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Ultrastructure and Host Specificity of Bacteriophages of *Streptococcus cremoris*, *Streptococcus lactis* subsp. *diacetylactis*, and *Leuconostoc cremoris* from Finnish Fermented Milk "Viili"

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"Viili," a fermented milk product, has a firm but viscous consistency. It is produced with traditional mesophilic mixed-strain starters, which have various stabilities in dairy practice. Thirteen morphologically different types of phages were found in 90 viili samples studied by electron microscopy. Ten of the phage types had isometric heads with long, noncontractile tails, two had elongated heads with long, noncontractile tails, and one had a unique, very long elongated head with a short tail. Further morphological differences were found in the tail size and in the presence or absence of a collar, a baseplate, and a tail fiber. To find hosts for the industrially significant phages, we examined the sensitivities of 500 bacterial isolates from starters of the viili. Seven of the phages attacked *Streptococcus cremoris* strains, three attacked *S. lactis* subsp. *diacetylactis* strains, and four attacked *Leuconostoc cremoris* strains. Some phages differed only in their host specificity. Hosts were not found for 4 of the 13 morphological types of phages.

The fermented milk product "viili" has a viscous but firm, even consistency. It is produced in 20 Finnish dairies, the total consumption being about 30 million liters per year. The traditional mixed-strain starters for fermentations contain mesophilic lactic streptococci, a few strains of *Leuconostoc cremoris*, and the mold *Geotrichum candidum*, which grows on the surface of the product. The strain compositions of traditional starters are not known. Characteristic of some of the streptococcal strains is encapsulation or production of loose slime and production of lipoteichoic acid or both at the fermentation temperature (17 to 20°C) of this milk product (11, 17).

Failures in the production of viili are not rare, and sometimes they are caused by bacteriophages (25), just as in fermentations in cheese factories (20, 32). In addition to lytic growth in the host strain, phage KSY1 was also shown to dissolve capsules of nonhost streptococcal strains, which continued to grow without capsules (25).

The aim of this work was to search and morphologically characterize phages found in viili samples and to find their host specificities for selecting phage-resistant starters with stable consistencies.

MATERIALS AND METHODS

Media. For the cultivation of bacteria and their phages, M17 agar and broth supplemented with Ca^{2+} (30) and KCA agar and broth (23) (modified as described in reference 34) were used. KCA medium was further modified by supplementation with 10 μM $MnSO_4 \cdot H_2O$ and the omission of calcium citrate-carboxymethyl cellulose. Some of the bacterial isolates were picked from Elliker agar (9).

Origin of phages. Samples of viili were collected from 20 dairies in Finland. Some of the samples were from failures in fermentations, and some were quality control specimens. Whey was separated from viili by filtration through filter paper or by centrifugation (3,000 × *g*, 20 min) and was then sterile filtered (0.45-μm pore size).

Isolation and identification of bacteria. Bacterial strains were isolated from seven starters for viili fermentation obtained from dairies. M17 and Elliker plates were incubated for 2 days, and KCA plates were incubated for 3 to 4 days. All the incubations were performed at 20°C. Lactic streptococci were grown in M17 or KCA medium, but leuconostocs were grown in KCA medium only. Identification of the host bacteria was done basically by the methods of Harrigan and McCance (13). Carbohydrate fermentation was tested with the API 50CHL system (API System S.A., La Balme les Grottes, 38390, Montalieu-Vercieu, France). For the API tests, the leuconostocs were grown in MRS broth (8), and the fermentation tests were performed in accordance with the instructions of the manufacturer; the streptococci were grown in M17 broth, and the fermentation tests were performed in M17 broth from which lactose and beef extract had been omitted. Bacterial strains were stored at 4°C in broth cultures or on agar plates and at −20°C in broth supplemented with 40% glycerol.

Determination of phage sensitivity of bacterial isolates. The phage sensitivity of each bacterial isolate was determined by a spot test on the media used for bacterial isolation. Soft agar was seeded with an overnight culture of bacteria and poured onto an agar plate. One drop (10 μl) of phage-containing whey was spotted onto the solidified soft agar. The wheys used in these tests contained all the morphological types of phages found by electron microscopy. The plates were incubated for 20 to 48 h, after which the inhibition of bacterial growth was examined. All the incubations were carried out at 20°C.

Isolation and growth of phages. Wheys were titrated by a double-layer technique (30) on bacterial strains which had shown a reaction in the spot test. Phages were isolated from one plaque and purified by replaquing at least twice in succession. Purified phages were grown in broth cultures.

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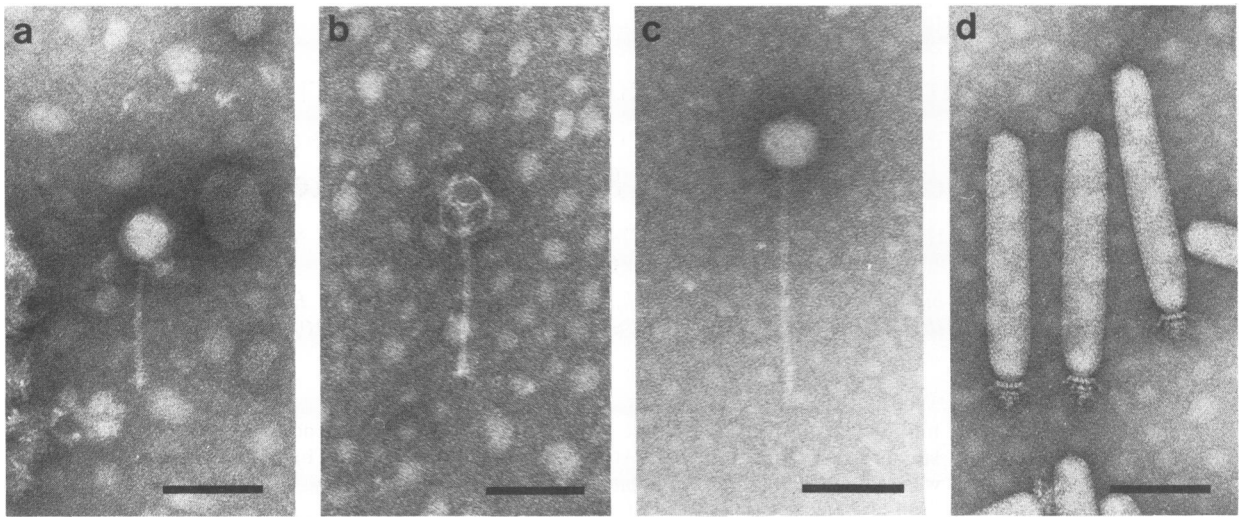

FIG. 1. Electron micrographs of negatively stained bacteriophages active against *S. cremoris*. (a) φ108; (b) φK27; (c) φ404; (d) φKSY2. Bars represent 100 nm.

An overnight culture of host bacteria was diluted 1:10 with broth, infected with one plaque, and incubated overnight. The culture was centrifuged, membrane filtered, and titrated. Phage stockes were stored at 4°C.

Electron microscopy. The wheys and isolated phages were negatively stained with 1% potassium phosphotungstate (pH 6.5) or 2% ammonium molybdate (pH 6.5). The grids were examined with a JEOL JEM-100B or JEOL JEM-100CX electron microscope at operation voltages of 80 and 60 kV, respectively. The magnification was calibrated with a cross-grating replica.

RESULTS

Origin of phages. During a period of 3 years, 90 whey samples were examined by electron microscopy; about 80% of them were shown to contain phages. Thirteen morphologically distinct types of phages were found (Fig. 1 to 4). Some of the differences were, however, quite slight, e.g., the presence or absence of a collar, length of a tail, etc. (Table 1). Ten of the phage types belonged to group B1, two belonged to group B2/B3, and one belonged to group C3 of Ackermann and Eisenstark (3). Phages belonging to groups B1, B2/B3, and C3 were found in 55, 9, and 5 whey samples, respectively. More than one type of phage was quite often found in a whey sample.

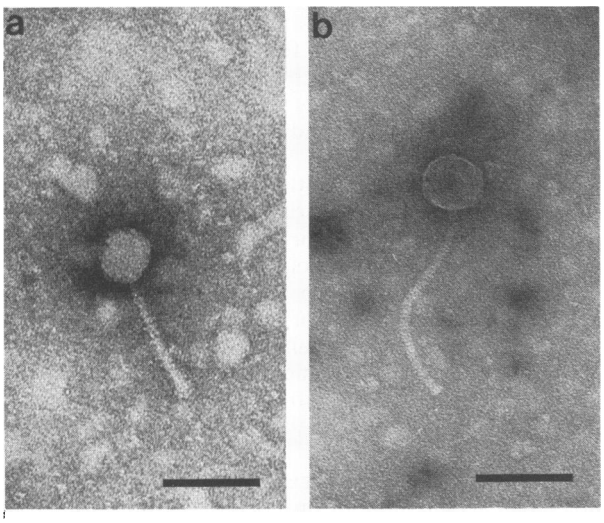

FIG. 2. Electron micrographs of negatively stained bacteriophages active against *S. lactis* subsp. *diacetylactis*. (a) φ105-5; (b) φ368. Bars represent 100 nm.

Ultrastructure of phages. Table 1 shows the morphological characteristics of the phages studied. φ108 (Fig. 1a) had an isometric head ranging from 45 to 55 nm. The relatively short tail (length 115 to 120 nm; width, 10 to 15 nm) had a striated structure (not very clear) and terminated in a small baseplate. A collar structure at the junction of the head and tail was seen in some pictures.

φK27 (Fig. 1b) and φK70 had isometric heads with a diameter of 55 to 60 nm. The tails were 130 to 145 nm long and faintly striated and had a diameter of 10 to 15 nm. The baseplate of φK27 was obscure or very small, but that of φK70 was distinct. φK27 had no collar, but a collar was occasionally observed on φK70.

φ336-11 and φ404 (Fig. 1c) were morphologically very similar. Their isometric heads had a diameter of 45 to 60 nm. The long, noncontractile tails (length, 240 to 260 nm; width, 10 to 12 nm) were flexible. The striation was not clear, and no collar or distinct baseplate could be demonstrated.

φKSY1 and φKSY2 (Fig. 1d) belonged to the rare morphological group with very long elongated heads and short tails. The lengths of the heads varied between 220 and 260 nm, and the widths varied between 45 and 55 nm. They had a tail structure with three collars above one another and spikelike structures originating from them. In the middle of the tail structure there was a central core of 5 nm. The length of the tail was 25 to 35 nm.

φ105-5 (Fig. 2a) and φ105-10 appeared to be morphologically similar, with isometric heads ranging from 50 to 60 nm. Their tails were thick, 16 to 20 nm, and striated with crossbarlike structures. The length of the relatively short tail varied between 120 and 130 nm. No collar could be seen, and a baseplate was seen in only a few pictures.

φ368 (Fig. 2b) had an isometric head of 50 to 60 nm. No collar was visible. The length of the tail varied between 185 and 200 nm, and the width varied between 11 and 15 nm. The tail was clearly striated and terminated in a small baseplate.

φ335 (Fig. 3a) and φ336 were morphologically identical.

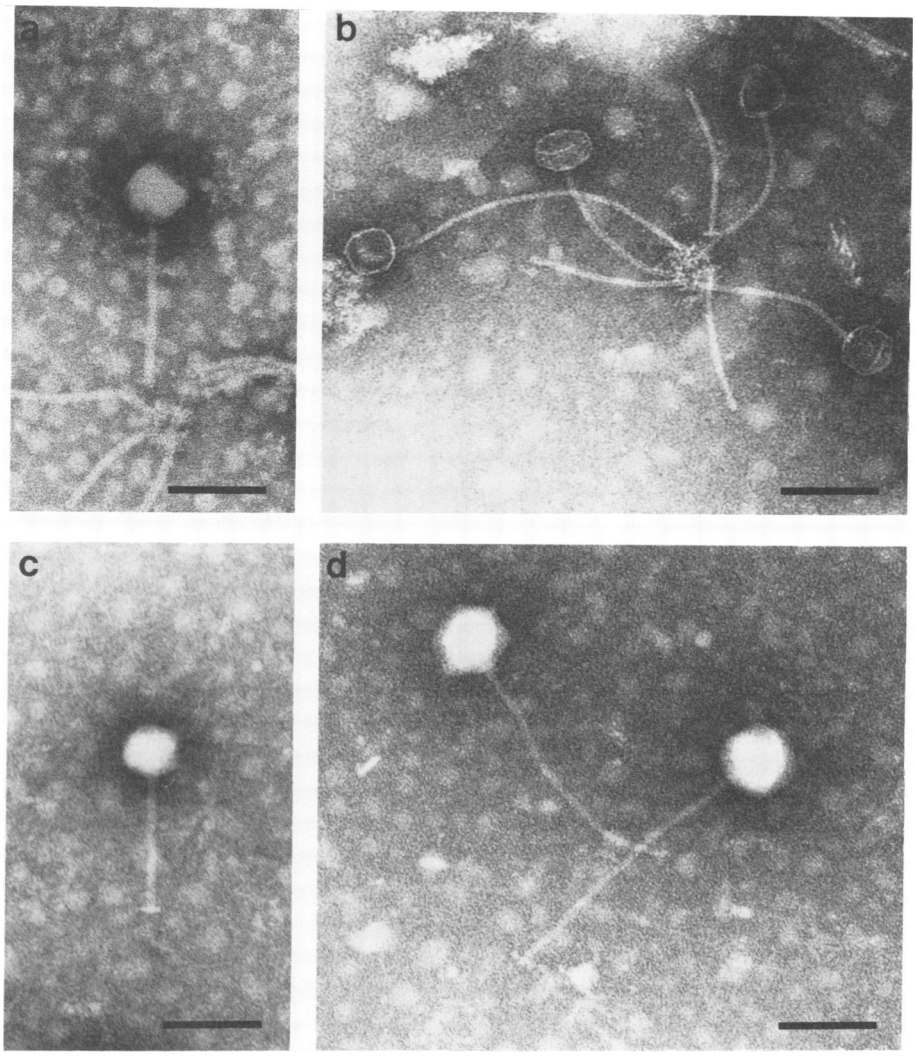

FIG. 3. Electron micrographs of negatively stained bacteriophages active against *L. cremoris* (a) φ335; (b) φ384; (c) φ400; (d) φ399. Bars represent 100 nm.

Head sizes were between 55 and 70 nm, and tail lengths varied from 165 to 190 nm. Sometimes exceptionally long tails (polytails) were observed, especially with φ384 (Fig. 3b), which was morphologically and by host specificity like φ336. The polytails had the same width, 11 to 16 nm, and structure as the normal tails but were about two times longer. No collar was seen. Segmentation of the tails was not very clear, but a channel running through the center was seen more clearly in particles with empty heads. The tails terminated in a small baseplate.

φ400 (Fig. 3c) had an isometric head of 50 to 60 nm. The tail was quite short, 130 to 150 nm, with a diameter of 11 to 16 nm. Occasionally, whiskers attached to a collarlike structure were observed (data not shown). The tail terminated in a baseplate but was not clearly segmented.

φ399 (Fig. 3d) had an isometric head with a diameter of 70 to 85 nm. The tail was quite thin (7 to 12 nm), flexible, and long (285 to 310 nm), and the striation was clear. On this phage no collar was observed, but a well-defined baseplate was seen.

A phage from whey sample 263 (Fig. 4a) had a large, elongated head. The width of the head varied between 45 and 55 nm, and the length varied between 110 and 120 nm. The tail was 210 to 225 nm long and 7 to 12 nm wide. Segmentation of the tail was not very clear. No baseplate was seen, but a collar was clearly visible.

A phage from whey sample 118 (Fig. 4b) was very similar to the phage from whey sample 263. The diameter of the head was 52 to 57 nm, and it was 105 to 115 nm long. The tail was flexible, 200 to 225 nm long and 10 to 13 nm wide. Neither baseplate nor collar was observed.

A phage from whey sample 7 (Fig. 4c) had clear crossbar-like structures on its tail. The isometric head was 45 to 55 nm in diameter. The tail was 205 to 215 nm long and 8 to 12 nm wide. A baseplate was observed, but no collar was seen.

A phage from whey sample 148 (Fig. 4d) had an exceptionally long tail fiber originating from a small baseplate at the end of the tail. The head of the phage was isometric and 50 to 60 nm. The length of the tail was 200 to 210 nm, and the width was 11 to 13 nm. The length of the very thin tail fiber was from 260 to even 430 nm. No collar was seen.

Host specificity of phages. Whey samples which contained all the morphologically distinct types of phages (Fig. 1 to 4) were used in tests of the phage sensitivity of 500 bacterial isolates. Growth of 130 isolates (26%) was inhibited by at least one of the whey samples. The inhibition was not wholly

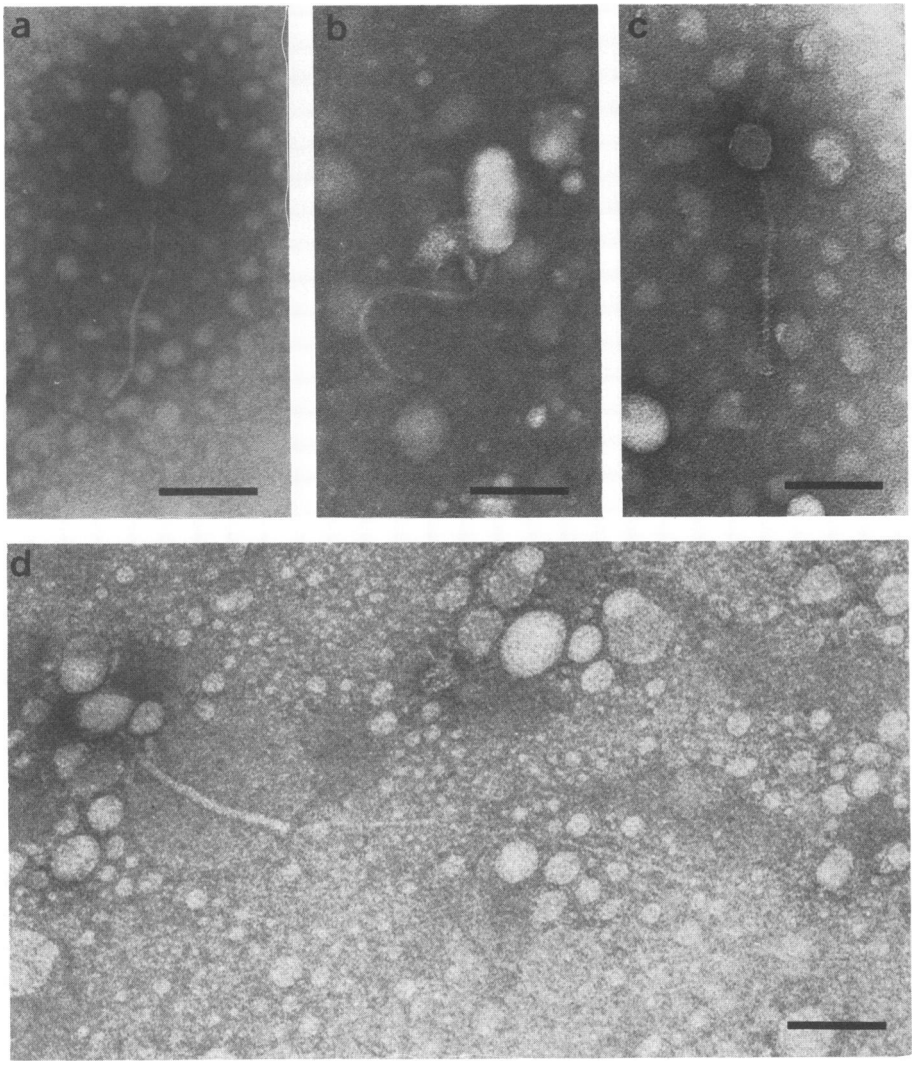

FIG. 4. Electron micrographs of negatively stained bacteriophages from different whey samples, with unknown host specificity. (a) Whey sample 263; (b) whey sample 118; (c) whey sample 7; (d) whey sample 148. Bars represent 100 nm.

due to phage growth, since single plaques were not always found in the subseqent titrations. However, 82% of the whey samples contained phages which could be propagated.

The host specificities of the purified phages are shown in Table 2. All these phages differed from each other in morphology or host specificity. Phages φK70 and φKSY2 had the same host specificity but differed from each other in their morphology (Fig. 1a and d, respectively), as did phages φ105-10 and φ368 (Fig. 2a and b, respectively).

Identification of host bacteria. Identification of the host bacteria is shown in Table 3. The basic identification was performed by conventional tests, i.e., formation of gas from glucose, reduction of litmus milk, production of ammonia from arginine, utilization of citrate, and production of acetoin in milk. Moreover, carbohydrate fermentation was tested with the API 50CHL system. Ribose, arbutin, salicin, maltose, and trehalose were fermented by *S. lactis* subsp. *diacetylactis* but not *S. cremoris* strains. Carbohydrate fermentation also differentiates *L. cremoris* from other *Leuconostoc* species, because *L. cremoris* uses only a few carbohydrates, i.e., galactose, glucose, *N*-acetylglucosamine, and lactose (12).

At present we have six *S. cremoris* strains, two *S. lactis* subsp. *diacetylactis* strains, and four *L. cremoris* strains which all have different phage sensitivities (Table 2).

DISCUSSION

The starter cultures for the production of viili contain both *S. lactis* subsp. *diacetylactis* and *L. cremoris* as citrate-fermenting and aroma-forming bacteria in addition to only lactic acid-forming *S. cremoris* and *S. lactis*. The traditional starter cultures are mixed-strain cultures with an unknown strain composition. Obviously, they contain many strains with different phage sensitivities, since disturbances in culture do not always cause a lack of fermentation. The appearance of phages in viili was not always an indication of a poor quality; they could also be found in good-quality products. This is normal when traditional mixed-strain starter cultures are used in the cheese industry (28). A broad strain composition may be a reason for the many distinct types of phages found in viili.

The most frequently found phages in viili had isometric heads and noncontractile tails and belonged to morphologi-

TABLE 1. Morphological characteristics of 18 phages found in viili

Phage	Head		Tail			
	Morphological type	Size (nm)	Length (nm)	Width (nm)	Collar[a]	Baseplate[a]
S. cremoris						
φ108	Isometric	45–55	115–120	10–15	±	+
φK27	Isometric	55–60	130–145	10–15	−	±
φK70	Isometric	55–60	130–140	10–15	±	+
φ336-11	Isometric	45–60	240–260	10–12	−	−
φ404	Isometric	45–60	240–260	10–12	−	−
φKSY1	Elongated	45–55 by 220–260	25–35	5[b]	+	−
φKSY2	Elongated	45–55 by 220–260	25–35	5[b]	+	−
S. lactis subsp. *diacetylactis*						
φ105-5	Isometric	50–60	120–130	16–20	−	±
φ105-10	Isometric	50–60	120–130	16–20	−	±
φ368	Isometric	50–60	185–200	11–15	−	+
L. cremoris						
φ335	Isometric	55–70	165–190	11–16	−	+
φ336	Isometric	55–70	165–190	11–16	−	+
φ399	Isometric	70–85	285–310	7–12	−	+
φ400	Isometric	50–60	130–150	11–16	+	+
Unknown host						
Whey sample 263	Elongated	45–55 by 110–120	210–225	7–12	+	−
Whey sample 118	Elongated	52–57 by 105–115	200–225	10–13	−	−
Whey sample 148	Isometric	50–60	200–210[c]	11–13	−	+
Whey sample 7	Isometric	45–55	205–215	8–12	−	+

[a] +, Presence; −, absence; ±, occasional presence.
[b] Central core.
[c] Long tail fiber present.

cal group B1 of Ackermann and Eisenstark (3). Phages of this type have been described earlier in many articles (2, 5, 16, 21, 29, 33). A comparison of the morphology of phages is, however, difficult because of the different negative stains, staining methods, and magnifications used. The latest method for determining the similarities of lactic streptococcal phages based on DNA homology was carried out by Jarvis (14). She found that isometric phages which differed only in the presence or absence of a collar and had tail lengths between 141 and 154 nm formed a phage species with high DNA-DNA homology, despite the host specificities of the phages. One the other hand, temperate and lytic phages with the same morphology did not have DNA homology (15). Our phages φ108, φK27, and φK70 (Fig. 1a and b) may belong to group a or b of Jarvis (15), although they had shorter tails (Table 1). φ404 (Fig. 1c) and φ336-11, with small isometric heads and tail lengths of 240 to 260 nm, resembled phage 964B isolated by Terzaghi (29). These phages also were the same size as phage P204 (21), the distinction in tail structure possibly being caused by a different staining procedure. φ105-5 (Fig. 2a) and φ105-10 had short, rigid tails and may belong to group c of Jarvis (14). This group was formed by two phages which had no DNA homology with other small isometric-head phages of group a or b.

TABLE 2. Host specificities of phages isolated from viili

Phage	Specificity[a] for indicated host bacterium:											
	S. cremoris						*S. lactis* subsp. *diacetylactis*		*L. cremoris*			
	KT17	ARH93	ARH86	SEPH11	HH11V	MLS96	KT5	KT10	SEPA1	P1	HA1	LFA6
φ108	+	−	−	−	−	−	−	−	−	−	−	−
φK70	−	+	+	−	−	−	−	−	−	−	−	−
φK27	−	−	+	−	−	−	−	−	−	−	−	−
φKSY2	−	+	+	−	−	−	−	−	−	−	−	−
φ336-11	−	−	−	+	−	−	−	−	−	−	−	−
φ404	−	−	−	−	+	−	−	−	−	−	−	−
φKSY1	−	−	−	−	−	+	−	−	−	−	−	−
φ105-5	−	−	−	−	−	−	+	−	−	−	−	−
φ105-10	−	−	−	−	−	−	−	+	−	−	−	−
φ368	−	−	−	−	−	−	−	+	−	−	−	−
φ335	−	−	−	−	−	−	−	−	+	−	+	−
φ336	−	−	−	−	−	−	−	−	+	−	−	−
φ399	−	−	−	−	−	−	−	−	−	+	−	−
φ400	−	−	−	−	−	−	−	−	−	+	−	+

[a] +, Plaque formation; −, no plaque formation.

TABLE 3. Physiological properties of host bacteria isolated from viili starters

Test	Test result[a] for indicated bacterium:											
	S. cremoris						*S. lactis* subsp. *diacetylactis*		*L. cremoris*			
	KT17	ARH86	ARH93	SEPH11	HH11V	MLS96	KT5	KT10	SEPA1	P1	HA1	LFA6
Growth at 10°C	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 40°C	−	−	−	−	−	−	+	+	−	−	−	−
Gas from glucose	−	−	−	−	−	−	−	−	+	+	+	+
Reduction of litmus	+	+	+	+	+	+	+	+	−	−	−	−
Utilization of citrate	−	−	−	−	−	−	+	+	+	+	+	+
Formation of acetoin	−	−	−	−	−	−	+	+	−	−	−	−
NH_4^+ from arginine	−	−	−	−	−	−	+	+	−	−	−	−
Fermentation[b]												
Ribose	−	−	−	−	−	−	+	+	−	−	−	−
D-Fructose	+	+	+	−	+	+	+	+	−	−	−	−
D-Mannose	+	+	+	+	+	+	+	+	−	−	−	−
Arbutin	−	−	−	−	−	−	+	+	−	−	−	−
Esculin	−	+	+	−	−	+	+	+	−	−	−	−
Salicin	−	−	−	−	−	−	+	+	−	−	−	−
Cellobiose	−	−	−	−	−	+	+	+	−	−	−	−
Maltose	−	−	−	−	−	−	+	+	−	−	−	−
Saccharose	−	−	+	−	−	−	−	−	−	−	−	−
Trehalose	−	−	−	−	−	−	+	+	−	−	−	−

[a] +, Positive result; −, negative result.

[b] All strains fermented galactose, D-glucose, *N*-acetylglucosamine, and lactose. No strain fermented glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, β-methylxyloside, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdalin, melibiose, inulin, melezitose, D-raffinose, amidone, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arablitol, L-arabitol, gluconate, 2-keto-gluconate, or 5-keto-gluconate.

Viili is produced only in Finland and in some parts of Sweden (19). The special, viscous starters are used mainly for the fermentation of viili, so this may be the reason for the appearance of some rare types of phages in viili. Phage KSY1 represented the first observation of this morphological type (25). Ackermann and Nguyen (4) found a phagelike particle from sewage which resembled φKSY1, but the origin and host specificity of this particle were not known. Also, Chopin and Rousseau (7) found morphological aberrations in two lactic streptococcal phages which showed some resemblance to φKSY1. However, φKSY1 is a mature, complete phage and not a morphological error. With respect to phage ecology, it would be interesting to know, however, if they have significant DNA homology with each other. Also, another phage, φKSY2, which differed from φKSY1 only in host specificity (Table 2), was found in viili.

Two types of phages with long elongated heads and long tails were found in our samples. They differed from each other in the presence or absence of a collar (Fig. 4a and b). The length-to-width ratio of these phage heads was between 2.0 and 2.15, so it is difficult to determine whether they belong to group B2 (length-to-width ratio, 1.2 to 1.4 and 1.8) or B3 (ratio, 3 to 3.4) of Ackermann and Eisenstark (3). However, the elongated phages found in viili differed from the frequently found prolate phages of lactic streptococci (group d of Jarvis [14]) and may therefore belong to group B3. A phage with a long elongated head and a long tail was induced earlier from a lysogenic strain of *Lactobacillus lactis* (18). The phage from *L. lactis* was somewhat bigger (head, 51 by 132 nm; tail, 282 nm) than the type B2/B3 phages in viili, but the morphology of their heads was similar. Hosts for our type B2/B3 phages have not been found. They could be temperate, or their host strains may not have been successfully isolated from mixed-strain starter cultures.

A few lactic streptococcal phages have a long tail fiber on their baseplates. These tail fibers have been found in virulent (21) and temperate (6, 31) phages of mesophilic lactic streptococci and in phages of *S. thermophilus* (1). The phage with long tail fiber in Fig. 4d may also be temperate, since no host was found.

Our collection contains four different phages of *L. cremoris*. Only a few reports of phages from dairy leuconostocs could be found (26, 27), although these bacteria are widely used as aroma-producing bacteria in starters for cheese and butter production. The phages of *L. cremoris* (Fig. 3a to d) did not differ in morphology from the isometric-head phages of lactic streptococci. Only one type, φ399 (Fig. 3c), was clearly bigger than the streptococcal phages found in this study. Polytails were found in one type, φ384 of *L. cremoris*. This observation was also made by Reinbold et al. (24), who found polytail formation in *S. thermophilus* phages. φ400 of *L. cremoris* resembled phage pro2 of *L. mesenteroides* described by Sozzi et al. (27) but did not have as large a baseplate as phage pro2. Sometimes φ400 also had whiskers. These structures are difficult to demonstrate (22), and they were only seen in some pictures.

Six of the streptococcal host strains were *S. cremoris*, and two were *S. lactis* subsp. *diacetylactis*. The absence of *S. lactis* strains was not unexpected, since the traditional viili starters contain *S. lactis* only as a minority (10). The encapsulation of the host bacteria was not studied, but at least some of them formed viscous cultures in milk.

The leuconostocs grew poorly if at all on M17 agar. On KCA agar and broth, growth was considerably improved by the addition of Mn^{2+} to the media. Because calcium citrate-carboxymethyl cellulose in KCA agar is not dissolved by *S. cremoris* and *S. lactis* it was omitted, and the plaques could be seen clearly. In our experience, KCA medium was good for the isolation of phages and their hosts from mixed-strain starters which also contained leuconostocs.

Our long-term research goal is to elucidate the reasons for instability in viili production. The main aim of this study was to isolate a collection of host bacteria for the phages present

in Finnish dairies, to establish a phage collection and, with the help of these phages, to select new, stable starters.

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