

# Identification and Analysis of a Novel Group of Bacteriophages Infecting the Lactic Acid Bacterium *Streptococcus thermophilus*

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## ABSTRACT

We present the complete genome sequences of four members of a novel group of phages infecting *Streptococcus thermophilus*, designated here as the 987 group. Members of this phage group appear to have resulted from genetic exchange events, as evidenced by their “hybrid” genomic architecture, exhibiting DNA sequence relatedness to the morphogenesis modules of certain P335 group *Lactococcus lactis* phages and to the replication modules of *S. thermophilus* phages. All four identified members of the 987 phage group were shown to elicit adsorption affinity to both their cognate *S. thermophilus* hosts and a particular *L. lactis* starter strain. The receptor binding protein of one of these phages (as a representative of this novel group) was defined using an adsorption inhibition assay. The emergence of a novel phage group infecting *S. thermophilus* highlights the continuous need for phage monitoring and development of new phage control measures.

## IMPORTANCE

Phage predation of *S. thermophilus* is an important issue for the dairy industry, where viral contamination can lead to fermentation inefficiency or complete fermentation failure. Genome information and phage-host interaction studies of *S. thermophilus* phages, particularly those emerging in the marketplace, are an important part of limiting the detrimental impact of these viruses in the dairy environment.

*Streptococcus thermophilus* is a globally employed dairy bacterium used in the production of a variety of cheeses and yoghurt. Having been safely consumed by humans for millennia, this bacterium is now a mainstay of the dairy industry due its favorable acidification and texturizing properties (1, 2). Despite advances in the available knowledge regarding dairy phage containment (3, 4) and *S. thermophilus* phage genetics and biology (5, 6), contamination of dairy production lines by *S. thermophilus*-infecting (bacteriophage) phages remains a persistent problem (for a review, see reference 7).

Classification of phages of *S. thermophilus* (reviewed by Mahony and van Sinderen [8]) has long been based on (i) morphology, i.e., as *Siphoviridae*, corresponding to group B as defined by Bradley (9), and (ii) a combination of the mode of DNA packaging (i.e., *cos* or *pac* site-containing) and major structural protein content (10). A variable genomic region thought to be (at least in part) responsible for host determination (VR2 region [11]) can also be used to categorize the majority of isolated *S. thermophilus* phages (12). More recently, however, a morphologically distinct and genetically divergent *S. thermophilus* phage named 5093, containing neither *cos*- or *pac*-defining structural elements nor a confirmed antireceptor-encoding gene, was described (13), prompting the creation of a third *S. thermophilus* phage group (here termed the “5093 group”). The genomic content of phage 5093 (containing several genes of nondairy streptococcal phage origin) highlights the genetic plasticity of *S. thermophilus* phages, thus explaining the appearance of such diverse phage lineages.

A total of 13 complete genome sequences of *S. thermophilus*-infecting phages have been published to date, with a large degree of conservation observed within the defined groupings. Phage groups have been defined as follows: (i) *cos* site-containing, with members Sfi19 and Sfi21 (lytic and temperate [14]), DT1 (lytic [15]), 7201 (lytic [16]), and Abc2 (lytic [6]); (ii) *pac* site-contain-

ing phages O1205 (temperate [17]), Sfi11 (lytic [18]), 2972 (lytic [19]), 858 (lytic [20]), ALQ13.2 (lytic [6]), and TP-J34 and TP-778L (temperate [5]); and (iii) the 5093 group archetype 5093 (lytic [13]).

Whole-genome sequencing of *S. thermophilus*-infecting phages has enabled their genome-wide, nucleotide level comparison and elucidation of their putative mechanisms of evolution. It was postulated (18) that the main modes of *S. thermophilus* phage evolution are represented by the rearrangement (or recombination) of discrete genomic modules, as well as by insertions, deletions, and point mutations—of which the latter is likely to function as a means to evade active clustered regularly interspaced short palindromic repeat (CRISPR) systems of their hosts (20). Consistent monitoring of phage populations in dairy plants in this manner is necessary to ensure that adequate knowledge-based rotational schemes are in place so as to avoid fermentation inconsistencies or even complete failure. This must initially include host sensitivity profiling and phage typing studies, yet may be extended

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to whole-phage-genome sequencing in the case of newly emerging groups and/or persistent or highly virulent phages.

Here, we present the complete genome sequences of four novel phages capable of infecting *S. thermophilus* ST64987, an industrial dairy starter strain. The 987 group phages were categorized as novel based on their recalcitrance to typing using a previously designed multiplex PCR protocol, their distinct morphology, and finally their genetic content, which differed from those of previously described groups of *S. thermophilus* phages. Comparative genomic analysis was performed for all four phages. The structural protein complement of one representative phage of this group was confirmed by mass spectrometry. The phages were further characterized by microscopic analysis and adsorption analyses, and the antireceptor of one phage was defined (as a representative) using an adsorption inhibition assay.

## MATERIALS AND METHODS

**Bacteriophage isolation, propagation, enumeration, and storage.** Bacterial strains were routinely grown from single colonies or reconstituted skimmed milk (RSM) stocks overnight at 30°C (*Lactococcus lactis*) or 42°C (*S. thermophilus*) in M17 broth (Oxoid, Hampshire, United Kingdom) containing 0.5% glucose (GM17; Sigma-Aldrich, St. Louis, MO, USA) for *L. lactis* or lactose (LM17; Sigma-Aldrich) for *S. thermophilus*. Phage enumeration was performed based on standard spot or plaque assay methods (21) in which LM17 broth was supplemented with 0.25% glycine (Oxoid), 10 mM CaCl<sub>2</sub> (Oxoid), and either 10 g/liter (solid agar base) or 4 g/liter (semisolid overlay) technical agar (Merck, Darmstadt, Germany). Industrially derived whey samples from dairy plants producing fermented milk products (such as cheeses and yoghurts) were obtained (stored at -20°C) and analyzed for the presence of phages against *S. thermophilus* using the spot and plaque assay methods mentioned above. These samples now form part of the DSM phage collection (Delft, Netherlands). Single plaque isolates were then propagated according to the method of Moineau et al. (22) in LM17 at 42°C. The lysed culture was filtered (0.45 µm; Sarstedt, Nümbrecht, Germany) and stored at 4°C for use in subsequent assays. Single plaque isolation and propagation were performed at least twice to ensure the purity of phage preparations.

**Bacteriophage purification and DNA preparation.** Individual phages were propagated in a 2-liter volume before concentration by polyethylene glycol (PEG) 8000 (Sigma-Aldrich) precipitation and purification using a discontinuous cesium chloride (CsCl; Sigma-Aldrich) block gradient as described by Sambrook et al. (23), using a Beckman 50 Ti rotor (Beckman Coulter, Brea, CA, USA). Phage DNA was prepared using a method adapted from Moineau et al. (22) and Sambrook et al. (23). Briefly, 20 µl proteinase K (20 mg/ml; Fisher Scientific, Waltham, MA, USA) was added to 500 µl of CsCl purified phage, and the mixture was heated at 56°C for 20 min. A sodium dodecyl sulfate solution (SDS; Sigma-Aldrich) was then added to a final concentration of 1.5% before heating at 65°C for 30 min. Potassium acetate was added to a final concentration of 1 M, and the mixture was placed on ice for 30 min. Centrifugation at 13,200 × g for 10 min was followed by two phenol-chloroform-isoamyl alcohol (25:24:1; Sigma-Aldrich) extractions and the addition of 0.1 volume of 3 M sodium acetate (pH 4.8; Lancaster Synthesis, Ward Hill, MA, USA) and 2.5 volumes of ice-cold 96% ethanol. Precipitated phage DNA was pelleted at 21,000 × g for 15 min and resuspended in 50 µl Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA [Sigma-Aldrich]; pH 7.5). Phage DNA was visualized on 1% agarose (Sigma-Aldrich) gels stained with Midori Green Advance DNA stain (Nippon Genetics Europe GmbH, Dueren, Germany) using the method of Sambrook et al. (23).

**DNA sequencing and *in silico* analysis.** Approximately 20 µg phage DNA was extracted and verified by nanodrop (Nanodrop 2000; Thermo Scientific) quantification. Confirmatory molecular identification (ID) tests were also conducted on the DNA extract prior to shipment to the contract sequencing facility (Macrogen Inc., Seoul, South Korea). At least

100-fold sequencing coverage was obtained using pyrosequencing technology on a 454 FLX instrument. The individual sequence files generated by the 454 FLX instrument were assembled with GSAssembler (454 Life Sciences, Branford, CT, USA) to generate a consensus sequence. Quality improvement of the genome sequence involved Sanger sequencing (Eurofins MWG, Ebersberg, Germany) of at least three PCR products across each entire genome to ensure correct assembly, double stranding, and the resolution of any remaining base conflicts occurring within homopolymer tracts. Genomes were annotated using a heuristic approach (Genemark) (24) and manually using the Basic Local Alignment Search Tool (NCBI) (25). Conserved protein domains (where relevant) were detected using Pfam (26), HHpred (27), and/or CDD (28). Complete genomes were visualized using Artemis (29). Phylogenetic trees were generated using the FigTree tool (<http://tree.bio.ed.ac.uk/software/figtree/>).

**Electron microscopic analysis.** Cesium chloride phage samples were dialyzed (as described above) and subjected to further purification by ultracentrifugation (and dialysis) according to the method of Briggler Marco et al. (30), using a Beckman VTi 65.2 rotor (Beckman Coulter). Dialysis was performed twice for 24 h and 45 min, respectively, against 2 liters of phage buffer (0.05 M Tris-HCl [pH 7.5], 0.1 M NaCl, 8 mM MgSO<sub>4</sub>) (30). Electron microscopy was performed as previously described by Casey and colleagues (31).

**Structural protein identification.** Phage protein extraction (including methanol-chloroform extraction), SDS-PAGE visualization, and preparation of phage structural protein samples were performed as described by Casey et al. (31). Electrospray ionization-tandem mass spectrometry (ESI-MS/MS) was performed as previously described (32, 33). The coverage levels of at least two unique peptides for each structural protein or 5% of the total protein length were used as cutoff values when identifying gene products as components of the viral particle (31).

**Adsorption assays.** Quantification of phage adsorption to bacterial strains was determined using a method adapted from Garvey et al. (34). A 10-ml volume of LM17 or GM17 broth was inoculated (2 to 4% depending on the strain) with the appropriate *S. thermophilus* strain (either ST64987 as the sample strain or ST67368 as the adsorption control strain) or *L. lactis* strain (LL64981 as the sample strain) from a fresh overnight culture and grown at 42°C or 30°C, respectively, until the optical density at 600 nm (OD<sub>600</sub>) reached a value between 0.5 and 0.54. A 700-µl volume of the growing culture was transferred to a microcentrifuge tube and centrifuged at 5,000 × g for 10 min to pellet the cells. The supernatant was removed, and the cells were resuspended in 700 µl of one-quarter-strength Ringer's solution (Merck). An equal volume of the appropriate phage lysate (diluted to an approximate titer of 10<sup>5</sup> to 10<sup>6</sup> PFU/ml) was added to the tube or to 700 µl one-quarter-strength Ringer's solution (Merck), which served as a negative control. The mixture was incubated at 30°C or 42°C for 12 min and centrifuged at 15,000 × g for 3 min to remove bacterial cells before 200 µl of the residual phage-containing supernatant was removed for enumeration as described above. Calculation of adsorption levels (as a percentage of the total number of phages present) was performed as follows: [(control phage titer - free phage titer in supernatant)/control phage titer] × 100.

**Antireceptor purification and adsorption inhibition assays.** The protein product of *ORF19*<sub>9871</sub> (where ORF is open reading frame), predicted to encode the phage antireceptor (termed here the receptor binding protein or RBP<sub>9871</sub>), was purified using a previously described method (35). Briefly, the *ORF19*<sub>9871</sub> gene was amplified using Phusion polymerase (New England BioLabs, Ipswich, MA, USA) and employing primers that incorporate a sequence encoding an N-terminal His<sub>6</sub> purification tag and appropriate restriction enzyme sites (namely RBP<sub>9871</sub>F, 5'-AGCAGCCC ATGGCACACCATCACCATCACCATTCTTCTGGTGAACATAAGAT AATTTAAGT-3', and RBP<sub>9871</sub>R, 5'-AGCAGCTCTAGATTAATAT ACTTGGATATGA-3') and cloned behind the nisin-inducible promoter of plasmid pNZ8048 (36). The ligation mixture was dialyzed against sterile distilled (sd) H<sub>2</sub>O for 10 min and introduced into electrocompetent *L. lactis* NZ9000 cells (36). Plasmid DNA was then extracted using a GeneJet

TABLE 1 Bacterial strains and bacteriophages used in this study

Bacterial strain or phage	Description	Origin
Bacterial strains		
ST64987	<i>S. thermophilus</i> host for phages 9871-4	DSM, Netherlands
LL64981	<i>Lactococcus lactis</i> subsp. <i>lactis</i> host for phage 98103	DSM, Netherlands
ST67368	<i>S. thermophilus</i> host for phage 3681, as adsorption control	DSM, Netherlands
NZ9000	Transformation host	Kuipers et al., 1998 (36)
Phages		
9871	Lytic phage of <i>S. thermophilus</i> ST64987	DSM, Netherlands
9872	Lytic phage of <i>S. thermophilus</i> ST64987	DSM, Netherlands
9873	Lytic phage of <i>S. thermophilus</i> ST64987	DSM, Netherlands
9874	Lytic phage of <i>S. thermophilus</i> ST64987	DSM, Netherlands
3681	Lytic phage of <i>S. thermophilus</i> ST67368, as adsorption control	DSM, Netherlands
98103	Lytic phage of <i>L. lactis</i> LL64981, as adsorption control	DSM, Netherlands

Plasmid Miniprep kit (Thermo Scientific) and subjected to Sanger sequencing (as described above) to verify the integrity of the DNA sequence. For target protein induction, NZ9000 strains containing the required plasmid were grown to an OD<sub>600</sub> of 0.2 prior to the addition of nisin (10 ng/ml) using Nisaplin (Danisco, Copenhagen, Denmark). Growth was continued for 3.5 h prior to cell lysis and sonication as per the method of Collins et al. (35), with the following modifications: the concentration of CaCl<sub>2</sub> (Sigma-Aldrich) in the lysis buffer (10 mM Tris, 300 mM NaCl, 10 mM CaCl<sub>2</sub>, 25 mg/ml lysozyme [Sigma-Aldrich]; pH 8) was increased to 50 mM, and a further 200 µl 1 M CaCl<sub>2</sub> was added to the lysed cells prior to sonication (Soniprep 150; MSE, London, United Kingdom) cycles. Sonicated cells were then centrifuged, and target protein purification was performed using an Ni-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) column (Bio-Rad, Hercules, CA, USA), using various concentrations of imidazole buffer (10 mM Tris-HCl, 50 mM CaCl<sub>2</sub>, 300 mM NaCl, 50 to 200 mM imidazole; pH 7.5) according to the manufacturer's instructions. Protein fractions were visualized by separation on a 12.5% SDS-PAGE gel at 160 V for 90 min. Fractions containing bands of the correct size with minimal contamination were dialyzed against 100 ml protein buffer (as above) three times for 40 min each to remove remaining imidazole. Dialyzed fractions were stored at 4°C for use in subsequent adsorption inhibition assays.

Adsorption inhibition assays were performed as described by Collins et al. (35), with the following modification: both the antireceptor incubation and phage adsorption temperatures were increased to 42°C. Adsorption to wild-type (WT) and antireceptor-incubated cells was calculated as described above. Adsorption inhibition, expressed as a percentage of phage adsorption to WT cells, was calculated as follows: [(% adsorption on WT - % adsorption on preincubated cells)/% adsorption on WT] × 100.

**Accession number(s).** Whole-genome sequence data for phages 9871, 9872, 9873, and 9874 are available in the GenBank database under the following accession numbers: [KU678389](#) (9871), [KU678390](#) (9872), [KU678391](#) (9873), and [KU678392](#) (9874).

## RESULTS AND DISCUSSION

**Isolation of phages.** The bacterial strains and phage isolates (Table 1), which formed part of a larger industrial starter strain and phage sample collection (an exception being NZ9000), were initially subjected to a phage-host survey to determine the host ranges of isolated phages. This was followed (in the case of the

TABLE 2 General characteristics of the genomes of phages 9871-4

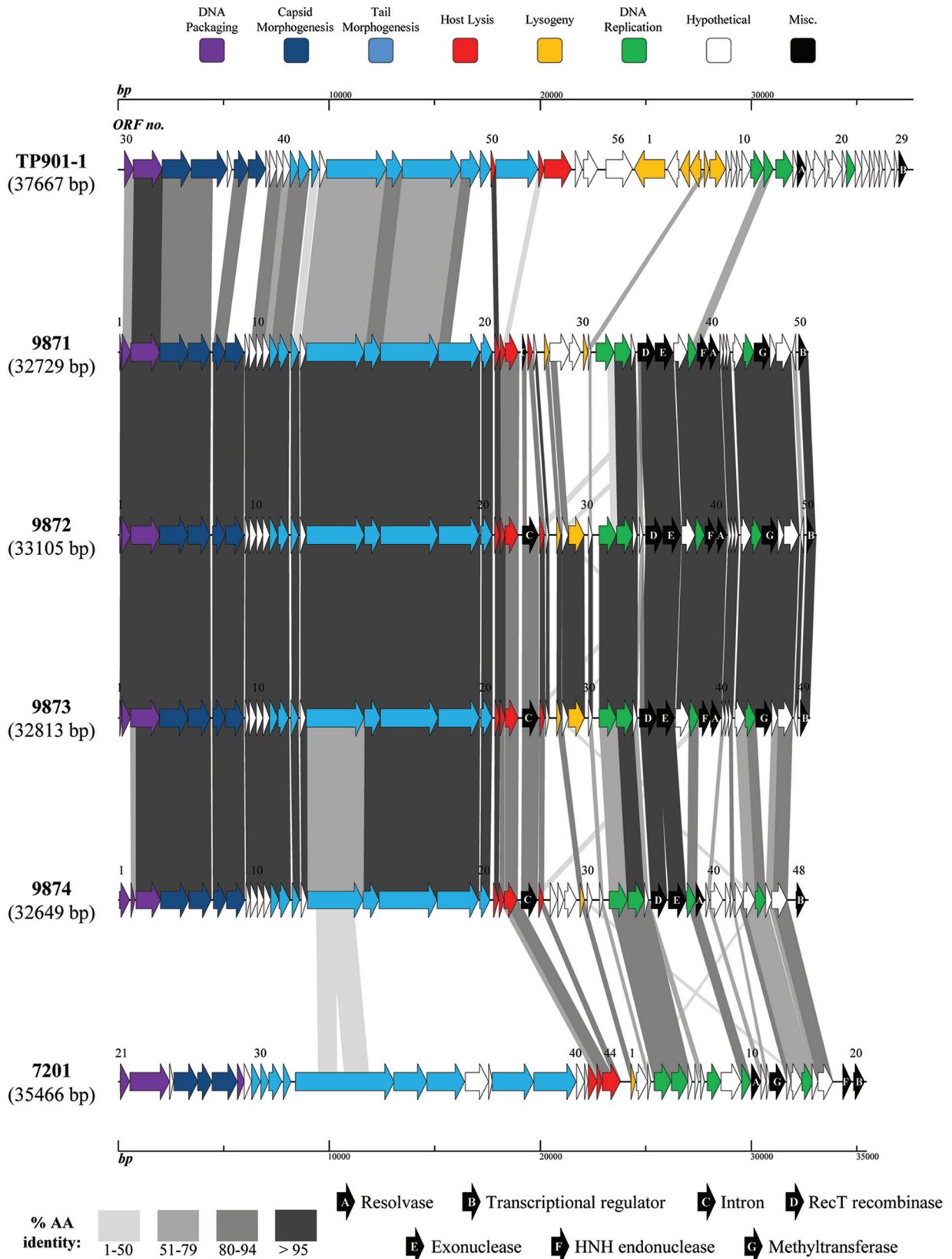
Characteristic	9871	9872	9873	9874
Length (bp)	32,729	33,105	32,813	32,649
No. of predicted ORFs	50	50	49	48
Coding (%)	92.2	91.4	91.7	89.0
GC content (%)	37.06	36.84	36.9	36.62

phages) by further characterization and genome sequencing of a representative selection, the results of which will be published elsewhere. The phage isolates characterized as part of the current study, named 9871, 9872, 9873, and 9874 (together referred to here as “9871-4” or the 987 phage group), originated from distinct dairy fermentation samples from a range of geographical locations and time points, specifically: Portugal in 2008 (isolate 9871), Slovakia in 2008 (isolate 9872), United Kingdom in 2009 (isolate 9873), and Australia in 2010 (isolate 9874). Of 90 industrial strains tested, phages 9871-4 were found to infect just a single strain (named ST64987), with subsequent experiments revealing the ability to cause low-level infection of a second strain (ST47795) by 9872, 9873, and 9874 only (data not shown). This observed narrow host range is typical of *S. thermophilus* phages (12, 37). The four phages were shown to reach a high titer during standard propagations (approximately 10<sup>9</sup> PFU/ml), and DNA could readily be extracted from both crude lysate and CsCl-purified preparations. However, a standard *cos* or *pac* phage-typing PCR (38) on either lysate or DNA preparations repeatedly failed to yield a product (data not shown). For this reason, these phages were identified as phage isolates that potentially belong to a novel group, and they were therefore subjected to genome sequencing.

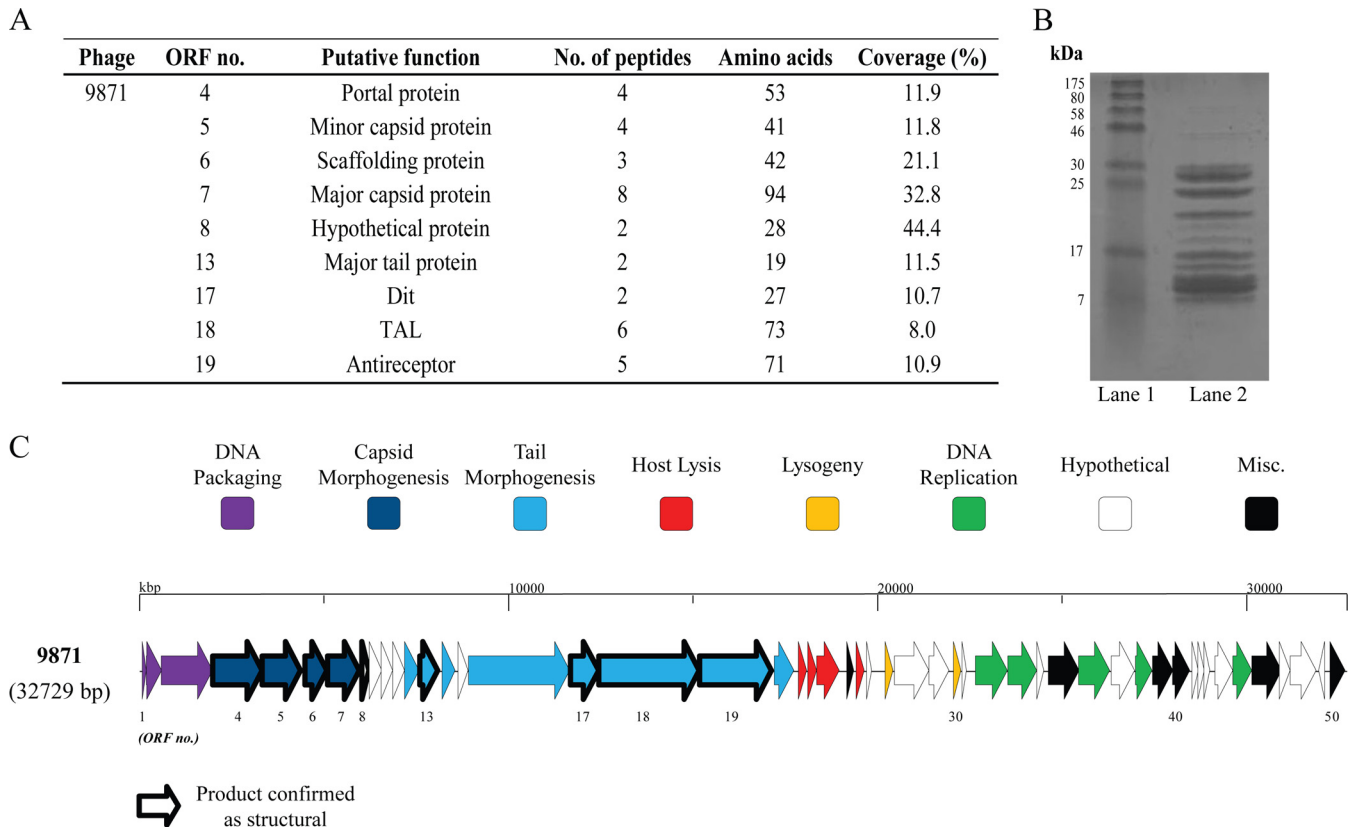
**Genome analysis. (i) General characteristics.** The salient genome characteristics of phages 9871-4 are outlined in Table 2, with a detailed list of top BLAST identities provided for phage 9871 (as a representative of the group, due to overall conservation of the four genome structures) in Table S1 in the supplemental material. Genome sizes ranged from 32.6 to 33.1 kilobase pairs (kbp), making these genomes the shortest thus far described for *S. thermophilus* phages. An initial analysis of the DNA sequences revealed a high level of nucleotide identity (greater than 90% across approximately one-third of the length of their genomes) with phage ul36 (39), and also with Tuc2009, TP901-1, and the archetype P335, which are related phages, all belonging to P335 subgroup II (40, 41). In contrast, the rightward end of each of the 9871-4 phage genomes appears to bear more similarity to *S. thermophilus* phage replication modules (42) (Fig. 1). Each module of this apparent “hybrid” 987 phage group is discussed below.

**(ii) Structural modules and structural protein determination.** The structural gene module in the four 987 group members in each phage, spanning the region starting at the putative portal protein-encoding gene to the predicted serine acetyltransferase-encoding gene, are remarkably conserved at the deduced amino acid level (Fig. 1). For this reason, structural proteins present in purified phage particles prepared from a cell lysate were determined by mass spectrometry for phage 9871 as a representative of the group (Fig. 2), and this phage alone will be discussed further (unless otherwise indicated). The deduced products of ORF<sub>49871</sub> to ORF<sub>89871</sub> were all confirmed as structural proteins and are presumed to be involved in phage head morphogenesis (based on their positions in the genome, as well as amino acid identities to





**FIG 1** Comparative analysis of the genetic organization and content of phages 9871-4 with archetypes TP901-1 (*L. lactis*-infecting phage species P335) and 7201 (*cos*-containing *S. thermophilus* phage). Predicted ORFs (indicated by arrows) and gene products (putative function indicated by color coding) are aligned with adjacent genomes according to percent amino acid identity (indicated by shaded boxes). Gene products considered to be notable are marked in black (see color and shade keys).



**FIG 2** Structural proteome analysis of phage 9871. (A) Deduced structural proteins (and corresponding ORF number) as identified by ESI-MS/MS (threshold, two unique peptides or 5% ORF coverage). No. of peptides, number of distinct polypeptide strings identified during the analysis; amino acids, the total number of amino acids identified in each protein; coverage, the number of amino acids identified expressed as a percentage of the number of amino acids in the entire proteome. (B) SDS-PAGE gel (12%) showing the structural protein profile of phage 9871. Lane 1, broad-range protein ladder (New England BioLabs); lane 2, phage 9871 protein extraction. (C) ORF schematic of phage 9871 highlighting confirmed structural protein-encoding genes (bold outline).

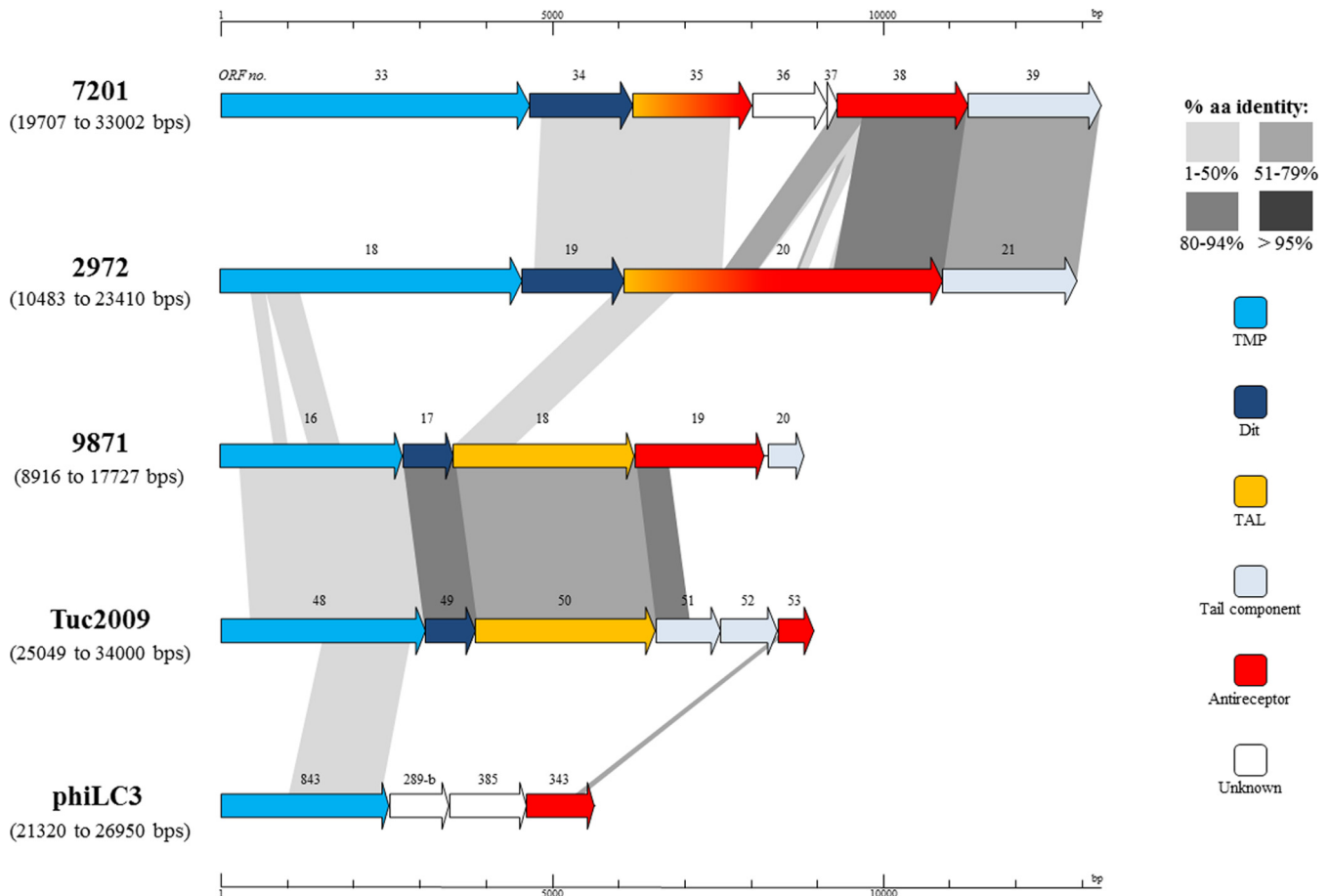
known phage head proteins [Fig. 2]). The proteins encoded by *ORF9*<sub>9871</sub>, *ORF10*<sub>9871</sub>, and *ORF11*<sub>9871</sub> were not detected during mass spectrometric analysis, possibly due to their low abundance in the 9871 particle. *ORF9*<sub>9871</sub> and *ORF10*<sub>9871</sub> appear to encode a so-called head-tail connector or adapter (43, 44), based on conserved phage head-tail connector domains (specifically, those present in proteins GP15 and GP16 of the well-characterized *Bacillus subtilis* phage SPP1) detected using CDD (*ORF9*<sub>9871</sub>) and HHPred (*ORF10*<sub>9871</sub>) (Fig. 1).

The defined tail morphogenesis gene cluster in phage 9871 commences with *ORF11*<sub>9871</sub>, which encodes a putative tail component (based on the presence of domains detected using CDD and Pfam). *ORF12*<sub>9871</sub> was not confirmed as a structural protein-encoding gene; however, a homologue of this gene product present in lactococcal phage TP901-1 has recently been annotated as the tail terminator protein (45), with apparently unchecked tail extension observed in mutant phages containing a stop codon in this gene. *ORF13*<sub>9871</sub> specifies the presumed major tail protein, with *ORF14*<sub>9871</sub> and *ORF15*<sub>9871</sub> encoding putative tail assembly chaperone proteins (46, 47) of *ORF16*<sub>9871</sub> (predicted to encode the tail tape measure protein). Indeed, *ORF14*<sub>9871</sub> shows evidence of a “slippery sequence” (5'-AAAAAAA-3'), a feature present in some genes involved in tail assembly that leads to an alternative frame translation (46) and production of an essential tail chaperone in bacteriophage λ (48). The product of *ORF16*<sub>9871</sub> (*TMP*<sub>9871</sub>) was

not confirmed as a structural protein, suggesting that it is present in small amounts in the phage particle.

*ORF17*<sub>9871</sub>, encoding the putative distal tail protein (the product of which was confirmed as a structural protein [Fig. 2]), is homologous to its functional equivalent in the lactococcal phage TP901-1, with the latter forming the core of the phage tail tip (49). The putative tail-associated lysin (TAL) is encoded by *ORF18*<sub>9871</sub> (confirmed as a structural protein [Fig. 2]) and shares significant amino acid similarity (particularly at the N terminus) with the corresponding genes in phages Tuc2009, TP901-1 (in which it was defined as the tail fiber), ul36, and P335 (39, 50–53). The location of the endopeptidase-encompassing domain (M23 family), including the catalytic His residue (residing within amino acid sequence ATGVHLHF, being the equivalent of VTGPHLHFH, where the His residue is underlined, in Tuc2009 and TP901-1 [54, 55]), in this protein appears to be conserved in phage 9871, based on CDD (28) search results. Previously, it has been reported that the TAL of *L. lactis* phage Tuc2009 undergoes autocleavage at a specific GGSSG\*GG amino acid sequence, where the asterisk (\*) indicates the cleavage site (54, 55). In *TAL*<sub>9871</sub>, this site appears to be replaced by AASGGGG, with underlined residues indicating amino acid substitutions relative to the site in *TAL*<sub>Tuc2009</sub>.

The final structural protein of phage 9871 as determined by mass spectrometry is the product of *ORF19*<sub>9871</sub>, which encodes the putative receptor binding protein, here referred to as *RBP*<sub>9871</sub>.



**FIG 3** Schematic representation of ORFs predicted to encode the tail proteins of *S. thermophilus* phage 7201 (*cos* containing), 2972 (*pac* containing), 9871 (987 group), and Tuc2009 and phiLC3 (*L. lactis* phage P335 group). Predicted ORFs (indicated by arrows) and gene products (putative function indicated by color coding) are aligned with adjacent genomes according to percent amino acid identity (indicated by shaded boxes).

While the “tripods” (as defined by Veessler et al. [49]) of *L. lactis* phages that are closely related to the 987 group phages are encoded by at least two genes (e.g., Tuc2009 [35, 56] and TP901-1 [57]), the baseplate in phage 9871 appears to be encoded by a single gene (for reasons outlined below), perhaps akin to the arrangement in several P335 phages of *Lactococcus lactis*, including BK5-T, LC3, and BM13 and Q33, which belong to the P335 subgroups I, III, and IV, respectively (41). Several *S. thermophilus* phages (including DT1) apparently share this arrangement, with a single antireceptor gene containing at least one variable region, (one of) which (termed ‘VR2’) was shown to be correlated to host specificity (11). Here, the N-terminal end of RBP<sub>9871</sub> shares a high level of amino acid identity (approximately 85%) with the N-terminal portion of the upper baseplate protein (BppU) of TP901-1, Tuc2009, P335, and ORF322 of ul36 and then appears to be extended (relative to BppU) at the C-terminal end. This composite arrangement is visualized in Fig. 3. A parallel beta helix domain at the C-terminal end of RBP<sub>9871</sub> (identified using Pfam) is a member of clan CL0268, members of which include glycosyl hydrolases, pectate lyases, pectin esterases, and *Salmonella* phage P22-like tail spikes. Similarly, using a CDD search, pectate lyase domains were found to be present toward the C-terminal end of the protein and bear similarity to glycosyl hydrolase family 28, members of which hydrolyze glycosidic bonds in the heteropolysaccharide pectin (58).

Taken together, these findings suggest that RBP<sub>9871</sub> has a carbohydrate binding function, leading us to hypothesize that this protein incorporates the receptor binding activities of the BppU and BppL proteins of TP901-1, where BppL is known to be responsible for host interaction and specificity (59). Importantly, the other three members of the 987 group each encompass an ORF19<sub>9871</sub> homologue, and these homologues exhibit near-complete nucleotide identity to each other, being consistent with the extremely narrow host range of these phages.

ORF20<sub>9871</sub> (highly conserved in the 987 group phages [Fig. 1]) was not detected during mass spectrometry, and its function is currently unknown, though it appears (using a BLAST search) to be related to a family of serine acetyltransferases. A search using the CDD database confirms the presence of the serine acetyltransferase domain as well as a sugar O-acetyltransferase domain of the NeuD family, identified as a sialic acid O-acetyltransferase in group B streptococci (60). O-acetylation has been shown to be present at precise locations of the sialic acid component of the capsular polysaccharide of group B *Streptococcus* (61). The presumed sugar interaction of the predicted O-acetyltransferase enzyme may thus be significant in the context of the outer cell layer encountered by the phage during host adsorption, in which the product of ORF20<sub>9871</sub> may perhaps play an accessory role in host recognition, similar to that exhibited by BppA in Tuc2009 (35,

62), particularly considering its proposed position in the tail morphogenesis module of the 987 group phage genomes.

**Lysis and lysogeny modules.** Approximately one-half of the currently sequenced *S. thermophilus* phages possess two distinct holin-encoding genes, being largely conserved, with the exception of phage 2972 (19). The 987 group phages also appear to possess two distinct holin-encoding genes, one gene product being closely related to holins found in *L. lactis* phages and one to those found in *S. thermophilus* phages (Fig. 1; see also Table S1 in the supplemental material). The lysin-encoding gene of 9871 (*ORF23*<sub>9871</sub>) is located immediately downstream of the holin-encoding genes and appears to be interrupted by a putative group I intron, a feature previously described in other phages of *S. thermophilus* (63). This is indicated by the presence of a predicted endonuclease-encoding open reading frame, known to be a feature of certain group I introns (64), as well as the presence of a 14-bp consensus sequence (surrounding the predicted intron splice site) correlated with intron possession (63) in all four phages, with various levels of nucleotide identity.

The predicted lysogeny modules present in the 9871-4 phages appear in each case to have been subjected to genetic decay and therefore to be redundant, based on the small size of the region relative to that of proven lysogenic phages (5) and the absence of certain genes (most notably, in this case, an integrase-encoding gene) typically associated with these modules in genuine temperate phages (65). These regions are commonly known as lysogeny “replacement” modules, which are a feature of lytic *S. thermophilus* phages (14, 17).

**Replication modules.** The gene products encoded by the individual replication modules present in phages 9871-4 (downstream of the lysogeny replacement modules) are largely conserved at the amino acid level (Fig. 1) and appear to belong to the “7201-like” grouping (66), which has previously been identified in phages 7201 (42), Abc2 (6), and more recently, 5093 (13). Despite this general conservation, however, the replication module of phage 9874 (more so than those of phages 9871 to 9873) is characterized by deletions, insertions, and point mutations, a common feature of this region in *S. thermophilus* phage genomes (18). Various genes encoding proteins of apparent non-streptococcal phage origin are positioned downstream of the replication module (detailed in the legend of Fig. 1; see also Table S1 in the supplemental material), including a RecT recombinase-encoding gene. Interestingly, these genes are often associated with exonuclease-encoding genes that together form so-called “recombination modules” (67). Indeed, this is the case for the 987 group phages, with the exonuclease-encoding gene being located immediately downstream of the recombinase-encoding genes (Fig. 1). Phages 9871, 9872, and 9873 are also predicted to encode a cytosine-5 methyltransferase (*ORF46*<sub>9871</sub>). In general, phage-encoded methyltransferases are thought to be an antidefensive response to the DNA-targeting activity of restriction-modification systems in bacterial hosts, but potentially they also function in other viral and cellular processes (for a review, see reference 68).

The proposed “terminal” ORFs are defined here as those ORFs preceding the small subunit of the terminase in the genomes of the 987 group phages (*ORF50*<sub>9871/9872</sub>, *ORF49*<sub>9873</sub>, or *ORF48*<sub>9874</sub>) (Fig. 1). These ORFs are defined as “terminal” based on homologues being present upstream of the defined *cos* site in several *cos*-containing phages of *S. thermophilus* such as DT1 (15), Sfi19 (14), 7201 (16), Sfi21 (14), and Abc2 (6), the *cos* site, in turn, being

located upstream of the small subunit of the terminase. The protein products of the terminal ORFs in the 987 group phages appear to be conserved in 9871 to 9873, with that of 9874 being divergent. A Pfam search using these proteins in phages 9871 to 9873 indicates that they belong to the DUF1492 family, which was recently found to be one of several major groups of “late transcriptional regulators” (Itr) in phages of Gram-positive bacteria (69). Similarly, transcriptional regulation appears to be the primary function of the product of *ORF48*<sub>9874</sub>, which shows approximately 50% amino acid identity with ArpU family transcriptional regulators of various streptococcal species (69).

**Morphological characteristics.** The morphology of the *Siphoviridae* family has been well documented (group B as defined by Bradley [9]), and siphophages infecting *L. lactis* and *S. thermophilus* exhibit the expected morphology, with icosahedral heads and noncontractile tails. Phages infecting *S. thermophilus* in general possess longer tails than their lactococcal counterparts (with an exception being the 949 group of lactococcal phages [40, 70]), their long tails being consistent with their long TMP-encoding genes (71). Upon electron microscopic analysis, it was found that phages 9871-4 exhibit icosahedral heads and relatively short tails (Fig. 4E shows exact dimensions). The distal tail-associated baseplate, which generally functions in the attachment of the phage to the bacterial cell (discussed above), is clearly visible (Fig. 4A to D). The presence and observed features of a baseplate are consistent with those previously observed in P335 species *L. lactis* phages such as Tuc2009 and TP901-1 (57, 71, 72) and indeed with the observed similarity between the tail structural gene products and those encoded by the 987 group phages. The measured head diameters are similar among each of the 987 group phages yet slightly larger than those previously reported for Tuc2009 and TP901-1; similarly, the tail lengths of the phages in the 987 group are within the same range relative to each other but slightly shorter than previously reported for those P335 phages (72-74). In keeping with this distinction between phages infecting *S. thermophilus* and *L. lactis* P335 group phages, the observed tail lengths of the 987 group phages are lower than those previously reported for *S. thermophilus* phages (71).

**Adsorption and adsorption inhibition assays.** Consistent with the genetic composition of the structural module (and, in particular, the antireceptor-encoding genes) of the four 987 group phages, it appears that these phages are able to adsorb to certain *L. lactis* strains as well as the primary *S. thermophilus* host. Ten *L. lactis* strains that are routinely combined with ST64987 in industrial fermentations were initially tested for phage adsorption (as described above), of which one (LL64981) appeared to adsorb all four 987 group phages at a level of approximately 50% (Fig. 5). For this assay, a negative-control phage (3681, a *cos*-containing phage infecting industrial *S. thermophilus* strain ST67368) was used to illustrate the specific adsorption affinity of strains ST64987 and LL64981 for the 987 group phages. Following the adsorption assay, a DNA transduction experiment was performed using this phage-strain combination, as well as a positive-control combination, according to a previously reported method (75); however, no confirmed *L. lactis* transductants were obtained (data not shown). Adsorption to both strains of *L. lactis* and *S. thermophilus* suggests that a common cell surface molecule is recognized by these hybrid phages (discussed further below), complemented by the observed genetic similarity of the tail tip regions of the 987 group phages to those regions in phages infecting *L. lactis*. Indeed, a phage infect-



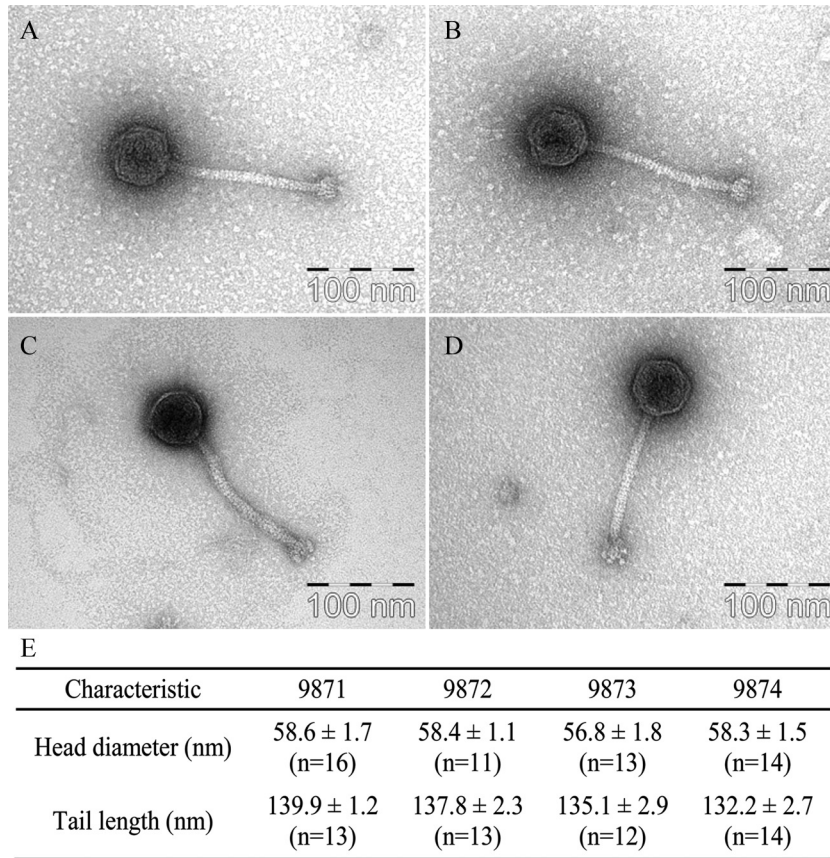


FIG 4 Uranyl acetate-stained transmission electron micrographs of phages 9871 (A), 9872 (B), 9873 (C), and 9874 (D) and discerned head and tail measurements (E).

ing *L. lactis* LL64981 (termed 98103) was also shown to exhibit adsorption affinity both to its host and to *S. thermophilus* ST64987 (Fig. 5).

In order to further investigate the interaction between the 987 group phages and their host(s), we performed a competitive phage adsorption inhibition assay using the presumed host

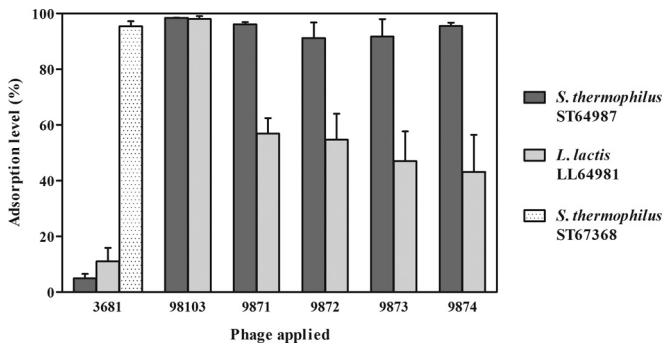
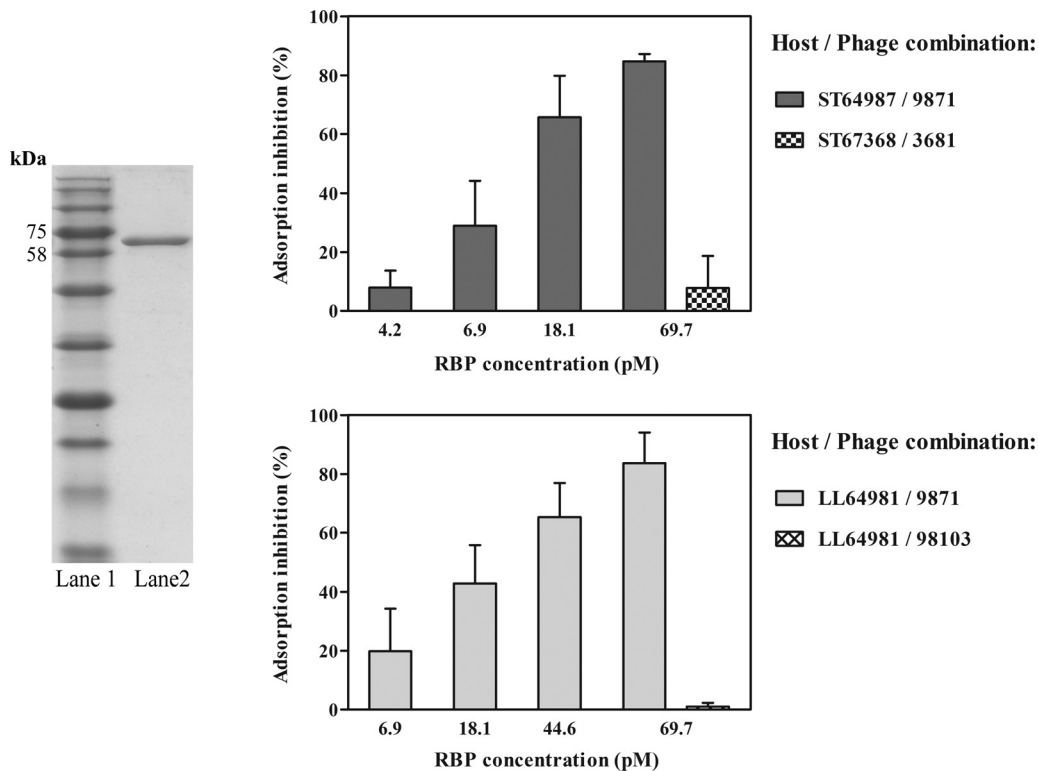


FIG 5 Adsorption analysis of phages 9871-4 on primary host *S. thermophilus* ST64987 and *L. lactis* LL64981 at an adsorption temperature ( $T_A$ ) of 42°C. Phage 3681 (a *cos*-containing lytic phage of *S. thermophilus*) is included as an adsorption-negative control for ST64987 and LL64981 and was found separately to adsorb optimally (>90%) to its primary host (*S. thermophilus* ST67368) at a  $T_A$  of 42°C. Phage 98103 (from the P335 phage group infecting *L. lactis* LL64981) was also shown to exhibit adsorption affinity to both strains. Comparable adsorption data for all strains were generated at a  $T_A$  of 30°C.

recognition protein RBP<sub>9871</sub>. This protein product is proposed to represent the antireceptor of this phage group based on the position of the encoding gene in all four phage genomes (Fig. 1) and its confirmed presence as a structural protein in the viral particle (Fig. 2A and C) and for reasons discussed in detail above. RBP<sub>9871</sub> was overexpressed and purified (Fig. 6, left, lane 2) and then used in adsorption inhibition assays (as described in Materials and Methods). Figure 6 clearly shows that RBP<sub>9871</sub>, when incubated with wild-type *S. thermophilus* and *L. lactis* cells, inhibits adsorption of phage 9871 to both strains in a dose-dependent manner (Fig. 6, right panels). Maximal (an average of approximately 80%) adsorption inhibition was achieved using a concentration of 69.7 pM in both cases, a concentration comparable to that observed by Collins et al. (35) using a lactococcal phage RBP and host combination. Any observed difference in the potency of the respective RBPs may possibly be accounted for by an increased (or decreased) amount of available binding sites present on the cell surface, particularly considering the differences in host genera (for *S. thermophilus* ST64987) and subspecies (for *L. lactis* subsp. *lactis* LL64981).

These adsorption inhibition data have a number of implications. First, it may be postulated that the cell surface target used by the 987 group phages is carbohydrate in nature, considering the putative carbohydrate-binding function of the antireceptor protein (discussed above) as well as the homology of the tail tip regions to phages of *L. lactis*, which target cell surface





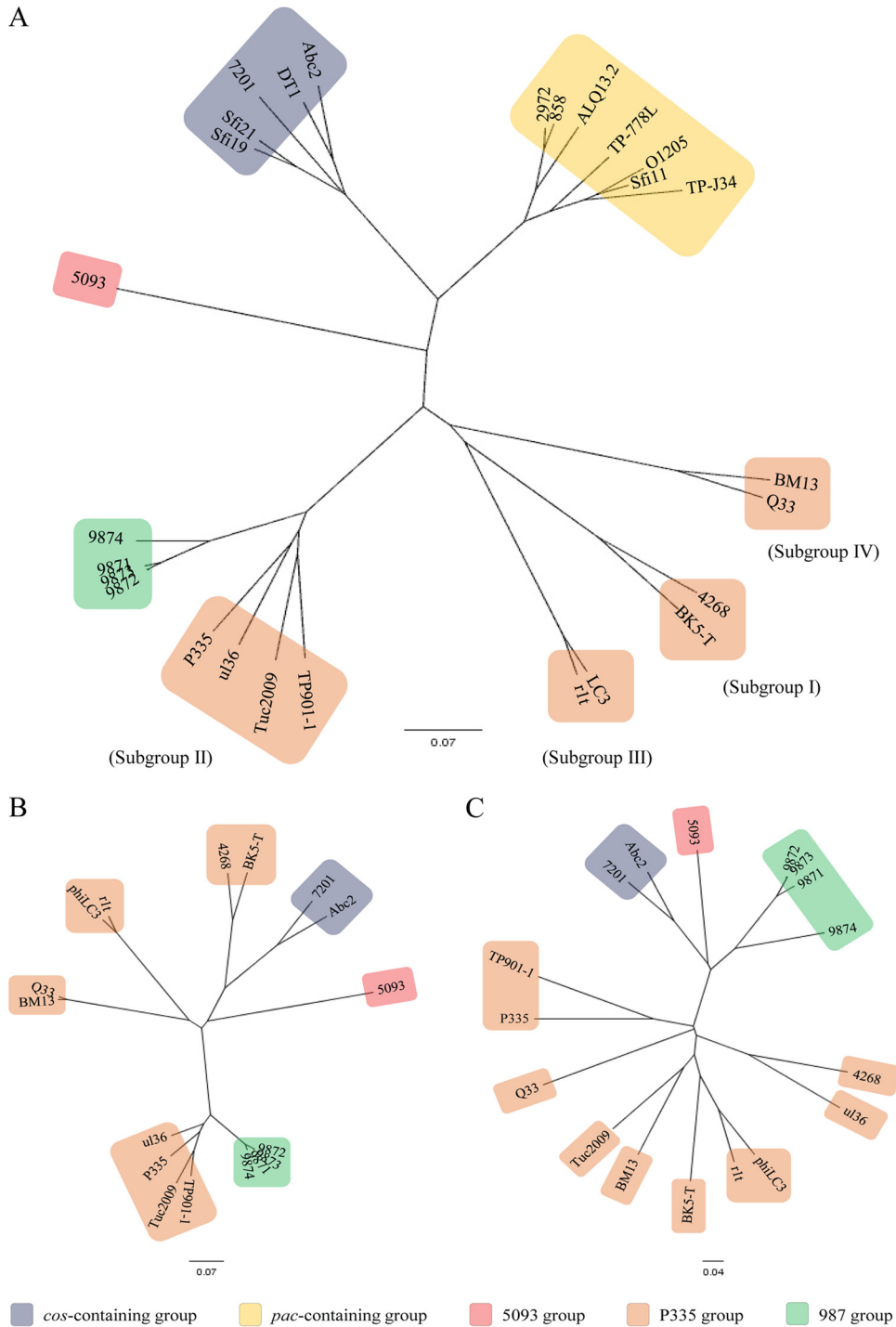
**FIG 6** Phage 9871 adsorption inhibition analysis using various concentrations of purified RBP<sub>9871</sub> on strains *S. thermophilus* ST64987 and *L. lactis* LL64981 by blocking assay. (Left) SDS-PAGE gel (12%) showing purified antireceptor of phage 9871. Lane 1, blue prestained protein standard, broad range (New England BioLabs); lane 2, purified 9871 antireceptor. (Right) Inhibition (%) of 9871 adsorption on ST64987 (top) and LL64981 (bottom).

carbohydrate moieties (76). These data also suggest that the phage targets expressed on the cell surface of *S. thermophilus* ST64987 and on *L. lactis* LL64981 are at least similar in nature. Considering the observed similarity between some *S. thermophilus* and *L. lactis* genes encoding exopolysaccharide (EPS) biosynthetic elements (1, 77), combined with the observed heterogeneity of the EPS clusters of *S. thermophilus* (1, 77, 78), this is conceivable. Furthermore, considering the genetic divergence between the antireceptors of the 987 group phages and those of previously sequenced phages such as DT1 (11) and phage 5093 (13), it is possible that alternative cell surface targets are recognized by these phages during the initial phage-host interaction.

**Evolutionary aspects.** Phages 9871-4 represent a group of *S. thermophilus*-infecting phages that is distinct from documented *cos*-containing and *pac*-containing phage groups, as well as the more recently discovered 5093 group. While phage 5093 appears to have acquired several genes from nondairy streptococcal phages, such as those infecting *Streptococcus pneumoniae*, *Streptococcus gordonii*, and *Streptococcus pyogenes* (13), a genetic crossover previously observed in prophages of *S. pyogenes* (79), the 987 group phages appear to have been the result of a genomic recombination event between a (temperate) P335 phage of *L. lactis* and an unknown *S. thermophilus* phage. Figure 7 shows the genetic distance between the currently known fully sequenced *S. thermophilus* phages (numbering 13), the 10 sequenced *L. lactis* phages of the P335 group, and the 4 phages of 987 group. Using this (unrooted) visualization, it appears that the 987 group phages are derivatives of the P335 subgroup of which Tuc2009, TP901-1, P335, and ul36 are members and are more closely related to this

group than to the other known phages that infect *S. thermophilus*. Considering the high level of nucleotide identity to these phages across the (generally) more-conserved structural regions, this is not surprising. Alignments of the structural modules (comprising the TerS-encoding gene to the holin-encoding gene [Fig. 7B]) and the replication modules (comprising the lysin-encoding gene to the terminal ORF [Fig. 7C]) of the 987 group with the relevant comparators, i.e., phages that also harbor group II/7201-like replication modules (42, 66), show a clear difference in clustering, indicating the diverse lineage of these respective modules in the 987 group phages.

The genetic/structural similarity of these phages to those of *L. lactis*—with the retention of the ability to infect *S. thermophilus*—may be considered a form of adaptive mosaicism, a known evolutionary strategy common to phages infecting a wide range of bacteria (80, 81). Due to the close association of *L. lactis* and *S. thermophilus* both in raw milk and in the dairy processing environment, gene transfer between (the phages of) these species has been the subject of speculation, with perhaps the most striking phage example of this phenomenon being observed in the case of phage BK5-T, a temperate phage of *L. lactis* H2L (82), which shares significant sequence similarity with *S. thermophilus* phage Sfi21 (83). Further examples of this phenomenon include the genomes of phage 1358 (infecting *L. lactis* SMQ-388), with homology to phages infecting *Listeria monocytogenes* (84), phage Q54 (infecting *L. lactis* SMQ-562), which appears to be a hybrid of the 936 and c2 lactococcal phage species (85), and phage 1706 (infecting *L. lactis* SMQ-450), proposed to be derived from a number of prophages of other *Firmicutes* (86). The mechanisms by which



**FIG 7** Unrooted phylogenetic tree showing the genetic relatedness between the 987 group phages, *cos*- and *pac*-containing *S. thermophilus*-infecting phages, as well as *L. lactis*-infecting phages of the P335 group (a color code for the respective groupings is provided). (A) Whole-genome nucleotide comparison; (B) structural module comparison with those *S. thermophilus* phages also harboring a group II/7201-like replication module; (C) replication module comparison with those *S. thermophilus* phages also harboring a group II/7201-like replication module.

such horizontal gene transfer events between phages occur have also been proposed. Moineau et al. (22) and Durmaz and Klaenhammer (87) have shown that lytic phages can evolve by acquiring segments of DNA from the host chromosome (including, potentially, remnant prophage) sometimes in response to pressure from

abortive infection (Abi) phage resistance systems (88). More recently, it has been shown that transduction in *L. lactis* is possible using *S. thermophilus* phages (75), clearly demonstrating that phage-mediated horizontal DNA transfer between these two species is possible. Furthermore, considering the rapid nature of

phage infection (reviewed by Quiberoni et al. [7]) and, in turn (by necessity), the acquisition of phage resistance, a common genetic lineage in phages of *S. thermophilus* and *L. lactis* phages may be reflected in the numerous phage resistance mechanisms of their hosts. Indeed, this has been shown by Sun and colleagues (89), who demonstrated that the superinfection exclusion (sie) phage resistance protein Ltp confers phage resistance to both *S. thermophilus* and *L. lactis* hosts, against their respective attacking phages (89). Such multigenus protection is indicative of coevolution of both phage and host, possibly accelerated by their continuous mutual exposure in the dairy environment.

The impact of genetic mosaicism in phages on the marketplace is illustrated by the 987 group phages above, which retain infective ability in *S. thermophilus*, despite having many genetic and morphological characteristics of phages of *L. lactis*. This is an important consideration in the dairy industry, an environment in which lactic acid bacteria are in close proximity on a regular basis and which may well present further examples of genetic mosaicism as an evolutionary strategy in dairy phages.

**Conclusions.** Here we report the complete genome sequences of four novel phages infecting the dairy bacterium *S. thermophilus*. Comparative genomic analysis revealed a high level of nucleotide identity to the replication modules of *S. thermophilus* phages and the structural modules of *L. lactis* phages, suggesting a relatively recent horizontal gene transfer or recombination event. These genome sequences represent a significant divergence compared to the previously published 13 *S. thermophilus* phage genomes, being highly mosaic in nature, and are the first members of this phage group to be sequenced. The structural protein complement of one of these phages (as a representative of the group) was determined and found to be similar to previously characterized phages of *L. lactis*. Morphological similarity to phages of *L. lactis* was also observed using electron microscopic analysis, in which short tails and clawlike baseplates were observed in all four members of the group.

Adsorption studies revealed the ability of members of this group of phages to adsorb to both their native *S. thermophilus* hosts and an *L. lactis* strain with which it is routinely combined in dairy fermentations, suggesting that certain cell surface molecules are shared between the genera. This finding also hints at the event by which the hybrid genomes of these phages may have begun to be replicated, possibly being facilitated by mutually expressed cell surface proteins in combination with a favorable *S. thermophilus* phage coinfection or prophage-mediated evolutionary event. The phage gene product responsible for this adsorption was defined by the use of purified protein to inhibit phage adsorption to both strains, providing a more detailed analysis of the initial phage-host interaction.

Genetic mosaicism is a common trait of bacteriophages and, in the context of dairy fermentations, may represent a new challenge to phage control methods, which usually consist of traditional bacteriophage-insensitive mutant (BIM) generation and rotational schemes. In light of the rapidly increasing genetic diversity being observed in phages of *S. thermophilus*, continual monitoring of phage populations in dairy productions will be necessary to ensure that BIM generation methods and knowledge-based rotational systems can be used effectively to ameliorate phage spoilage of industrial fermentations.

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