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Two Groups of Bacteriophages Infecting *Streptococcus thermophilus* Can Be Distinguished on the Basis of Mode of Packaging and Genetic Determinants for Major Structural Proteins

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A comparative study of 30 phages of *Streptococcus thermophilus* was performed based on DNA restriction profiles, DNA homology, structural proteins, packaging mechanisms, and host range data. All phages exhibited distinct DNA restriction profiles, with some phages displaying similarly sized restriction fragments. DNA homology was shown to be present among all 30 phages. The phages could be divided into two groups on the basis of their packaging mechanism as was derived from the appearance of submolar DNA fragments in restriction enzyme digests and the presence (*cos*-containing phages) or absence (*pac*-containing phages) of cohesive genomic extremities. Interestingly, the 19 identified *cos*-containing phages possessed two major structural proteins (32 and 26 kDa) in contrast to the remaining 11 *pac*-containing phages, which possessed three major structural proteins (41, 25, and 13 kDa). Southern hybridization demonstrated that all *pac*-containing phages tested contain homologs of the genes encoding the three major structural proteins of the *pac*-containing phage O1205, whereas all *cos*-containing phages tested exhibit homology to the gene specifying one of the structural components of the *cos*-containing phage Φ7201. Fifty-seven percent of the phages (both *cos* and *pac* containing) possessed the previously identified 2.2-kb *Eco*RI fragment of the temperate *S. thermophilus* phage Sfi18 (H. Brüssow, A. Probst, M. Frémont, and J. Sidoti, Virology 200:854–857, 1994). No obvious correlation was detected between grouping based on packaging mechanism and host range data obtained with 39 industrial *S. thermophilus* strains.

Streptococcus thermophilus is a component of thermophilic dairy starter cultures used in the manufacture of yogurt and several types of cheeses. As observed for other dairy lactic acid bacteria, these streptococci are often susceptible to phage attack, which can result in slow lactic acid fermentation and the production of inferior products. Most previous reports on the characterization and classification of *S. thermophilus* phages were based on morphology, host range, DNA restriction patterns, and genome homologies. To date, all phages isolated from *S. thermophilus* belong to the *Siphoviridae* family corresponding to group B as defined by Bradley (5). At the DNA level and on the basis of protein profiles, no more than two (2, 19), three (18), or four (11) phage types could be distinguished. There does not appear to be any correlation between observed DNA homology and host range (2, 21). This is in agreement with Ackermann et al. (1), who pointed out that host range is not a very reliable criterion for phage classification purposes due to inherent variability of this property. The limited value of host range data for classification purposes was also noticed by Brüssow and Bruttin (8), who proposed a classification of *S. thermophilus* phages into four lytic groups based on host range and type-specific antisera. These authors also reported on the characterization of a DNA fragment from ΦSFi11 which was specific for their lytic group II.

An alternative approach for classifying *S. thermophilus* phages may originate from the analysis of phage genomes and would make use of their evolutionary descent. Mercenier (16) proposed that all *S. thermophilus* phages are derived from a common ancestor. Corroborating this idea is the cloning of a DNA fragment from ΦS1 which hybridized to all *S. thermophilus* phage isolates (7). The diversity in phage genomes observed by different researchers may result from multiple rearrangements occurring within the phage population. It has been proposed by Brüssow et al. (6) that evolution of *S. thermophilus* phages takes place by means of module exchange between phage genomes, a hypothesis which was proposed previously for other bacteriophages as well (4). The former study involving 41 *S. thermophilus* phages revealed the presence of a conserved DNA fragment of 2.2 kb present in 59% of phage genomes analyzed and for which no function could be assigned (6). Diversity of phage genomes can also result from site-specific deletions occurring in defined regions of the virion DNA (9).

In this study, we have analyzed 30 *S. thermophilus* phages based on DNA restriction profiles, DNA homology, structural proteins, packaging mechanism, and host range data. In addition, we have investigated the diversity of phage genomes and the presence or absence of certain genes and DNA fragments.

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This analysis was performed with phages obtained from different collections to render a general relevance to the results.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. Thirty plaque-purified bacteriophages and their *S. thermophilus* hosts were used in this study in addition to 36 industrial *S. thermophilus* strains which were obtained from Quest International (Naarden, The Netherlands) (Table 1). *S. thermophilus* strains were routinely grown at 43°C in Elliker broth (Difco Laboratories, Detroit, Mich.) supplemented with 10 g of beef extract (Difco) liter^{-1} and 19 g of β-glycerophosphate (Sigma Chemical Co., St. Louis, Mo.) liter^{-1}. Twenty-eight phages which had been isolated from yogurt or cheese whey samples, usually as a result of an abnormal fermentation pattern, were obtained from various collections (Table 1). All of these behaved as virulent phages except for phage CNRZO1205 (also designated O1205), which had been isolated as a temperate phage (11). In addition, two virulent phages (ΦQ7 and ΦQ10) were isolated from yogurt whey supplied by Quest International and were propagated on strain 90461. Enumeration of phage was achieved by plaque assays as described previously (11) with the addition of 100 mM glycine to the growth medium to enhance plaque formation.

Determination of host range. The sensitivity of 36 industrial *S. thermophilus* strains and three strains described previously (17) to 27 phages from our collection was determined in the supplemented Elliker broth described above containing 10 mM $CaCl_2$. Duplicate cultures were infected with phage at a multiplicity of infection higher than 1 and incubated at 32 and 43°C. At regular intervals, the turbidity of phage-infected cultures was compared with that of the corresponding controls (the same culture to which no phage had been added) by visual examination. Three sequential subcultures were systematically prepared when lysis appeared to be delayed or absent. Inoculation of these subcultures took place after the control culture of the particular strain had entered the stationary growth phase.

Phage purification and concentration. Phage particles were isolated by the protocol reported by Fayard et al. (11) with the following modifications. Following polyethyleneglycol precipitation, the pellet containing phage particles was resuspended in 15 ml of TMN buffer (10 mM Tris-HCl, 10 mM $MgSO_4$, 0.3 M NaCl [pH 7.4]) and left overnight at 4°C. Phage particles were further purified on a CsCl step gradient (20).

DNA isolation. Phage DNA was isolated as reported by Fayard et al. (11).

Amplification of DNA by PCR. DNA samples were amplified in a thermal cycler (Perkin-Elmer, Norwalk, Conn.) programmed for 30 cycles, each consisting of a denaturation step at 94°C for 2 min, a primer annealing step at 50°C for 1 min, and a primer extension step at 72°C for 1 min. Three-hundred picomoles of each primer was used per PCR. Amplification of the three genes encoding the major structural proteins (designated here as *mpL-1205*, *mpM-1205*, and *mpS-1205*) from the genome of phage O1205 (GenBank accession number U88974) and the gene specifying the small major structural protein of phage Φ7201 (GenBank accession number AF001793; designated here as *mpS-7201*) was achieved with primers designed on the basis of the DNA sequence of these genes (23) and had the following nucleotide compositions: 5′ ACTTTGGGTGAGTC TAT 3′ and 5′ AACTTTAGTTTGAACGT 3′ for amplification of *mpL-1205*; 5′ ATGGCTGATAACAATGA 3′ and 5′ ATTCCTGCAGGCGTAGT 3′ for amplification of *mpM-1205*; 5′ ATGCCAACAACAACAAT 3′ and 5′ TAACGAG TGGCAATGCT 3′ for amplification of *mpS-1205*; 5′ ATGGCAATTGTAGGT TTG 3′; and 5′ GCTGTAGAAGTAACTAG 3′ for amplification of *mpS-7201*. Primers based on the published sequence from the conserved *S. thermophilus* DNA fragment (6) had the following sequences: 5′ GCCATTCTTAACGAG 3′ (nucleotide positions 301 to 315) and 5′ CGCTGACAAACCACC 3′ (nucleotide positions 1452 to 1466).

Southern blotting and dot blot assays. DNA fragments were separated on an agarose gel and transferred to Hybond-N$^+$ nucleic transfer membranes (Amersham, Buckinghamshire, United Kingdom) by Southern blotting (22). Alternatively, 1 μg of undigested phage DNA in 500 μl of NaOH (0.5 M)-EDTA (10 mM) solution was transferred to Zeta-Probe blotting membranes (Bio-Rad, Richmond, Calif.) by means of a Bio-Rad dot blot apparatus as described in the instructions of the manufacturer. DNA probes were labeled and used for hybridization experiments with the enhanced chemiluminescence kit according to the high-stringency conditions specified by the supplier (hybridization and washing steps at 42°C and in 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]; Amersham).

SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (14). CsCl-purified phages (approximately 10^{10} PFU per ml) were dialyzed against 100 volumes of TMN buffer, and a 100-μl aliquot was boiled for 10 min with an equal volume of SDS-PAGE loading buffer (50 mM Tris-HCl, 3% SDS, 1% β-mercaptoethanol, 20% glycerol, 0.7% bromophenol blue [pH 6.8]). Proteins (10 μl) were separated through an SDS-containing polyacrylamide gel (12.5%) with a mini-Protean II system (Bio-Rad). Electrophoresis was initiated at 100 V until samples had run through the stacking gel (approximately 45 min). The voltage was subsequently increased to 150 V, and electrophoresis was continued until the tracking dye had reached the bottom of the gel (approximately 2.5 h). Proteins were stained with Coomassie brilliant blue R-250 (Sigma).

Electron microscopy. Phages were negatively stained with 2% uranyl acetate and examined in a JEOL (London, United Kingdom) 1200 EX transmission electron microscope at an accelerating voltage of 80 kV.

RESULTS

Electron microscopy. Phages were isolated as described in Materials and Methods, and a select number were taken for electron microscopic examination based on differences in DNA restriction and protein profiles. As reported for other *S. thermophilus* phages, all phages examined appeared to be small isometric-headed phages with a long noncontractile tail (Fig. 1).

Comparison of restriction endonuclease patterns of phage DNAs. DNA of 30 *S. thermophilus* phages was subjected to restriction analysis with the enzymes *Eco*RV, *Hin*dIII, and *Pvu*II. All phages exhibited distinct profiles (Fig. 2 shows restriction profiles for 11 representative phages). In some cases, however, similar restriction profiles were observed, which usually involved phages obtained from a common source. For example, the *Eco*RV digests of Φ7201, Φ7203, Φ7205, Φ7206, Φ7209, and Φ8FN (NIZO, Ede, The Netherlands) showed the presence of seven to nine equally sized fragments, five of which were present in all six phage genomes (data not shown). A similar observation was made for Φ83 and Φ117 (CRBGC collection, Toulouse, France), which appeared to share 12 of 13 *Eco*RV fragments (Fig. 2, lanes E and F). Another example included phages from different sources, i.e., ΦQ1, ΦQ3, ΦQ7, and ΦQ10 from Quest International and Φ4FN from NIZO. *Eco*RV digestion of genomic DNA of any of these phages generated 12 to 13 distinct DNA fragments, 9 of which appeared to be common to all five phages (data not shown).

The size of each of the phage genomes ranged between 30 to 43 kb and was calculated by adding the estimated molecular weights of the *Eco*RV- or *Hin*dIII-generated fragments of each of the 30 phages. By the use of various restriction enzyme digests, it was found that all phage genomes contain submolar fragments (results not shown). Heating for 10 min at 80°C or treatment with 25% formamide resulted, in the case of 19 phages, in the loss of one band and the appearance of two smaller bands in a restriction profile, indicating that these genomes were packaged by a *cos*-type mechanism (3) (these phages were designated as *cos*-containing phages [Table 2]). For the remaining 11 phages, the observed submolar bands did not disappear after either of these treatments, which suggested that these do not contain cohesive ends and that they therefore use a *pac*-type packaging mechanism (3) (these phages were designated as *pac*-containing phages [Table 2]).

Host range. Twenty-seven phages of our collection were tested for their infectivity towards 36 industrial strains as well as strains SMQ119, SMQ174, and SMQ174 at 32 and 43°C (see Materials and Methods). Table 3 illustrates the results obtained with the 23 most sensitive strains of this collection (strains which were shown to be sensitive to more than 2 of the 27 phages tested). This phage typing system allowed the differentiation of most *S. thermophilus* strains on the basis of varyious phage sensitivities, although it proved to be impossible to derive a sensible phage classification from these results. Seven strains (strains 4038, 4054, 4116, 4134, 4141, 4145, and 4147) were found to be resistant to all 27 phages, whereas nine strains (strains 90729, SMQ173, SMQ174, 4021, 4067, 4076, 4097, 4137, and 4149) were sensitive to only 1 or 2 phages. Eleven strains were shown to be sensitive to more than half of the phages. Strains sensitive to both *cos*- and *pac*-containing phages were also identified (see Tables 2 and 3). Furthermore, for certain strains, phage sensitivity was affected by growth

TABLE 1. Bacterial strains and bacteriophages

Bacterial strain or bacteriophage	Relevant characteristics	Source or reference[a]
S. thermophilus strains		
CNRZ440	Host for st2	INRA collection
CNRZ447	Industrial strain used in yogurt production; host for 447-B4	INRA collection
CNRZ887	Industrial strain used in yogurt production; host for Φ1	INRA collection
CNRZ1151	Traditional gruyere cheese starter; host for c20	INRA collection
CNRZ1205	Traditional strain used in yogurt production; lysogenic for O1205	11
CNRZ1575	Industrial strain used for cheese production; host for BaS19	INRA collection
CNRZ1589	Traditional Emmenthal cheese starter; host for BaS265	INRA collection
SMQ-119	Industrial strain used for yogurt production; host for ΦQ1 and ΦQ3	17
SMQ-173	Industrial strain used for mozzarella production; host for ΦQ5	17
SMQ-174	Industrial strain used for mozzarella production; host for ΦQ6	17
B106	Host for Φ7201, Φ7203, Φ7205, Φ7206, Φ7209, P4, Φ4FN, and Φ8FN	NIZO collection
S0	Host for P0	18
124	Host for 124/44	IMK collection
71	Host for 71/45	18
St29	Host for Φ83	CRBGC collection
St56	Host for Φ117	CRBGC collection
St 31	Host for Φ31	ISLC collection
St 33	Host for Φ33	ISLC collection
St 45	Host for Φ45	ISLC collection
St 47	Host for Φ47	ISLC collection
90461, 90462, 90726, 90727, 90728, 90729, 90730, 90731, 90732, 4011, 4012, 4018, 4019, 4021, 4035, 4038, 4039, 4041, 4043, 4044, 4052, 4053, 4054, 4062, 4063, 4067, 4076, 4078, 4097, 4116, 4134, 4137, 4141, 4145, 4147, and 4149	Industrial strains used for yogurt production; strain 90461 is host for ΦQ7 and ΦQ10	Quest
S. thermophilus phages		
CNRZ9125(c20)	Phage isolated from yogurt whey	INRA collection
CNRZ9132(st2)	Phage isolated from yogurt whey	INRA collection
CNRZ9128(447-B4)	Phage isolated from Emmenthal whey	INRA collection
CNRZ9149(Φ1)	Phage isolated from Emmenthal whey	INRA collection
CNRZ9154(BaS19)	Phage isolated from cheese whey	INRA collection
CNRZ9164(BaS265)	Phage isolated from Emmenthal whey	INRA collection
CNRZO1205	Temperate phage induced from CNRZ1205	11
ΦQ1	Phage isolated from yogurt whey	17
ΦQ3	Phage isolated from yogurt whey	17
ΦQ5	Phage isolated from mozzarella whey	17
ΦQ6	Phage isolated from mozzarella whey	17
ΦQ7	Phage isolated from yogurt whey	This study
ΦQ10	Phage isolated from yogurt whey	This study
Φ4FN	No information available	NIZO collection
Φ8FN	No information available	NIZO collection
Φ7201	No information available	NIZO collection
Φ7203	No information available	NIZO collection
Φ7205	No information available	NIZO collection
Φ7206	No information available	NIZO collection
Φ7209	No information available	NIZO collection
P4	No information available	NIZO collection
PO	Phage isolated from cheese whey	18
124/44	Phage isolated from yogurt whey	IMK collection
71/45	Phage isolated from yogurt whey	18
Φ83	No information available	CRBGC collection
Φ117	No information available	CRBGC collection
Φ31	No information available	ISLC collection
Φ33	No information available	ISLC collection
Φ45	No information available	ISLC collection
Φ47	No information available	ISLC collection

[a] INRA, Domaine de Vilvert, Jouy-en-Josas, France; NIZO, Ede, The Netherlands; IMK, Institut für Mikrobiologie, Bundesanstalt für Milchforschung, Kiel, Germany; CRBGC, Centre de Transfert en Biotechnologie, Microbiologie, Toulouse, France; ISLC, Instituto Sperimentale Lattiero Caseario, Lodi, Italy; Quest, Quest International, Bioproducts-cultures, Naarden, The Netherlands.

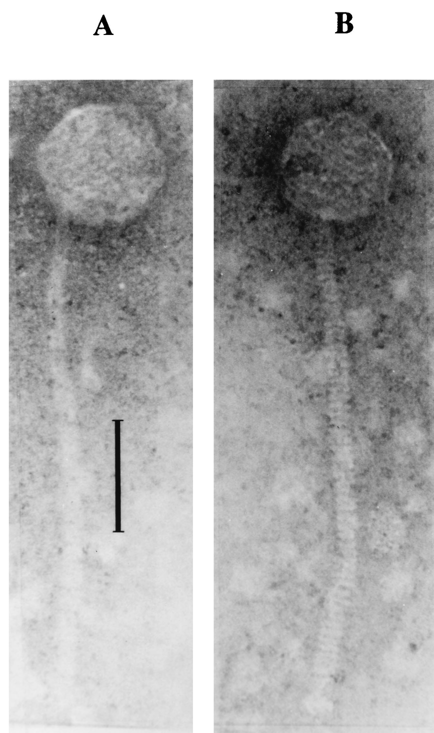

FIG. 1. Electron micrograph of two representative phages infecting *S. thermophilus*, i.e., *pac*-containing phage O1205 (A) and *cos*-containing phage Φ7201 (B). Bar, 50 nm.

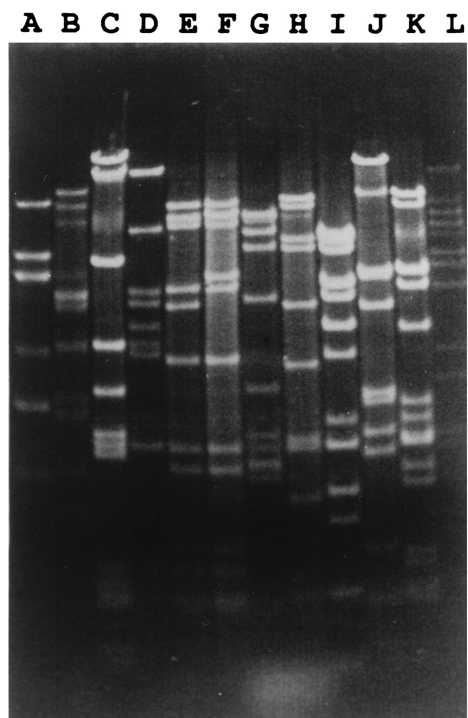

FIG. 2. *Eco*RV digests of *S. thermophilus* bacteriophage DNAs. Lanes: A, Φ31; B, Φ33; C, Φ45; D, Φ47; E, Φ83; F, Φ117; G, P0; H, 124/44; I, 71; J, CNRZ9128(447-B4); K, CNRZO1205; L, lambda DNA digested with *Bst*EII.

temperature, which would suggest the presence of temperature-sensitive phage resistance systems.

Dot blot hybridization analysis. The presence of homologous DNA sequences among the 30 phage DNAs was determined by a dot blotting procedure using genomic DNA from phage Φ7201 or O1205 as a probe. The latter two phages have been obtained from two different collections (NIZO and INRA, respectively) and exhibit very distinct features. Φ7201 is a lytic, *cos*-containing phage, whereas O1205 is a temperate phage containing a *pac* site. Both of these probes hybridized with DNA samples from all phages of our collection, showing that all *S. thermophilus* phages are genetically related (Fig. 3B and C [phages Φ1 and BaS265 are not shown in this figure]). Further examination of our results revealed that the 11 *pac*-containing phages generated a significantly stronger hybridization signal with the O1205-derived probe than with Φ7201 DNA (Fig. 3B). Similarly, 19 *cos*-containing phages appeared to exhibit a more extensive homology with Φ7201 genomic DNA than with O1205 DNA (Fig. 3C). Identical results were obtained with a probe which corresponded to the *cos*-containing phage ΦQ5.

Protein composition. Phage particles were purified as described in Materials and Methods and used to determine the structural protein content of each phage by SDS-PAGE analysis. Two distinct profiles were observed among the 30 phages tested (Fig. 4). Phage particles derived from 19 different phages were shown to contain two major protein bands with approximate molecular sizes of 32 and 26 kDa (for examples, see Fig. 4A, lanes 2 to 4, and Fig. 4B, lane 2). The remaining 11 phages consisted of three major proteins with estimated molecular sizes of 41, 25, and 13 kDa (for examples, see Fig. 3A, lanes 5 and 6). Both profiles occasionally varied slightly

TABLE 2. *cos*- and *pac*-containing phages, structural proteins, and presence of the SFi18 conserved module

Phage type	Phage	Presence of Sfi18 conserved module
cos-containing phages (two major structural proteins)	Φ83	+
	Φ117	+
	PO	+
	Φ71	+
	Φ124	+
	Φ47	+
	st2	+
	BaS19	+
	Q5	+
	Φ7201	−
	Φ7203	−
	Φ7205	−
	Φ7206	−
	Φ7209	−
	Φ8FN	−
	Φ33	−
	c20	−
	BaS265	−
	Q6	−
pac-containing phages (three major structural proteins)	P4	−
	Φ31	−
	Φ1	−
	Φ4FN	+
	Φ45	+
	O1205	+
	447-B4	+
	Q1	+
	Q3	+
	Q7	+
	Q10	+

TABLE 3. Host range of 23 industrial strains

Phage	Sensitivity of strain(s)[a]																				
	90461 and 90462	90726	90727	90728	90730	90731	90732	SMQ 119	4011	4012	4018	4019 and 4035	4039	4041	4043	4044	4052	4053	4062	4063	4078
Φ7201	1-1	1-1	1-1	0-0	0-1	0-0	0-0	1-1	0-0	1-1	1-1	1-1	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0
Φ7203	1-1	1-1	1-1	0-0	0-0	0-0	0-0	1-1	0-0	1-1	1-1	1-1	1-1	0-1	0-0	1-1	0-0	0-0	0-0	0-1	0-0
Φ7206	1-1	1-1	1-1	0-0	0-0	0-0	0-0	1-1	0-0	1-1	1-1	1-1	0-1	0-0	0-0	0-1	0-0	0-0	0-0	0-0	0-0
Φ7205	1-1	1-1	1-1	0-0	0-0	0-0	0-0	1-1	0-0	1-1	1-1	1-1	1-1	0-0	0-0	1-1	0-0	0-0	0-0	0-1	0-0
Φ8FN	1-1	1-1	1-1	0-0	0-0	0-0	0-0	1-1	0-0	1-1	1-1	1-1	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0
Φ7209	1-1	1-1	1-1	0-0	0-0	0-0	0-0	1-1	0-0	1-1	1-1	1-1	1-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0
P4	1-1	1-1	1-1	0-0	0-0	0-0	0-0	1-1	0-0	1-1	1-1	1-1	1-1	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-1
Φ4FN	1-1	1-1	1-1	0-0	0-0	0-0	0-0	1-1	0-0	1-1	1-1	1-1	1-1	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-1
Q1	1-1	1-1	1-1	0-0	0-0	0-0	0-0	1-1	0-0	1-1	1-1	1-1	1-1	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-0
Q3	1-1	1-1	1-1	0-0	0-0	0-0	0-1	1-1	0-0	1-1	1-1	1-1	1-1	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-0
Q5	1-1	1-1	1-1	1-1	0-0	0-0	0-0	1-1	0-0	1-1	1-1	1-1	1-1	0-0	0-0	0-0	1-0	0-0	0-0	0-1	0-0
Q7	1-1	1-1	1-1	0-0	0-0	0-0	0-0	1-1	0-0	1-1	1-1	1-1	1-1	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-0
Φ83	0-0	0-0	0-0	0-0	1-1	1-1	0-0	0-0	1-1	0-0	0-0	0-0	0-0	1-1	1-1	0-0	1-0	1-1	1-1	1-1	0-0
Φ117	0-0	0-0	0-0	1-0	1-1	1-0	0-0	0-0	1-1	0-0	0-0	0-0	0-0	1-1	1-1	0-0	1-0	1-1	0-1	1-1	0-0
c20	0-0	0-0	0-0	0-0	1-0	1-0	0-0	0-0	1-1	0-0	0-0	0-0	0-0	1-1	1-0	0-0	1-0	1-0	0-0	0-0	0-0
st2	0-0	1-1	1-1	1-1	1-1	1-1	1-1	1-1	1-1	1-1	0-1	1-1	1-1	1-1	1-1	0-1	1-0	1-1	1-1	1-1	0-0
447-B4	0-0	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0
Φ1	0-0	1-1	0-0	0-0	0-0	0-0	0-0	0-0	0-0	1-1	0-1	1-1	1-1	0-0	0-0	0-1	0-0	0-0	0-0	0-0	0-0
BaS19	0-0	0-0	0-0	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0
BaS265	0-0	0-0	0-0	0-0	0-0	1-0	1-1	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	1-0	0-0	0-0	0-0	0-0
PO	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-1
124/44	0-0	0-0	0-0	1-1	0-0	0-0	0-0	0-0	1-1	1-1	1-1	1-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0
71/45	0-0	1-1	1-1	0-0	0-0	0-0	0-0	0-0	1-1	1-1	1-1	1-1	1-1	0-0	0-0	1-1	0-0	1-1	0-0	0-1	0-0
Φ31	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0
Φ33	0-0	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0
Φ45	0-1	1-1	1-1	1-1	0-0	1-0	0-0	1-1	0-0	0-1	0-1	1-1	0-0	0-0	0-0	0-1	0-0	0-0	0-1	0-1	0-0
Φ47	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-0	0-0	1-1	0-1	0-0	1-1	0-0	0-0	1-1	0-0	0-0	0-0	0-0	0-0

[a] The designations 1 and 0 indicate that a given strain is sensitive or resistant to the phage used in the experiment, respectively. The first and second numbers given for each phage-host combination represent the results obtained at 43°C and 32°C, respectively.

(±1 kDa) from phage to phage. For example, a variation in the sizes of the 41- and 13-kDa proteins was observed for a few phages (data not shown). For instance, phage P4 was shown to harbor a protein of lower molecular weight than the 13-kDa molecule present in O1205 (Fig. 4A, lanes 5 and 6).

Following CsCl gradient purification, most of the phage preparations yielded an additional phage particle band of a lower density, which indicated the presence of defective particles. These were identified as phage heads based on the following observations: the ability to isolate DNA from these particles and the predominant presence of phage heads in preparations examined by electron microscopy (data not shown). SDS-PAGE analysis showed that these defective particles consisted primarily of the 32-kDa (in the case of group I phages) or the 41-kDa (in the case of group II phages) proteins, which would identify each of these proteins as the major capsid components of the two phage groups (Fig. 4B, lanes 2 and 3).

Comparison of packaging mechanism and structural protein content reveals two phage groups. Two distinct groups of phages could be identified on the basis of the observation that the apparent packaging mechanism (exemplified by the presence of a *cos* or *pac* site) of a given phage always coincided with a specific structural protein content. Upon examination, it was found that all *cos*-containing phages possessed two major structural components while all *pac*-containing phages contained three major structural proteins (Table 2; Fig. 4). The close genetic relationship among members of one phage group also appeared to be illustrated by strong dot blot hybridization signals (Fig. 3). The latter result suggested the presence of conserved DNA regions specific for each group, and given the observed correlation between packaging mechanism and structural protein content, this conserved region could conceivably encompass the genes specifying structural proteins and the packaging machinery.

Genes encoding major structural proteins are specific for either *cos*- or *pac*-containing phages. From the sequencing data of the *pac*-containing phage O1205 (GenBank accession number U88974) and the *cos*-containing phage Φ7201 (GenBank accession number AF001793) (23), the genes encoding the three major structural proteins of phage O1205 (*mpL-1205*, *mpM-1205*, and *mpS-1205*) and one of the two major structural proteins of phage Φ7201 (*mpS-7201*) were identified. PCR primers were designed to amplify DNA fragments which almost completely (in the case of *mpS-7201*) or exactly (for *mpL-1205*, *mpM-1205*, and *mpS-1205*) encompassed these genes. Correctly sized PCR fragments of 897, 497, 340, or 606 bp were obtained for the *mpL-1205*, *mpM-1205*, *mpS-1205*, and *mpS*-7201 genes, respectively, with O1205 or Φ7201 phage DNA as a template. Each fragment was used separately as a probe to detect homology to *Eco*RV digests of the 30 genomic phage DNAs. An example of the results obtained for the 30 phages is shown in Fig. 5 for 12 representative phages. Hybridization patterns using *Eco*RV-digested O1205 DNA were consistent with the presence of an *Eco*RV site within the *mpL-1205* gene and with the organization of the three genes in the order *mpS-1205*, *mpL-1205*, and *mpM-1205* (23). The genomic DNA of the 11 *pac*-containing phages hybridized to each of the three O1205-derived probes, while no signal was observed for the 19 *cos*-containing phages (see Fig. 5B to D for representative data obtained with 12 phages). The observed smearing of the hybridizing signal may be caused by hybridization of the

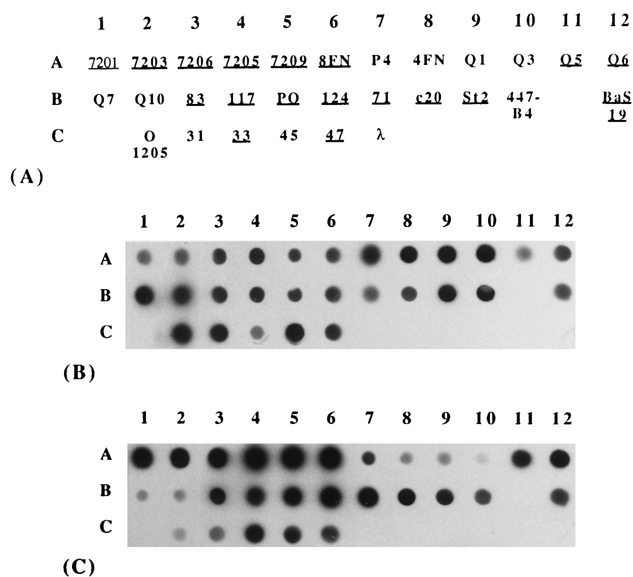

FIG. 3. Dot blot hybridizations between *S. thermophilus* phage DNAs, showing the different level of homology correlating with the mode of packaging. (A) Identity of phage DNAs on the blot. The *cos*-containing phages are underlined. (B and C) Dot blot hybridizations of O1205 (*pac*-containing) and Φ7201 (*cos*-containing) DNAs, respectively.

probe to submolar DNA fragments which had arisen by so-called headful cleavage (3). The hybridization patterns also indicated that the three genes encoding the structural proteins of *pac*-containing phages are, as observed for O1205, clustered on each of the phage genomes.

The Φ7201-derived probe generated a hybridization signal(s) (the *mpS-7201* gene contains an *Eco*RV site which appears to be conserved in only a few phages) with the genomic DNA of all 19 *cos*-containing phages, whereas no signal was detected with any of the *pac*-containing phages (Fig. 5E). Only in the case of the *cos*-containing phage st2 was the obtained signal significantly weaker than the signals obtained from other *cos*-containing phages (Fig. 5E, lane 10). However, a clear signal was obtained after extended exposure or when hybridization was performed at lower stringencies (results not shown), indicating that this gene may have undergone genetic diversion.

From these results, it was concluded that the packaging mechanism and genetic determinants for the major structural proteins are conserved within all *S. thermophilus* phages tested.

Presence of the conserved DNA fragment from the phage ΦSFi18 genome. Brüssow et al. (6) have described a DNA fragment from the ΦSfi18 genome, which was shown to be conserved in 24 of the 48 phages (59%) tested. This conserved DNA consisted of a 2.2-kb *Eco*RI fragment containing two open reading frames of unknown function. Sequence comparison demonstrated that the conserved fragment is also present in the genome of the *pac*-containing phage O1205 (23). PCR amplification of a 1.15-kb fragment internal to the conserved region encompassing parts of both open reading frames was performed with O1205 DNA as a template. Homology between this amplified fragment and other phage genomes was determined by Southern hybridization (see Materials and Methods). Seventeen of the 30 phages tested (57%) hybridized to the PCR fragment (Fig. 5A and F). Among the positive signals were DNA samples derived both from *cos*- or *pac*-containing phages (Table 2).

Lysogeny. Fifty-one *S. thermophilus* strains of the UCC collection were examined with a dot blotting procedure to determine if they contained (parts of) lysogenic phages. To maximize the possibility of detecting prophage DNA, a probe consisting of a mixture of two different phage DNAs, the *cos*-containing Φ7201 and the *pac*-containing O1205, was used. Only the lysogenic strain CNRZ1205 (11), used as a positive control, gave a signal (results not shown). This result indicated that these strains are unlikely to contain (defective) prophages.

DISCUSSION

Several attempts have been made to classify the phages infecting *S. thermophilus* (2, 7, 11, 15, 18). All phages analyzed to date have been shown to belong to group B as defined by Bradley (5) and are genetically related as demonstrated by DNA-DNA hybridization experiments. Thus, unlike lactococcal phages (12), hybridization analysis does not allow the differentiation of *S. thermophilus* phages into species, and it has been generally accepted that *S. thermophilus* phages are all derived from a common ancestor (16). Nevertheless, depending on the extent of homology between *S. thermophilus* phages and based on some other parameters, various numbers of subgroups have been identified (2, 7, 8, 11, 15, 18).

In this work, we have initiated a more meaningful and reliable division of *S. thermophilus* phages primarily on the basis of genetic data. The 30 phages of this study originated from seven different sources. At the protein level, the phages could be classified in two groups. The first phage group contained two major structural proteins (32 and 27 kDa), whereas the second group possessed three (43, 25, and 15 kDa). Although classification of *S. thermophilus* phages on the basis of similar protein profiles had been suggested before (2, 18), identical molecular weights of proteins do not necessarily reflect an evolutionary relatedness. However, our results have unambiguously demonstrated the genetic relationship within the two groups of *S. thermophilus* phages based on the presence of the genes encoding their major structural components. No DNA homology was found between the specific DNA regions encoding the respective major structural proteins of each of the two phage groups, although all phage genomes exhibited some DNA homology regardless of whether they possessed two or three structural proteins. Interestingly, all the phages belonging to the group of two major structural proteins are *cos*-containing phages (67%), while all the *pac*-containing phages possess three major proteins. Therefore, our results indicate a strict correlation between the presence of a particular set of major structural proteins and the mechanism of DNA packaging. Since the buildup of the DNA packaging machinery is determined by structural components (e.g., terminase proteins and portal protein) (3), this link may not be very surprising. Apparently, at least two different groups of *S. thermophilus* phages have evolved, each containing a genetic module harboring structural genes which determine not only the principal protein composition of the phage but also the mechanism of DNA packaging. More groups of *S. thermophilus* phages may exist since there is at least one report (13) describing phages which appear to contain four major protein bands, although two of these display an apparent molecular weight similar to that of the two smallest structural proteins of the *cos*-containing phages described in this paper. In any case, it seems that bacteriophages infecting *S. thermophilus* originated from at least two ancestors which had different packaging mechanisms, each with an accessory set of structural genes. It is conceivable that the subsequent exchange of genetic modules gave rise to the current variety of phages encountered for *S. thermophilus*

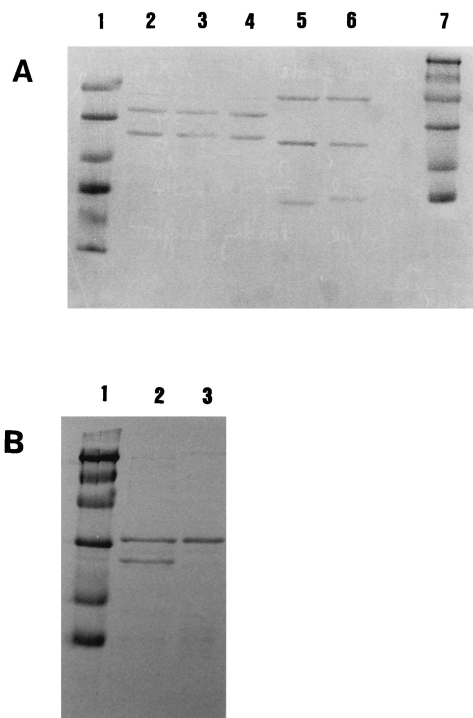

FIG. 4. SDS-PAGE of structural proteins of *S. thermophilus* bacteriophages. (A) Lanes: 1 and 7, protein markers of high molecular sizes (in descending order from top to bottom, 97.4, 69, 46, 30, 21.5, and 14.3 kDa) and low molecular sizes (in descending order from top to bottom, 46, 30, 21.5, 12.5, 6.5, and 3.4 kDa); 2, Φ7201; 3, Φ7208; 4, Φ7209; 5, P4; 6, O1205. (B) Lanes: 1, high molecular size markers (same as those for lane 1 in panel A); 2, Q5; 3, Q6 heads. The SDS-polyacrylamide gel (12.5% [wt/vol]) was stained with Coomassie brilliant blue R-250.

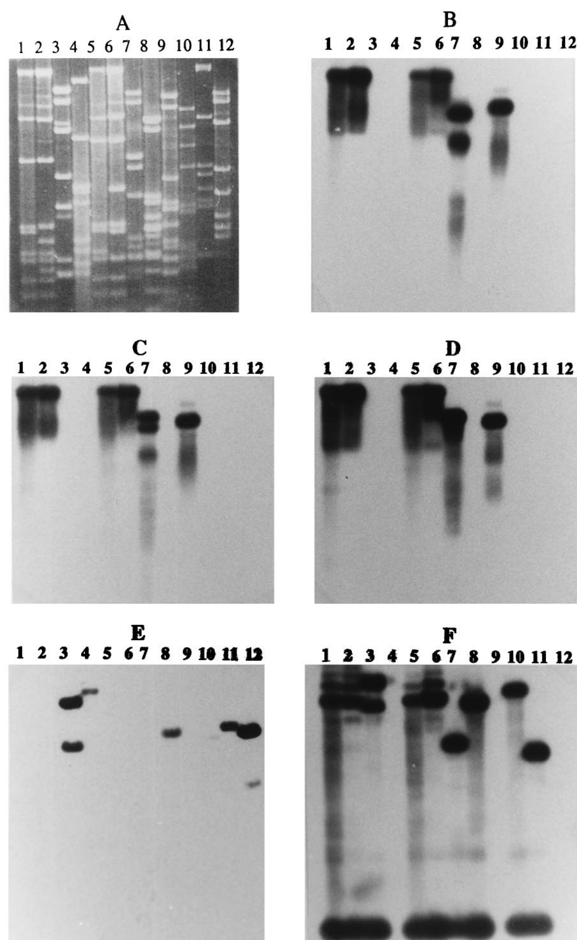

FIG. 5. Agarose gel electrophoresis of the *Eco*RV-generated DNA fragments of the phages (A) and the corresponding Southern blots hybridized with O1205 or Φ7201 PCR-amplified fragments encompassing the structural genes *mpS-1205* (B), *mpL-1205* (C), *mpM-1205* (D), *mpS-7201* (E), or the conserved Sfi18 fragment (F). Lanes: 1, ΦQ1; 2, ΦQ3; 3, ΦQ5; 4, ΦQ6; 5, ΦQ7; 6, ΦQ10; 7, O1205; 8, BaS19; 9, Φ1; 10, st2; 11, Φ47; and 12, Φ7201 (*cos*-containing phages are underlined).

which are all genetically related but which have retained their original division with respect to packaging and structural protein composition. The finding that the conserved 2.2-kb *Eco*RI fragment described by Brüssow et al. (6) can be present in either *cos*- or *pac*-containing phages would substantiate this notion. Further study and identification of genetic modules and their function will allow a more detailed grouping of *S. thermophilus* phages and is expected to expose the relative ease with which phages in general are capable of changing their genetic makeup.

Lysogenic phages of *S. thermophilus* prove to be quite related to their lytic counterparts (8; present study), and this situation therefore differs from that found with temperate lactococcal phages (12). In *S. thermophilus*, lysogeny appears to be rare (2, 7, 8, 10, 11, 15, 18), and it was therefore not surprising that only 1 (CNRZO1205) of 51 strains tested appeared to be lysogenic.

There is little or no correlation between host range and our genetically based grouping system, which is in agreement with previous attempts to link DNA homology and host range (2, 21). Apparently, host range is determined by parameters which do not appear to reflect genetic relationships between *S. thermophilus* phages. To unravel these parameters would represent a major step towards understanding the interactions which take place between the phage and its host and may be one of the routes which will lead to the design of rational solutions to phage attack in thermophilic starter cultures.

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