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A Specific Sugar Moiety in the *Lactococcus lactis* Cell Wall Pellicle Is Required for Infection by CHPC971, a Member of the Rare 1706 Phage Species

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ABSTRACT *Lactococcus lactis* is a Gram-positive bacterium widely used as a starter culture for the production of different dairy products, especially a large variety of cheeses. Infection of lactococcal starter cultures by bacteriophages is one of the major causes of fermentation failure and often leads to production halt. Lactococcal bacteriophages belonging to the c2, 936, and P335 species are the most commonly isolated in dairy plants and have been extensively investigated in the past three decades. Information regarding bacteriophages belonging to less commonly isolated species is, on the other hand, less extensive, although these phages can also contribute to starter culture infection. Here, we report the nucleotide sequence of the newly isolated *L. lactis* phage CHPC971, belonging to the rare 1706 species of lactococcal phages. We investigated the nature of the host receptor recognized by the phage and collected evidence that strongly suggests that it binds to a specific sugar moiety in the cell wall pellicle of its host. An *in silico* analysis of the genome of phage CHPC971 identified the hypothetical genes involved in receptor binding.

IMPORTANCE Gathering information on how lactococcal bacteriophages recognize their host and proliferate in the dairy environment is of vital importance for the establishment of proper starter culture rotation plans and to avoid fermentation failure and consequent great economic losses for dairy industries. We provide strong evidence on the type of receptor recognized by a newly isolated 1706-type lactococcal bacteriophage, increasing knowledge of phage-host interactions relevant to dairying. This information can help to prevent phage infection events that, so far, are hard to predict and avoid.

KEYWORDS bacteriophage, *Lactococcus lactis*, dairy, phage receptor, phage-host interaction

Lactococcus lactis is a Gram-positive lactic acid bacterium (LAB) that is widely used in the dairy industry as a starter culture for the production of fermented foods that include quark, buttermilk, sour cream, and a wide variety of cheeses. It produces lactic acid, which causes the rapid acidification of milk, and other organic compounds that contribute to the development of the characteristic taste and texture of the end products (1–4).

Specific combinations of *L. lactis* starter strains are used in dairy production processes in order to obtain products that meet consumers' demands (5–8). A specific starter culture combination, however, cannot be continuously used in the same industrial setting due to the ubiquitous presence of bacteriophages in dairy processing lines (9). Infection of starter cultures by these bacterial viruses is, in fact, one of the biggest

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causes of milk fermentation failure and leads to important economic losses for the dairy industry (10).

A solution routinely employed to circumvent this problem is to adopt a starter culture rotation plan in which cultures with comparable metabolic properties but different phage sensitivity profiles are used in rotation (11). However useful it is in reducing phage proliferation, this strategy does not always prove to be entirely sufficient due to the continuous adaptation of bacteriophages to their host resistance mechanisms (9, 12, 13).

Because of the threat they represent to the dairy industry, *L. lactis* bacteriophages have been extensively investigated over the last 3 decades, with increasing interest in the identification of the host receptors recognized by the phages and, conversely, in the characterization of host recognition devices of the phage particles (14–16). The most recent taxonomic classification separates the known lactococcal bacteriophages into ten different species, namely, 936, c2, P335, KSY1, Q54, 1358, P087, 949, P034, and 1706 (17). Phages belonging to the first three species are the most commonly isolated in dairy environments, where they represent the main cause of *L. lactis* culture infection and fermentation failure. For this reason, they have been studied in more detail than members of the remaining groups (18).

Nevertheless, if the right conditions arise, rarer lactococcal bacteriophages can proliferate and even cause fermentation failure (19–21). Information regarding the replication cycle, host receptors, and structure of these phages is still quite limited, requiring new and detailed information gathering on these types of phages. Bacteriophage 1706 is the namesake of its species and is an example of such a rarely encountered lactococcal phage (21). Its genome organization and life cycle have been elucidated, but no information is available on the host receptor recognized by any of the phages belonging to this species (21, 22).

Here, we report a study on a new member of the 1706 phage species, named CHPC971, which was isolated from a cheese whey sample derived from a factory based in the United States. We investigated the nature of the possible host receptor recognized by phage CHPC971 and show that it most probably binds to a specific sugar moiety. Furthermore, we analyzed the genome of the phage to get a better insight into the genes coding for the putative receptor binding protein and to identify its differences and similarities with other members of the 1706 phage species.

RESULTS AND DISCUSSION

Isolation of bacteriophage-insensitive mutants for studying phage-host interaction. Bacteriophage CHPC971 was isolated from an industrial cheese whey. Genome sequencing showed that it belongs to the rare 1706 species of lactococcal phages (see below). In a procedure aimed at obtaining phage-resistant mutants of industrial starter strains, *L. lactis* CH_LC01 was initially tested for its sensitivity to phage CHPC971 and, in addition, that to phage CHPC1174 (belonging to the P335 species). The strain initially proved to be sensitive to both phages, and bacteriophage-insensitive mutants (BIMs) of bacteriophage CHPC1174 could be isolated after challenging the strain with this phage. One of these BIMs was selected and named *L. lactis* CH_LC02. Strain CH_LC02 was resistant to phage CHPC1174 but still sensitive to phage CHPC971. After infection of strain CH_LC02 with phage CHPC971, four BIMs (resistant to phages CHPC1174 and CHPC971) were isolated and named CH_LC07, CH_LC08, CH_LC09, and CH_LC10. All of the BIMs were isolated at a frequency of 1×10^{-6} (calculated by dividing the CFU/ml of visible BIMs after appropriate incubation time by the CFU/ml of the culture used for the double-layer agar overlay test).

It is known that phage resistance in BIMs is often due to mutations in genes coding for the host receptor recognized by the phages (23). Since nothing is known about the nature of the receptor recognized by phages of the 1706 species, the four BIMs (CH_LC07 through CH_LC10) were further analyzed to fill this knowledge gap.

Genomic and phenotypic characterization of the four isolated BIMs. Genomic DNA of strains CH_LC01, CH_LC02, and the four BIMs (CH_LC07 through CH_LC10) was

TABLE 1 Mutations identified in the glycosyltransferase gene and rate of adsorption of phage CHPC971 to the indicated strains

Strain	Mutation[a]	Amino acid change[b]	Adsorption of phage CHPC971 (% ± SD)[c]
CH_LC01			89 ± 6
CH_LC02			79 ± 25
CH_LC07	599 G→T	Trp200Leu	10 ± 15
CH_LC08	917 C→A	Ser306*	0 ± 8
CH_LC09	461 G→A	Gly154Glu	10 ± 12
CH_LC10	544 C→T	Gln182*	0 ± 17
UC509.9			0 ± 14
SMQ562			6 ± 13

[a]Nucleotide change and position relative to A of AUG start codon of the glycosyltransferase gene are indicated.
[b]Amino acid change and its position in glycosyltransferase (GT). *, stop codon.
[c]Experiments were performed as independent triplicates, and the corresponding standard deviation of the independent results set is indicated.

sequenced and a single-nucleotide polymorphism (SNP) analysis was carried out to identify mutations in strain CH_LC02 and in the four BIMs relative to their parent strain, *L. lactis* CH_LC01. Among the several SNPs identified, one type of mutation was particularly interesting, as it was the only one detected in all four BIM strains but not in strain CH_LC02 (Table S1), and it could thus be linked to the resistance developed by the four BIMs toward phage CHPC971. The mutation entailed a single-nucleotide change in a putative glycosyltransferase (GT) gene (locus_tag E6O52_09585), the position of which was different in each BIM. Two of the mutations led to a truncated protein product, while the other two involved amino acid changes (Table 1). It is not known what specific role this putative GT enzyme and its mutated derivatives might play in *L. lactis*, but *in silico* analysis revealed that its gene is present in the operon encoding the cell wall polysaccharide (CWP) that covers *L. lactis* cells (24).

Phenotypic analyses of the four BIMs revealed that the strains tend to form a precipitate after overnight growth in LM17 and that their growth is delayed for at least 1 h compared to that of strains CH_LC01 and CH_LC02 (Fig. S1).

When the expression vector pMGG36c, harboring the original GT gene of CH_LC01, was introduced in each of the four BIMs, growth in liquid medium was restored to the level of that of strain CH_LC01 with no noticeable precipitate formation (data not shown). Moreover, each of the complemented strains was found to be sensitive to phage CHPC971 to the same extent as their sensitive parental strain CH_LC01 (Fig. S2). These data confirm that the mutations in the GT gene are responsible for the resistance of the four BIMs to phage CHPC971. Since it is known that sugar moieties in the CWP are used as receptors by many *L. lactis* bacteriophages (25, 26), we proceeded by examining whether this also might be the case for phage CHPC971.

Phage CHPC971 receptor is of a saccharidic nature. A phage adsorption assay using phage CHPC971 permissive and resistant strains was performed to first confirm that the resistance mechanism of the four BIMs (CH_LC07 through CH_LC10) involved changes in a receptor for the phage. As shown in Table 1, phage CHPC971 shows a low to very low adsorption rate to all four BIMs compared to those of its sensitive strains, CH_LC01 and CH_LC02. To confirm these findings via a different assay, the interaction between phage CHPC971 and the various bacterial strains used in this study was visualized by fluorescence microscopy. As shown in Fig. 1, the phage is able to adsorb to *L. lactis* strains CH_LC01 and CH_LC02, while no to very little adsorption to the four BIMs was observed.

Glucose, galactose, rhamnose, and glucosamine are the prevalent sugar components of the *L. lactis* CWP (24). In order to identify which sugar plays a key role in phage CHPC971-host interaction, a competition assay was performed in which phage infection was monitored in LM17 medium with a high concentration of one of the four sugars. A delay in the decrease of viable cells would indicate that the examined sugar competes with the host receptor for phage binding and suggest that it is part of the

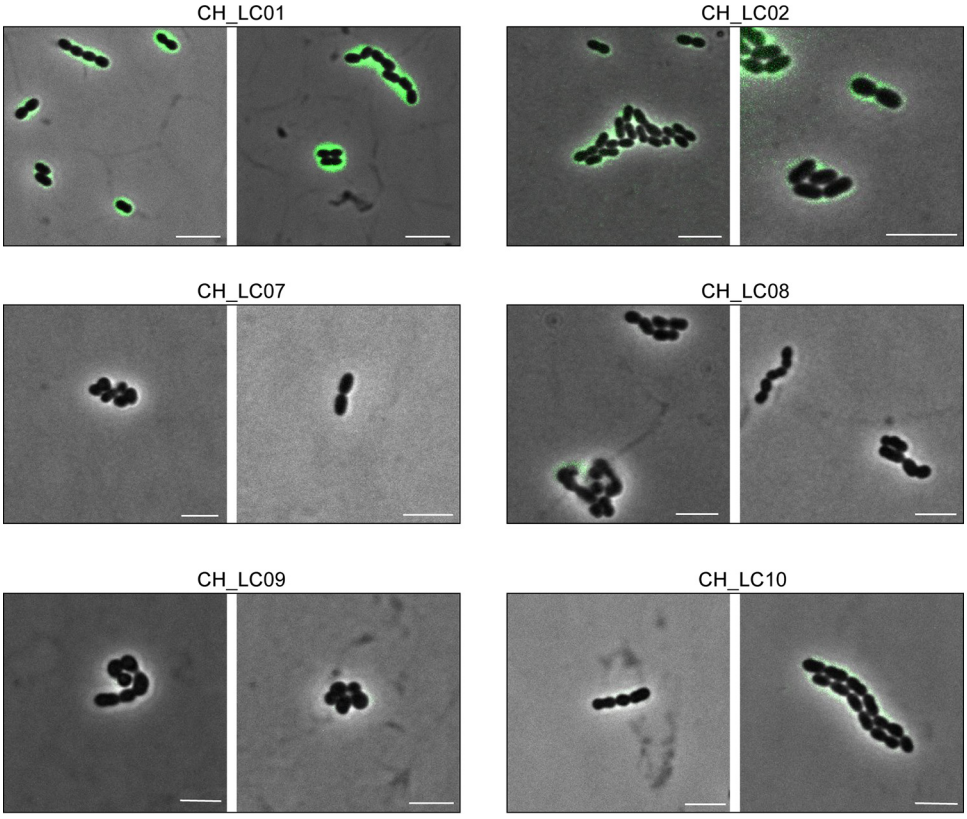

FIG 1 Fluorescence imaging of phage CHPC971 infecting permissive hosts *L. lactis* CH_LC01 and CH_LC02 and the four BIMs. Fluorescently labeled phage CHPC971 was added to cells of all 6 strains in their early exponential growth phase at an MOI of >1. Two images are presented for each strain, the name of which is reported at the top of each pair of images. Bar, 5 μm.

sugar motif recognized by the phage. As shown in Fig. 2, glucose, galactose, and glucosamine at final concentrations of 250 mM did not delay the infection of strain CH_LC01 by phage CHPC971. On the other hand, at the same final concentration, rhamnose almost completely prevented phage infection, suggesting that this sugar is very important in the recognition of the host by this phage.

The findings presented above strongly suggest that the mutations in the GT gene affect the receptor of phage CHPC971 or at least one of the molecules that is recognized during the process of the phage adsorbing to its permissive hosts. The data also indicate that phage CHPC971 binds to a saccharidic molecule and that rhamnose is predominantly involved in this interaction.

Relation between phage CHPC971 adsorption and the CWP of the host. Three variations of the CWP operon have been identified in *L. lactis*; they differ in their gene content and give rise to three different pellicle types (types A, B, and C) that vary in sugar composition (25). The CWP operon has a conserved overall structure with a region of highly conserved genes followed by a variable region that is typical for each of the three pellicle types. A set of primers for multiplex PCR has been previously designed to distinguish CWP operons (25). Following this approach, we analyzed the four BIMs (CH_LC07 through CH_LC10) and their parent strain CH_LC01 and established that they all possess a type A CWP operon (data not shown). The specific sugar composition of the lactococcal CWP has been elucidated for the *L. lactis* strains MG1363, 3107, and SMQ388, all coding for a type C CWP (24, 26, 27); UC509.9, which codes for a type A CWP; and IL1403, which codes for a type B CWP. The last two types of CWP were revealed to be rhamnose rich (28, 29).

The host range of phage CHPC971 was tested against an array of *L. lactis* dairy isolates and dairy isolate-derived laboratory strains representing all three CWP types. As

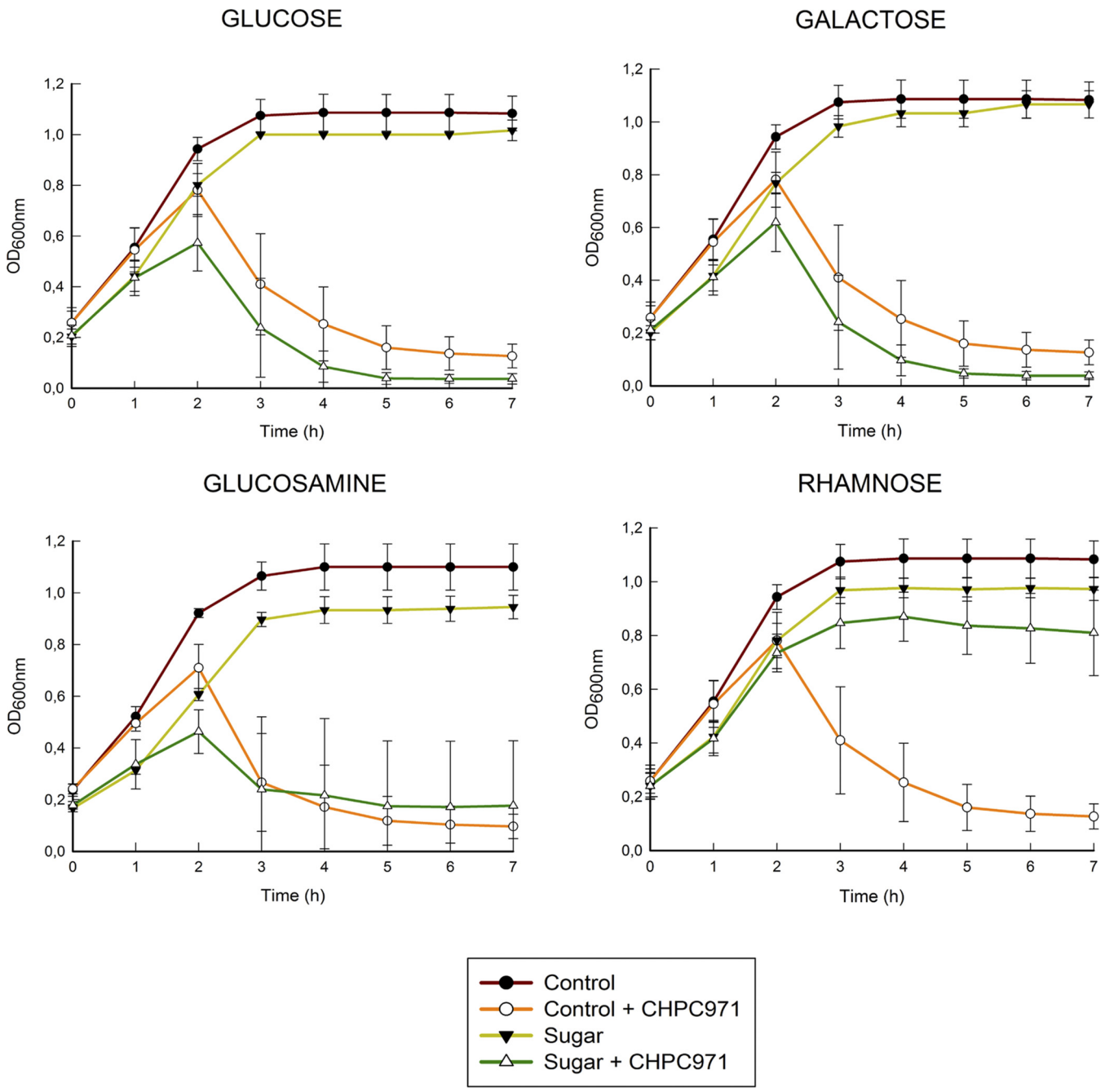

FIG 2 Sugar competition assay. Growth of *L. lactis* CH_LC01 started with a 3% inoculum of an overnight culture at 30°C in LM17 broth with the indicated sugar at 250 mM concentration. The cultures were infected in their early exponential growth phase (OD_{600}, ca. 0.2) with 10^6 PFU/ml of phage CHPC971, after which growth was monitored at hourly intervals. Each point represents the mean of three independent replicate experiments. Error bars indicate standard deviations.

shown in Table 2, only strains that possess a type A CWP are sensitive to phage CHPC971, a result that is in accordance with the proposed involvement of rhamnose in phage-host interaction.

Four strains with a type A CWP are resistant to phage CHPC971, two of which, UC509.9 and SMQ562, were tested in an adsorption assay. As shown in Table 1, adsorption of phage CHPC971 to both strains was close to zero. This result suggests that, in these cases, the host receptor of the phage has also changed. An *in silico* analysis of the proteins encoded by the CWP operons of strains UC509.9 and SMQ562 revealed that their glycosyltransferase enzyme, which is mutated in the four BIMs, is

TABLE 2 Host range of phages CHPC971 and 1706

L. lactis strain	CWP type[a]	Subspecies	Infected by indicated phage[b]	
			CHPC971	1706
C10	A	*lactis*	+	+
UL8	A	*lactis*	+	+
SMQ86	A	*lactis*	+	+
SMQ384	A	*lactis*	+	+
SMQ385	A	*lactis*	+	+
SMQ450	A	*lactis*	−	+
CH_LC01	A	*lactis*	+	−
158	A	*cremoris*	−	−
UC509.9	A	*cremoris*	−	−
SMQ562	A	*lactis*	−	−
AM1	A	*cremoris*	−	−
IL1403	B	*lactis*	−	−
Bu2-60	B	*lactis*	−	−
229	B	*lactis*	−	−
ML8	B	*lactis*	−	−
MG1363	C	*cremoris*	−	−
SK11	C	*cremoris*	−	−
3107	C	*cremoris*	−	−
184	Unknown	*lactis*	−	−

[a]CWP type was determined by multiplex PCR (25).
[b]+, infected; −, not infected.

identical to that of strain CH_LC01. However, apart from a putative membrane protein (encoded by open reading frame [ORF] E6O52_09620 in strain LC_CL01) that showed a high amino acid variability among the CWP of all the strains of our collection (data not shown), an array of amino acid changes is present in several other proteins encoded by the respective CWP operons of strains UC509.9 and SMQ562, including two glycosyltransferases (Fig. S3). Although involvement of the individual mutations in the CWP operons of strains UC509.9 and SMQ562 in phage resistance was not experimentally verified, these results, in combination with those obtained for the four BIMs, make it tempting to speculate that phage CHPC971 preferably infects strains possessing a type A CWP. A more comprehensive analysis involving additional strains with type B and type C CWP is required to confirm this hypothesis.

***In silico* analysis of the phage CHPC971 genome and its relation with other members of the 1706 species.** The CHPC971 phage genome contains 54,381 bp with a G+C content of 34% and a total of 73 predicted open reading frames (ORFs). It shows more than 90% nucleotide (nt) identity with the genome of bacteriophage 1706, the reference phage of the 1706 species of rare lactococcal phages. The general organization of the genomes of the two phages is conserved, but both contain mutually unique ORFs, all of which encode putative proteins of unknown function (Fig. 3A).

The product of ORF23 (Porf23; GenBank accession number QCW07625.1) of phage CHPC971 shows 55% amino acid (aa) identity with that of ORF21 of phage 1706. The latter protein was previously annotated as a hypothetical receptor binding protein (RBP) on the basis of its 71% aa identity with the hypothetical RBP of the P335-type phage phismq86 (21). Porf23 shares high amino acid sequence identity with other lactococcal phage tail-associated proteins (Table S2). An HHpred analysis revealed that the C terminus of Porf23 is highly similar to that of the RBPs of the *L. lactis* phages Tuc2009 and WRP3 (data not shown). The latter two proteins contain a sugar binding domain and have been proven to bind to a sugar receptor in the CWP of their *L. lactis* hosts (30, 31). Although a sugar-binding domain could not be identified in Porf23, these findings suggest that ORF23 most probably codes for the RBP of phage CHPC971.

An ORF unique to phage CHPC971 is located close to ORF23 (ORF21, see Fig. 3A). Other *L. lactis* and *Streptococcus thermophilus* phages (temperate *L. lactis* phages and, in the case of *S. thermophilus*, both temperate and lytic phages) (32–35) encode putative proteins with amino acid sequences almost identical to that of Porf21

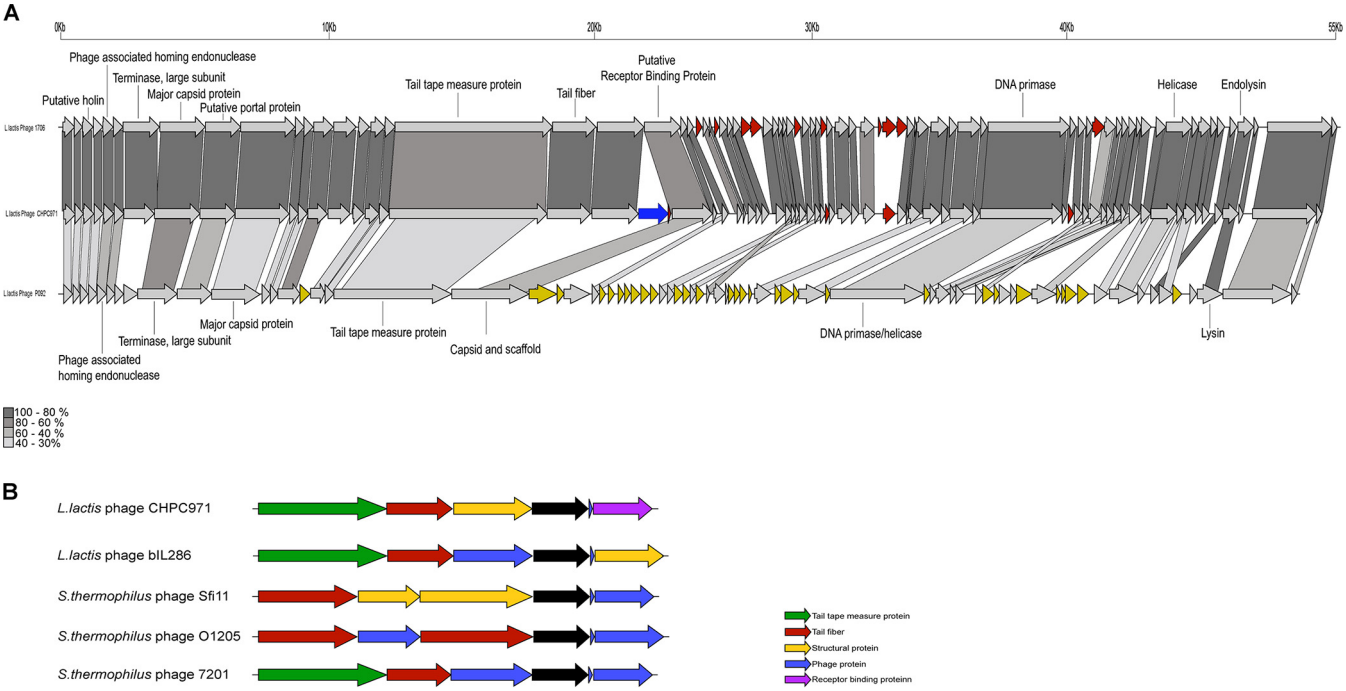

FIG 3 Comparison of bacteriophage genomes. (A) Comparison of the complete genomes of *L. lactis* phages 1706, CHPC971, and P092. Gray boxes between the gene arrows show the percentage of amino acid identity of the respective gene products, for which the gray scale is given at the bottom left corner. Percentages are intended for the following comparisons: phage 1706 versus phage CHPC971, phage CHPC971 versus phage P092. Red arrows, unique open reading frames (ORFs) of phage 1706 or CHPC971. Yellow arrows, ORFs of phage P092, the product of which shows significant amino acid identity only with ORF products of phage P078, P118, or P162. Blue arrow, ORF21 of phage CHPC971. Annotations for predicted ORFs of phages 1706 and CHPC971 coincide and are given at the top of the figure. Annotations for predicted ORFs of phage P092 are given at the bottom of the figure. The image was generated with Easyfig (42) and subsequently manually modified. (B) Comparison of the area of the genome coding for tail structural proteins of phage CHPC971 with that of other *L. lactis* and *S. thermophilus* phages. ORF21 of phage CHPC971 and the corresponding ORFs of the other phages with high nucleotide similarity to it are shown as black arrows. The legend in the lower right corner indicates the annotation of all the other ORFs shown in the panel. The image was generated with SEED Viewer (http://rast.nmpdr.org/seedviewer.cgi) and subsequently manually modified.

(GenBank accession number QCW07623.1). Their respective ORFs share over 90% nt identity and are always positioned next to or near genes specifying the tail tape measure protein or the tail fiber protein (Fig. 3B). Although the function of the encoded proteins has not been experimentally confirmed, these *in silico* results make it tempting to speculate that they may be involved in phage-host recognition. The low amino acid identity between the predicted RBPs of phages CHPC971 and 1706, together with the Porf21 of phage CHPC971 possibly being involved in host recognition, might explain the minor host range differences between the two phages (Table 2). This setup would be similar to that reported for the two P335-type phages Tuc2009 and TP901-1. These two phages have similar baseplate structures, but Tuc2009 encodes an extra baseplate protein, BppA, to which host range differences between the two phages have been ascribed (36).

In a previous study, the genomes of four bacteriophage isolates derived from raw milk samples, P078, P092, P162, and P118, were compared to that of phage 1706 (22). The four phages showed a ≥95% nt identity among each other and have the same genome organization. Although this genome structure is similar to that of phage 1706, the shared nucleotide identity with the latter genome is much lower (Fig. 3A), and they are thus classified as being distantly related to 1706 (34). We conducted an *in silico* analysis to compare phage CHPC971 with the four raw milk phages, choosing one of them, P094, as a reference.

Phages CHPC971 and P094 show 55% nt identity, while only 37 of the 73 putative proteins of phage P092 have amino acid identities higher than 30% (E value, $<10^{-5}$) with the predicted phage CHPC971 proteins (Fig. 3A). This result is very similar to that already reported for the comparison of phages P092 and 1706 (22). Thirty of the

predicted proteins of phage P094 have significant amino acid similarity with the putative products of the other raw milk phages, P078, P162, and P118, but not with any other (putative) protein present in the NCBI database. The genomes of all of the 1706-type phages carry a number of small ORFs of unknown function with some, albeit limited, similarity with ORFs of (pro)phages of a number of different bacterial genera, including *Ruminococcus*, *Clostridium*, *Streptomyces*, *Faecalibacterium*, and *Rhodococcus* (data not shown). The predicted RBP of phage CHPC971, Porf23, shares 41% aa identity only with the C-terminal end of Porf19 of phage P092; proteins with significant shared amino acid identity with phage CHPC971 Porf21 are not specified by P092. Moreover, a BLASTp analysis of phage P094 proteins revealed that none has any significant similarity with known bacteriophage proteins involved in host recognition.

The work presented here provides new and useful insights into phage-host interaction in the dairy environment and could help improve starter culture rotation strategies. The four isolated BIMs comply with European legislation regarding the use of genetically modified organisms (GMOs) in food products and could thus, in principle, be used in the dairy industry to avoid infection by phage CHPC971 and related phages. Their aggregation phenotype, which could lead to precipitation of the bacteria to the bottom of the production vat and result in pH differences between the top and the bottom of the fermenter, and their lower growth rates do not concur with large-scale dairying and would have to be carefully investigated and amended before any of these isolates are employed as a commercial starter culture.

MATERIALS AND METHODS

Bacterial strains, phages and culture conditions. The bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 3. All *L. lactis* strains were grown at 30°C in M17 liquid medium (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 0.5% lactose (LM17) or 0.5% glucose (GM17) based on the bacterial metabolic requirements. When necessary, 5 μg/ml of chloramphenicol was added. CFU were estimated by plating serial dilutions of a liquid culture of the strain to be tested on LM17 agar (1.5%) plates.

Bacteriophages were propagated by infecting a 10-ml culture of the indicator strain in its early exponential growth phase (optical density at 600 nm [OD_{600}] = 0.3 to 0.5) with a single plaque in LM17 supplemented with 10 mM $CaCl_2$ and 10 mM $MgCl_2$. The sample was incubated at 30°C until visible cell lysis had occurred and was subsequently centrifuged at 3,500 $\times$ *g* for 10 min in a 5804R tabletop centrifuge (Eppendorf, Hamburg, Germany) to eliminate cell debris. The supernatant was filter sterilized using a 0.45-μm filter (Minisart NML, Sartorius, Germany) and stored at 4°C.

PFU and bacteriophage host range were determined using the double agar overlay plaque assay as previously described (37) with the following modifications: bottom and top agar layers contained 1% and 0.4% agar, respectively. $CaCl_2$ and $MgCl_2$ were added to the medium at a final concentration of 10 mM each. Glycine was added to the medium at a final concentration of 0.5% (wt/vol) to facilitate plaque counting, as previously described (38). Bacteriophage lysates were diluted in TBT buffer (100 mM NaCl, 10 mM $MgCl_2$, and 50 mM Tris-HCl [pH 7]).

To isolate bacteriophage-insensitive mutants (BIMs), 100 μl of an appropriately diluted overnight-grown culture of the host strain was mixed with 100 μl of phage lysate (titration, $>10^8$ PFU/ml) to reach a multiplicity of infection (MOI; ratio of PFU over CFU) of $\geq$1. The bacterium-phage mixture was plated in a double-layer agar overlay test as previously described (37), incubated for 24 to 48 h at 30°C, and subsequently monitored for the presence of phage-insensitive colonies.

Bacteriophage adsorption assay. LM17 broth (10 ml) was inoculated with 3% of an overnight culture of the test strain and incubated at 30°C until an optical density at 600 nm (OD_{600}) of approximately 0.5 was reached. Cells (200 μl) were mixed with a bacteriophage preparation to yield a final concentration of 10^5 PFU/ml in a final volume of 1 ml of LM17 containing 10 mM $CaCl_2$. A control tube without cells was also prepared. Samples were incubated at 30°C for 15 min to allow adsorption of the phage to the host cells and then centrifuged at 15,000 $\times$ *g* for 10 min in a 157.MP microcentrifuge (Ole Dich Instrumentmakers ApS, Hvidovre, Denmark). Supernatant (800 μl) was collected and the titer therein of nonadsorbed phages was calculated via a double-agar overlay plaque test as described previously. Each test was conducted in triplicate, and the percentage of phage adsorbing to the bacterial cells was calculated as follows:

$$\frac{\text{control titer} - \text{nonadsorbed phage titer}}{\text{control titer}} \times 100$$

Microscopy analysis. Bacteriophage DNA was stained with SYBR gold nucleic acid stain (Invitrogen, Carlsbad, CA). To this end, the original SYBR gold solution supplied by the manufacturer was diluted 100-fold in dimethyl sulfoxide and stored at −20°C in the dark. One ml of a bacteriophage stock (10^9 PFU/ml) was mixed with 5 μl of the diluted SYBR gold stock and incubated at 4°C in the dark for at least 12 h. Bacterial host cells in early exponential growth phase (OD_{600} = 0.3 to 0.5) growing in LM17 broth were mixed with fluorescently labeled phages in a 1:5 (vol/vol) ratio at an MOI of >1; $CaCl_2$ was added

TABLE 3 List of strains, bacteriophages, and plasmids used in this study

Lactococcus lactis strain, bacteriophage, or plasmid	Subspecies	Description or origin[a]	Reference or source
Strains			
CH_LC01	*lactis*	Dairy starter strain	This work
CH_LC02	*lactis*	Derivative of CH_LC01	This work
CH_LC07	*lactis*	Derivative of CH_LC02	This work
CH_LC08	*lactis*	Derivative of CH_LC02	This work
CH_LC09	*lactis*	Derivative of CH_LC02	This work
CH_LC10	*lactis*	Derivative of CH_LC02	This work
MG1363	*cremoris*	Laboratory model strain; plasmid-free derivative of dairy isolate NCDO712	43
AM1	*cremoris*	Dairy starter strain	44
SMQ86	*cremoris*	Dairy starter strain	45
SMQ384	*cremoris*	Dairy starter strain	46
SMQ385	*cremoris*	Dairy starter strain	46
SMQ450	*cremoris*	Dairy starter strain	17
SMQ562	*cremoris*	Dairy starter strain	17
ML8	*cremoris*	Dairy starter strain	47
3107	*cremoris*	Dairy starter strain	48
158	*cremoris*	Dairy starter strain	49
UC509.9	*cremoris*	Dairy starter strain	25
SK11	*cremoris*	Dairy starter strain	50
184	*lactis*	Dairy starter strain	25
UL8	*lactis*	Dairy starter strain	25
IL1403	*lactis*	Laboratory model strain; plasmid-free derivative of dairy isolate CNRZ157	25
C10	*lactis*	Dairy starter strain	25
229	*lactis*	Dairy starter strain	25
Bu2-60	*lactis*	Dairy starter strain	51
Bacteriophages			
CHPC1174		Whey sample, USA	This work
CHPC971		Whey sample, USA	This work
Plasmids			
pMG36c		pWV01-based expression vector carrying the strong lactococcal promoter P_{32}; Cm^r	39
pMGG36c		pMG36c carrying the GT gene from *L. lactis* CH_LC01	This work

[a]Cm^r, chloramphenicol resistance.

to the mixture at a final concentration of 10 mM. Immediately after mixing bacterial cells and phages, 3 μl of the infection mixture was placed on an agarose-coated microscopy slide for microscopic observation. To this end, a solution of agarose at a final concentration of 1% (wt/vol) in 1× phosphate-buffered saline (PBS) was prepared. Molten agarose (300 μl) was spread over the microscopy slide and allowed to solidify at room temperature before spotting the infection mixture. Micrographs were obtained with a Delta Vision Elite inverted epifluorescence microscope (Applied Precision, GE Healthcare, Pittsburgh, PA). A standard fluorescence filter, fluorescein isothiocyanate (FITC), was used to visualize the signal from the SYBR gold label: excitation was at 475/28 nm and emission was monitored at 523/36 nm. A 100× phase-contrast objective was used in combination with SoftWorX 3.6.0 software (Applied Precision, Inc., Issaquah, WA) to operate the microscope and to perform imaging of bacteria and bacteriophages.

Sugar competition assay. LM17 broth (10 ml) was inoculated with 3% of an overnight culture of the test strain and incubated at 30°C until the OD_{600} was approximately 0.2. Subsequently, $CaCl_2$ (10 mM final concentration) and glucose, galactose, glucosamine, or rhamnose, each at a final concentration of 250 mM, were added. Bacteriophages were then added at 10^6 PFU/ml. Control tubes consisted of inoculated LM17 medium containing only the phage, only the sugar, or neither of the two. Bacterial growth was monitored by measuring the OD_{600} of the samples at hourly intervals in a Genesys 20 scientific spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Each test was conducted in triplicate.

DNA techniques. Cell wall polysaccharide (CWP) typing of all the strains used in this study was performed by multiplex PCR as described earlier (25) using the CWP primer set mentioned in Table 4.

To perform full-genome sequencing, total DNA of the selected strains was isolated using the DNA DNeasy blood and tissue kit following the protocol for Gram-positive bacteria (Qiagen, Hilden, Germany). The genomes were sequenced on an Illumina MiSeq platform with 2 × 150-bp paired-end sequencing (Illumina, San Diego, CA). Mutations identified by single-nucleotide polymorphism (SNP) analysis were verified using PCR with the primers indicated in Table 4, followed by Sanger sequencing (Macrogen, Amsterdam, The Netherlands).

TABLE 4 Primers used in this study

Locus tag of the targeted gene (amplicon size [bp])	Primer name[g]	Sequence (5′→3′)	Reference or source
UC509_0206[a] (442)	Type A CWP Fw	GTGCCTATGCTCCGTTAGTC	
	Type A CWP Rv	CGAGGGCCAATCTCTTTACC	25
LLKF_205[b] (183)	Type B CWP Fw	GATTCAGTTGCACGGCCG	
	Type B CWP Rv	AGTAAGGGGGCGGATTGTG	25
llmg_0226[c] (686)	Type C CWP Fw	AAAGCTCATCTTTCCCCTGTTGT	25
	Type C CWP Rv	GCACCATAGTCTGGAATAAGACC	
dTDP-glucose 4,6-dehydratase (891)	Control Fw	GTACACTATGTTTATAACAATCATCCAG	25
	Control Rv	GCAAACCAGATTCAAAGTCAGTATG	
Glycosyltransferase (693)[f]	GT Fw[d]	GGGCTCAATCTATCTGAAGG	
	GT Rv[d]	TCCGTAAGTTTGCGTCCTCG	This work
Glycosyltransferase (1,001)	GT Fw[e]	GACTGAGCTCGTAAAGGAAAATGATGATAAATAAAG	
	GT Rv[e]	GATCCTGCAGTCCCTTTATTTGCAAACTAGTTTTG	This work

[a]Locus tag of the reference genome of strain *L. lactis* UC509.9.
[b]Locus tag of the reference genome of strain *L. lactis* IL1403.
[c]Locus tag of the reference genome of strain *L. lactis* MG1363.
[d]Used for sequencing of the glycosyltransferase gene for validation of the SNPs detected in the four BIMs (CH_LC07 through CH_LC10).
[e]Used to amplify the glycosyltransferase gene from strain CH_LC01 for cloning into pMG36c and subsequent validation of the obtained vector, pMGG36c.
[f]The amplicon size is that obtained when using the primers on strain CH_LC01.
[g]Fw, forward; Rv, reverse.

For the complementation of the mutated phenotype of the four BIMs, the wild-type glycosyltransferase (GT) gene was amplified from *L. lactis* strain CH_LC01 total DNA using the primers indicated in Table 4. Plasmid pMG36c was used as the cloning vector (39). Both the vector and the GT DNA fragment were digested using the fast-digest enzymes SacI (recognizing the GAGCT^C site) and PstI (recognizing the CTGCA^G site) (Thermo Fisher Scientific, Waltham, MA, USA), purified using the NucleoSpin gel and PCR cleanup kit according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany) and ligated using T4 DNA ligase (Thermo Fisher Scientific, Waltham, MA, USA). The ligation mixture was purified using a NucleoSpin gel and PCR cleanup kit according to the instructions of the manufacturer and then introduced by electrotransformation into *L. lactis* MG1363 (40) using a Gene Pulser electroporation system (Bio-Rad, Richmond, CA, USA). Selection of transformants was performed on GM17 plates containing 0.5 M sucrose (SGM17) and 5 μg/ml chloramphenicol. The resulting plasmid, pMGG36c, was isolated, sequenced to confirm the proper nucleotide sequence of the GT gene using the primers indicated in Table 4, and subsequently introduced into the relevant strains via electroporation using the protocol mentioned above. Transformants were selected on SLM17 plates containing 5 μg/ml chloramphenicol.

Bioinformatics analyses. Nucleotide sequence reads were trimmed, analyzed, and assembled using CLC Genomics Workbench 10.1.1 (Invitrogen, Thermo Fisher Scientific). The assembled contigs were annotated using Rapid Annotations using Subsystems Technology tool kit (RASTtk) (41). SNP analyses of wild-type and phage-insensitive *L. lactis* mutants were performed with CLC Genomics Workbench 10.1.1 (Invitrogen, Thermo Fisher Scientific). Online protein analyses were performed using HHpred (https://toolkit.tuebingen.mpg.de) and BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard parameters.

Accession number(s). The complete genomic sequences of *L. lactis* strain CH_LC01 and bacteriophage CHPC971 are available at GenBank under accession numbers SSHI00000000.1 and MK779875, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01224-19.

SUPPLEMENTAL FILE 1, PDF file, 2.1 MB.

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