the *L. lactis* host and another control in which only the host and the phages were added into the BCP medium. After the incubation period, the lactic acid production by *L. lactis* strains (without phages) resulted in a color change (from purple to yellow) of the medium containing a pH indicator (bromocresol purple). Conversely, the presence of phages led to cell lysis and limited acid production, and thus the color of the medium remained purple. If the antibody properly neutralized the phage, *L. lactis* was able to produce lactic acid and the pH indicator turned yellow. This assay was performed in two independent experiments for all of the 50 lactococcal phages tested.

Isolation of nonneutralized phage mutants. For all lactococcal phages neutralized by VHH5, the isolation of nonneutralized phage mutants was carried

TABLE 2. Data collection and refinement statistics

Statistic	Value
Data collection	
% of data > 1 σ (overall/last shell) ^a	97/90
Overall I/ σ (I) (overall/last shell) ^a	6.3/2
Resolution limits (Å)	1.73–40
R-merge (%) (overall/last shell) ^a	8.5/29
Refinement	
No. of protein/glycerol/solvent atoms	5,789/36/1,142
Resolution limits (Å)	1.73–40
R/R-free value (%)	17.5/19.6
RMSD on bonds (Å) and angles (°) ^b	0.11/1.3
Mean B-factor (Å ²) for protein	19.4
Mean B-factor (Å ²) for glycerols (sites 1/2)	17.8/27.6

^a Last shell, 1.73 to 1.78 Å.^b RMSD, root mean square deviation.

out. Following the standard neutralization assay, the cultures were centrifuged, and the supernatant was filtered on a 0.45- μ m Acrodisc. The phage titer was determined (27), and a second neutralization assay was performed using 10⁴ phages from the above filtrate. Three to five neutralization assays were needed to obtain nonneutralized phages in which no color change occurred in the presence of VHH5. Phages were then plated, and individual plaques were isolated and retested for neutralization by VHH5. Phage adsorption tests were carried out for wild-type and mutant phages as reported elsewhere (12). Adsorption assays were performed in three independent experiments.

DNA manipulation and fingerprinting analysis. Phage DNA was isolated using a Lambda mini kit (QIAGEN) with the following modifications. The phage pellet was resuspended in buffer L3 containing 1 mg/ml of proteinase K and then incubated for 30 min at 65°C. In the final step, the DNA pellet was dissolved in 50 μ l of 10 mM Tris (pH 8.0). DNA was digested with EcoRI and EcoRV as recommended by the manufacturer (Roche Diagnostics). Restriction fragments were heat-treated at 65°C for 10 min, electrophoresed on agarose gel (0.7%) in Tris-acetate-EDTA buffer, stained with ethidium bromide, and visualized under UV light. Phage restriction fragment length polymorphism (RFLP) patterns were analyzed with Molecular Analyst Fingerprinting Plus version 1.6 software (Bio-Rad), using the unweighted-pair group method using average linkages.

DNA sequencing and analysis. The genes coding for the receptor-binding protein of wild-type lactococcal phages and nonneutralized phage mutants were sequenced on both strands by primer walking using synthetic oligonucleotide primers (Invitrogen) and phage genomic DNA as a template. DNA sequencing was performed using an ABI Prism 3100 apparatus from the service center at the Université Laval. Computer-assisted DNA and protein analyses were performed using the Genetics Computer Group Sequence Analysis software package version 10.3 (11). The multiple sequence alignment was achieved with ClustalW, available at EMBL-EBI (<http://www.ebi.ac.uk/clustalw/>). For a few phages (712, bIL170, P008, and sk1), the RBP gene was already available in GenBank but was still confirmed by sequencing. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0 (20). The bootstrap test for phylogeny was used and replicated 2,000 times.

Crystallographic study of RBP. Crystallization of the RBP was described elsewhere (34). The 1.7-Å data set was collected at beam line ID14-4 (ESRF, Grenoble). Data were integrated and reduced using MOSFLM and SCALA (6). Refinement was performed with REFMAC5 (30) alternated with rebuilding using Turbo-Frodo (33), using the 2.3-Å RBP structure as a starting point (2BSD). Statistics are presented in Table 2. The high resolution and the low R and R-free values indicate the excellent quality of the structure.

Surface plasmon resonance. All surface plasmon resonance experiments were performed using a Biacore 1000 instrument (Biacore Inc., Piscataway, NJ) at 25°C. The buffer for the continuous-flow pump was HBS-P (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% [vol/vol] surfactant P-20) supplemented with 0.05 mM EDTA; the buffer for the sample pump was HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% [vol/vol] surfactant P-20). The buffers were degassed before use. The general strategy followed in the Biacore experiment was to take advantage of the presence of a His tag in the native and mutant RBPs and the absence of a His tag in the VHH5 molecule. The chip Ni-nitrilotriacetic acid (Ni-NTA) was first saturated with Ni²⁺ by washing it with 0.5 μ M NiCl₂ (20 μ l at 20 μ l/min). Then, the RBP solution (at 190 nM, 40 μ l at 10 μ l/min) was

passed, followed by the VHH5 solution (100 nM, 40 μ l at 10 μ l/min). Regeneration was achieved by washing the flow cell with 350 mM EDTA (20 μ l at 20 μ l/min). Step 1 was run with the command Inject, followed by Extraclean; steps 2 and 3 were run with the command Kinject; step 4 was run with the command Quickinject, followed by Extraclean. The four-step procedure described above was performed identically for the wild-type RBP, the mutants (Thr165Met, Trp201Arg, Val217Ile, and Asn236Lys), and the control injection of running buffer instead of VHH5.

Fluorescence quenching experiments. Fluorescence experiments were carried out on a Varian Eclipse spectrofluorimeter using a quartz cuvette in a right-angle configuration; the light path was 0.4 and 1 cm for the excitation and emission, respectively. The interaction of RBP with saccharides was monitored by recording the quenching of the intrinsic protein fluorescence upon addition of ligand aliquots. The excitation wavelength was 290 nm, and emission spectra were recorded in the range of 320 to 400 nm. The excitation slit was 5 nm while the emission slit was 10 nm for a protein concentration of 1 μ M. A moving-average smoothing procedure was applied, with a window of 3 nm. Titrations were carried out at room temperature with 1 μ M protein in 10 mM phosphate buffer Na₂Na₂, 50 mM NaCl, pH 7.5. The fluorescence intensities at the maximum of emission (346 nm) for different concentrations of quencher were corrected for the buffer contribution before plotting and further analysis. The affinity was estimated by plotting the decrease of fluorescence intensity at the emission maximum as $100 - (I_i - I_{\min})/(I_0 - I_{\min}) \times 100$ against the quencher concentration; I_0 is the maximum of fluorescence intensity of the protein alone, I_i is the fluorescence intensity after the addition of quencher (i), and $I_{\min}$ is the fluorescence intensity at saturating concentration of quencher. The K_d values were estimated using Prism 3.02 (GraphPad Software, Inc.) by nonlinear regression for a single binding site with the equation $Y = B_{\max} \times [X/(K_d + X)]$, where $B_{\max}$ is the maximal binding and K_d is the concentration of ligand required to reach half-maximal binding.

Nucleotide sequence accession numbers. All RBP gene sequences were deposited in the GenBank database (see Table 1 for accession numbers).

Protein structure accession number. The coordinates and structure factors for the RBP have been deposited in the Protein Data Bank of the Research Collaboratory for Structural Bioinformatics (<http://www.rcsb.org/pdb/>) as entry 1ZRU.

RESULTS

Neutralization of 936-related phages by VHH5. It was previously shown that VHH5 binds to the RBP of the lactococcal phage p2, a member of the 936 group (8, 23). The addition of VHH5 to milk also prevents phage infection and fermentation failure during the manufacture of cheese (8, 23). In order to investigate the neutralizing potential of the VHH5 antibody, 50 lactococcal phages of the 936 group were selected and tested in a neutralization assay (Table 1). All of these phages were isolated from various geographic areas and are distinct, based on the analysis of their host range as well as the EcoRV and EcoRI restriction profiles (RFLP) of their genomes (data not shown). Of the 50 phages tested, 12 phages were neutralized by VHH5 (Table 1). The 38 other lactococcal phages were not neutralized by the antibody and were still able to infect their respective host cells (Table 1). These results indicate a significant diversity of the RBP epitope and suggest a limited efficiency of VHH5 for phage control in an industrial framework.

Isolation of nonneutralized phages. From an evolutionary standpoint, phages are known to adapt to their local environment. Thus, the isolation of phage mutants that were no longer neutralized by VHH5 was carried out. Repeated infection experiments of the 12 phages in the presence of VHH5 were performed and led to isolation of nonneutralized mutants derived for 11 of these 12 phages (Table 3). No nonneutralized mutants were isolated for phage HD12, while two different mutants were identified for phage p2. Three to five challenges were needed to obtain high-titer mutant phages. The escape

TABLE 3. Differences in RBP genes and deduced proteins of phage mutants

Phage	Base replacement	Amino acid change
Q50.g1	494 C→T	165 T→M
sk1.g1	601 T→C	201 W→R
jj50.g1	601 T→C	201 W→R
HD10.g1	601 T→C	201 W→R
HD22.g1	601 T→C	201 W→R
p2.g1 ^a	601 T→C	201 W→R
HD8.g1	649 G→A	217 V→I
HD20.g1	649 G→A	217 V→I
HD9.g1	708 C→G	236 N→K
HD11.g1	708 C→G	236 N→S
HD21.g1	708 C→G	236 N→K
p2.g6	708 C→A	236 N→K

^a Five other phage mutants with an identical mutation were isolated. No phage mutant was obtained with phage HD12.

phage mutants were no longer neutralized by VHH5 but had the same RFLP patterns as the neutralized wild-type phages (data not shown). These results suggest point mutations within the epitope specificity of VHH5 to the receptor-binding protein. Adsorption of the escape phage mutants to their hosts was also measured and found to be similar to the adsorption of the wild-type phages (data not shown). Altogether, these data show that most phages can rapidly evolve to evade recognition by VHH5 without affecting their host range.

Analysis of the RBP of lactococcal phages. In order to investigate the epitope recognition of VHH5, the RBP gene of each of the 50 wild-type lactococcal phages and the nonneutralized phage mutants was sequenced and analyzed (Fig. 1 and Table 3). A single mutation was found to be sufficient to avoid neutralization by the antibody fragment. In all mutants, the point mutation led to an amino acid change within the C-terminal region of the RBP. Overall, four different mutations were observed (Table 3).

The deduced RBPs from 50 phages range in size from 252 to 308 amino acid residues. The alignment of the multiple RBP sequences revealed that the N terminus is highly conserved, as the 50 shoulder domains (residues 1 to 141) shared 50.4% identity (71/141 amino acids). In fact, the first 120 amino acids were particularly conserved (56.7% identity), while the next 21 residues were less conserved. It was previously predicted that this conserved N-terminal part of the protein may be involved in protein-protein interactions with other phage tail proteins (12, 17). The last 21 residues are probably slightly less conserved to allow connection to the neck domain.

The neck (residues 142 to 163) and head (C-terminal) domains were more diverse, possibly reflecting the differences observed in host recognition (data not shown). However, in most cases, a specific neck domain was associated with a particular head domain. One could argue that the rigid neck structure serves as a linker between the larger shoulder and head domains. Nonetheless, the 50 RBPs could be classified in at least 10 groups based on their overall identities (Fig. 2). The 12 phages neutralized by VHH5 were classified in one large group of 18 RBPs sharing almost 80% identity. Comparative analysis of the 12 RBPs recognized by VHH5 and the 6 non-recognized RBPs allowed the identification of a few amino acid discrepancies between both groups of RBP (data not shown).

Finally, the other 32 phages have a distinct C-terminal RBP compared to the above group, explaining their nonneutralization by VHH5 and leading to the identification of 9 other groups of RBPs. Recently, Dupont et al. (13) reported six different types of RBP genes of lactococcal 936 species bacteriophages and developed a PCR assay to detect them. They suggested that the PCR assay could be used in cheese plants for phage detection. Here, we showed that there are many more RBP groups within this lactococcal phage species, and perhaps this variability may limit the usefulness of such a PCR assay.

Complex of the RBP with neutralizing llama VHH5. We have already reported that, overall, the water-accessible surface area of the RBP of phage p2 covered in the interaction with VHH5 is 825 Å², with 647 Å² and 188 Å² for monomers A and B, respectively. The interaction surface involves many charged or semipolar residues, but nonpolar residues are also observed (Table 4). The VHH5 domain interacts with the RBP in a semilateral way, as observed in several camelid VHH complexes with haptens or proteins (9, 31). Facing the RBP surface, the VHH5 interacting surface is also rather polar and formed by a mixture of charged or semipolar residues (Table 4).

The isolation of p2 phage escape mutants reported above can be rationalized in view of the interaction surfaces observed in the RBP/VHH5 complex. The four observed mutations were localized in the RBP structure and spotted on the interaction surface (Fig. 3A). The first mutation appearing in sequence (Thr165Met) is located outside the interaction area, whatever monomer is taken into account (Fig. 3A). In contrast, the three other mutations involve residues belonging to the interacting area. The Trp201Arg mutation is very drastic, as Trp 201 establishes three optimal van der Waals contacts with VHH5 residues Tyr 59, Phe 103, and Ser 105. Visual inspection of models of this mutant with molecular graphics clearly indicates that the mutation Trp201Arg is no longer compatible with binding to VHH5, due to the severe clashes it introduces between both partners. Val 217 is in van der Waals contact with VHH5 residue Ser 30. The Val217Ile mutation leads to a clash between both partners that does not seem severe, since only an extra methyl group is introduced. Asn 236 establishes two strong hydrogen bonds with VHH5 residues Ser 30 and Asn 31. Therefore, the replacement of Asn 236 with a lysine destroys this arrangement and introduces a severe steric conflict between the lysine and VHH5 residues facing it.

Binding of RBP escape mutants to llama VHH5. In order to correlate and quantify the viral and structural molecular data obtained on the escape phage mutants, we expressed the four mutated RBPs (Table 3) as well as the wild-type RBP from p2 and tested their affinity for VHH5 by plasmon resonance. In this technique, direct or indirect attachment of a protein to the chip produces an increase of the plasmon resonance (y axis in Fig. 4). The six-His-tagged RBP molecules, wild type or mutant, were attached to a Ni-NTA chip, and the tagless VHH5 was injected in a second step (Fig. 4). The difference resonance units (DRU) observed after injection of p2 RBP varied between 4,600 and 4,800 for wild-type p2 and mutant Val217Ile, while they were around 5,300 for mutant Thr165Met, 9,800 for Trp201Arg, and 10,200 for Asn236Lys. As the concentrations of the different RBPs were identical, the DRU differences after RBP injections are due to different adsorptions on the chips.

FIG. 2. Phylogenetic tree of the receptor-binding proteins of 936-like phages. The tree was generated by the MEGA program. The numbers associated with the branches refer to bootstrap values (confidence limits) resulting from 2,000 replicate resamplings. The scale represents the number of amino acid substitutions per site. Phage names in bold indicate that they are neutralized by VHH5.

TABLE 4. Interactions of the receptor-binding domain with VHH mono5^a

Receptor-binding protein	VHH	Distance (Å) and bond ^a
201 Trp CD1	103 Phe CD1	3.54
202 Asn N	103 Phe O	2.96, H-bond
202 Asn OD1	99 Arg NH2	3.11, H-bond
202 Asn ND2	101 Gly O	3.04, H-bond
207 Trp CZ2	99 Arg NH1	3.67
217 Val CG2	30 Ser OG	3.19
232 His NE2	55 Tyr OH	2.90, H-bond
234 Asp OD2	31 Asn ND2	2.97, H-bond
234 Asp OD2	55 Tyr OH	2.51, H-bond
236 Asn ND2	30 Ser OG	2.54, H-bond
236 Asn ND2	31 Asn OD1	3.03, H-bond
238 Asn ND2	100 Ser OG	2.76, H-bond
240 Ser OG	101 Gly CA	3.36
242 Thr OG1	31 Asn OD1	3.41, H-bond
242 Thr OG1	101 Gly O	2.52, H-bond
244 Trp NE1	31 Asn ND2	3.04, H-bond
225B Ala CB	55 Tyr CE2	3.53
256B Arg NH2	54 Asp O	2.99, ionic

^a "H-bond" indicates the presence of a hydrogen bond. "ionic" indicates an ionic bond. All other interactions are nonpolar van der Waals bonds.

The DRU observed after injection of VHH5 was between 2,000 and 2,350 for the wild type and the mutants Val217Ile and Thr165Met and 160 and 40 for the mutants Trp201Arg and Asn236Lys, respectively. Control injection of buffer instead of VHH5, repeated two times, resulted in a DRU of 117. Control injection of VHH5 on the Ni-NTA chip saturated with Ni²⁺ gave a negligible DRU (17); the RU level after regeneration with 350 mM EDTA was reproducible (11,600 to 11,700). The wild-type molecule is able to bind VHH5, as a sharp increase of the DRU that was followed by a very slow decrease was observed (Fig. 4). The Thr165Met mutant has the same behavior as the wild-type protein and binds to VHH5. In contrast, both Asn236Lys and Trp201Arg do not exhibit a DRU increase upon VHH5 injection and therefore do not bind VHH5. The Val217Ile mutant exhibits a large DRU increase upon VHH5 injection. However, the DRU decrease is faster than those observed with the wild-type or Thr165Met proteins, indicating a larger kinetic off rate of the ligand and thus lower affinity of the former mutant.

Structure of the RBP in complex with glycerol. In this study, the resolution of the RBP structure was extended from 2.3 to 1.7 Å. In addition to the better quality of the map and the model (Table 2), six glycerol molecules originating from the cryocooling liquor (two per monomer) were clearly identified in the electron density map (Fig. 3C and D). One glycerol

molecule is located in the head domain, with an average B-factor of 17.8 Å², while the other is located at the upper face of the shoulders, with an average B-factor of 27.6 Å². The head domain tightly binds glycerol, as three hydrogen bonds were established between the His 232 and Asp 234 side chains and the glycerol O-1 atom and between the Arg 256 (from the other monomer) guanidyl group and the glycerol O-2 atom. Furthermore, the hydrophobic face of glycerol packs nicely against the Trp 244 side chain, an interaction often observed with sugars (Fig. 3C). The glycerol binding is looser at the shoulders site, as a unique hydrogen bond was established between the Met 33 carbonyl group and the glycerol atom O-1, although a water molecule network also participates in binding. The Trp 43 B side chain is located at 3.8 Å, but no stacking occurred (Fig. 3D).

The interaction of p2 RBP with VHH5 covers a large area. However, a very specific interaction is observed within the glycerol-binding site. The Tyr 55 from the VHH CDR2 penetrates deeply in the glycerol-binding site (Fig. 3E and F) at the exact position of glycerol, stacked between Trp 244a and Arg 256b. The OH group from Tyr 55 superimposes with the OH-1 of glycerol and establishes similar hydrogen bonds with His 232 and Asp 234 (Fig. 3F).

RBP in complex with glycerol and saccharides in solution.

The binding of glycerol to RBP in solution was also analyzed. Considering the close vicinity of Trp residues to glycerol molecules (Trp 244 and 43), we performed fluorescence quenching experiments. Increasing quantities of glycerol were added to a 1 μM solution of RBP. A decrease of Trp fluorescence was observed and registered. A good fit between experimental data and the theoretical curve could be obtained when a unique binding site was taken into account. The same experiment was repeated with 1-phospho-glycerol (D/L racemic) and glucopyranosyl saccharides, galactose, and muramyl dipeptide (MurNAc-D-Ala-D-iGln). The *K_d* constants observed with the four saccharides (Table 5) range from 0.26 to 0.13 μM.

In order to discriminate between the two glycerol-binding sites, we cloned and expressed the head domain (residues 164 to 264) and repeated the fluorescence quenching experiments with this construct. We also observed a fluorescence quenching curve that could be interpreted by taking into account a unique binding site. Furthermore, the *K_d* constants, ranging from 0.17 to 0.14 μM, are identical (within experimental error) to the native RBP *K_d* constants (Table 5). We conclude that the titrated site is located in the head domain. Surprisingly, the *K_d* values are very close for all sugars, including glycerol. This suggests a lack of specificity and that the extra hydroxyl groups introduced by the glucopyranosyl saccharides compared to

FIG. 3. Views of the RBP/VHH5 interacting surface and of the saccharide binding sites. (A) The RBP head domains are colored as in Fig. 1A. The interacting surface comprises the white area and the three red zones labeled W201, V217, and N236. The red zones identify the four escape mutations. The mutation at position 165 is located outside of the interaction area. The green line separates the interaction areas belonging to two different monomers. (B) The RBP interacting surface is depicted in spheres and colored in white, except for the side chains of the residues interacting with VHH5, which are colored and labeled. *, residues belonging to the second monomer; **, the positions of the escape mutants. (C) Electron density map (2fo-fc) of the glycerol-binding site in the head domain. (D) Electron density map (2fo-fc) of the glycerol-binding site in the shoulder domain. (E) Stereo view of the protruding VHH5 Tyr 55 in the glycerol-binding site of native RBP. VHH5 is represented in sticks colored according to atom type. The surface of Tyr 55 is displayed as a grid. The RBP molecular surface is colored light brown. (F) Stereo view of the superposition of VHH5 Tyr 55 with glycerol. The RBP/glycerol complex is colored blue (RBP) and pink (glycerol). The RBP/VHH5 complex is colored in blue. The hydrogen bonds between the glycerol OH-1 and Asp 234 or His 232 are displayed as dashed lines.

FIG. 4. Complexation study of RBP and mutants with VHH5 by SPR. Wild-type and mutant RBPs were injected and fixed on a Ni-NTA chip (first RU increase). VHH5 was then injected, producing an increase in RU when bound. Note the faster decrease of the RU value with Val217Ile compared to wild-type RBP.

glycerol have no or little effect on binding. Visual inspection of models of their complexes with RBP confirms that the extra hydroxyl groups are facing the bulk solvent and do not interact with the RBP molecule (data not shown). In contrast, the phosphate group of the glycerol phosphate is well placed to establish an ionic bond with Arg 256b. Surprisingly, the K_d value of glycerol phosphate is only slightly smaller than that of glycerol.

Soaking with these various saccharides followed by cryocooling and data collection yielded again the glycerol in the electron density maps. Indeed, the large excess of glycerol used in cryocooling was able to displace any bound sugar. No diffraction was observed, unfortunately, when no or other types of cryoprotectants were used.

DISCUSSION

Because phages are omnipresent in the dairy environment, considerable efforts in the last decades were aimed at controlling their proliferation rather than trying to eradicate them (26). In many countries, lactococcal phages of the 936 species are the most predominant. Therefore, several targeted anti-936 strategies have been implemented over the years, while others, such as the use of a phage-neutralizing heavy chain antibody fragment, have been envisioned. In spite of these

extensive efforts, “phage attacks” remain today the most common cause of slow milk fermentation, and this is mainly due to phage diversity and evolution. Here, we have convincingly shown that VHH5, which binds to the RBP and prevents phage infection, confers only a limited protection against 936-like phages, as only 12 out of the 50 phages tested could be neutralized. Moreover, escape phage mutants could readily be isolated from phage lysates. The latter observation was somewhat unforeseen, as it was assumed that a mutation in the RBP (to avoid neutralization by VHH5) could lead to phage mutants no longer able to infect the host strain. Obviously, this was not the case. It remains to be seen if other heavy chain antibody fragments, capable of neutralizing both the wild-type phages and the escape mutants, can be selected.

The competitive advantage for the lactococcal 936-like phage mutants to escape neutralization by VHH5 is profitable only if the mutations do not alter significantly their binding to the host receptors. Hence, the mutations should not involve residues located in the receptor-binding site. This was observed here, since none of the four essential residues of the binding site were mutated (Fig. 5). The observation of a genetically stable receptor-binding site raises the possibility of using small molecules targeting specifically this site with an antiviral therapy.

The structure of the complex of phage p2 RBP with neutralizing VHH5 has made it possible to identify the interaction area. Since VHH5 prevents phage infection, the interaction area should contain the receptor-binding site or at least part of it. Analysis of the deduced amino acid sequences of the 12 RBPs neutralized by VHH5 indicated that all of the binding site residues are conserved, with two exceptions (Fig. 5). First, the phage Q50 has a histidine at position 217 instead of a valine. Second, the phages HD8 and HD20 have a valine instead of an alanine at position 225. The former difference might introduce a clash in the contact area of Q50 with VHH5. When isoleucine was replaced by valine in other phages, they could escape to VHH5 neutralization. However, SPR data

TABLE 5. K_d values of the interaction between p2 head domain and saccharides

Saccharide	K_d (μ M)	
	RBP	Head domain
Glycerol	0.26 ± 0.05	0.18 ± 0.04
Galactose	0.17 ± 0.03	0.16 ± 0.03
MurNAc-dipeptide	0.12 ± 0.03	0.14 ± 0.03
Phosphoglycerol	0.13 ± 0.03	ND ^a

^a ND, not determined.

FIG. 5. Sequence alignments of the binding domain of 12 phages belonging to the lactococcal 936 species and neutralized by VHH5. The residues belonging to the interaction area are identified by black filled circles. Equals signs identify escape mutant positions. Gray filled circles identify binding site residue positions. The sequence of a 936-like phage (bIL170, last line) not neutralized by VHH5 is given for comparison.

have indicated that this residue is less sensitive, in molecular terms, than the two other neutralizing mutants. The differences observed with phages HD8 and HD20 seem compatible with VHH5 binding. Ala 225 is buried in a crevice between two subunits (Fig. 3B). It faces Tyr 55 from VHH5, whose side chain can slightly rotate to release enough space for the extra methyl groups to fit (data not shown). SPR experiments with mutated RBP confirm the structural analysis. Surprisingly, the Thr165Met mutant does not belong to the RBP interaction area with VHH5 and exhibits a native-like behavior. Perhaps this mutation affects the binding of the RBP with other phage structural proteins, somehow leading to nonrecognition by VHH5 in vivo. In contrast, the three other mutant RBPs do not bind to VHH5 at all or bind with lower affinity.

It has been shown recently that lactococcal phages belonging to the 936 species, such as bIL170 and ϕ 645, bind to cell wall polysaccharides found at the surface of *L. lactis* (14). Moreover, Geller et al. (16) suggested that the 936-like phages sk1, jj50, and Q64 might not require a host membrane protein or lipoteichoic acid and that cell wall components are sufficient for the initial steps of phage infection. Here, a glycerol molecule was located in the X-ray structure, bound to His 232, Asp 234, Arg 256b, and Trp 244, residues conserved in the list of phages under study (Fig. 3C). Fluorescence quenching experiments confirm this binding site with high affinity to saccharides. The nature of the receptor-binding site is in agreement with polysaccharides acting as receptors at the *L. lactis* surface. It is reminiscent of sugar-binding sites, such as those observed in lectins (3), which often associate hydrophobic patches of aromatic residues, to accommodate the saccharide's nonpolar face, and polar residues functioning as hydrogen bond donors or acceptors, bringing specificity (4). All saccharides tested here yielded lower K_d values than what is generally observed with monosaccharides. However, these K_d values are restricted within a narrow range and are all close to that of glycerol, making it difficult to identify the natural saccharide receptor. Nonetheless, phosphoglycerol, a component of teichoic or lipoteichoic acids, exhibits an excellent affinity for RBP, a fact that points to teichoic or lipoteichoic acid as a candidate receptor.

In conclusion, we showed that VHH5 has a limited efficacy, since it could neutralize only 12 of the 50 lactococcal phages tested and phages can easily mutate to avoid recognition by the

heavy chain antibody fragment. Moreover, we have extended the resolution of the RBP structure to 1.7 Å and proven the saccharidic nature of the bacterial receptor recognized by lactococcal phages of the 936 species. We propose that teichoic or lipoteichoic acid might be the phage receptor in the *L. lactis* cell wall. Experiments are under way to validate this hypothesis.

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