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Isolation of *Streptococcus lactis* Bacteriophages and Their Interaction with the Host Cell

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Phages may cause lysis of lactic acid bacteria used in cheese production. Three virulent bacteriophages specific for *Streptococcus lactis* subsp. *lactis* C2 were isolated and purified from cheese whey. They showed distinct plaque sizes, and although they had similar morphology by electron microscope examination, their dimensions were slightly different. The phage heads were elongated and hexagonal in shape, and the flexible tails appeared periodically cross-striated. They were DNA phages based on the acridine orange test. On infection, phage was adsorbed on the bacterial surface by the free end of the tail. After 80 min of incubation at 25°C, the phage heads appeared empty, slightly collapsed, and possessed a visible hollow tube through which the genetic material had been injected.

Most lactic acid bacteria used as starter cultures for milk fermentation may be infected and destroyed by different bacteriophages (1, 8, 16). When virulent phages lyse the host cells, microbial acidification fails, causing variations in acid production during the cheese manufacturing process (15).

The isolation and classification of specific phages for lactic streptococci are of importance to know the prevalent types which occur in the cheese production environment. This knowledge will facilitate development of a suitable system of phage-resistant strains.

In this communication, three bacteriophages active against *Streptococcus lactis* subsp. *lactis* C2, isolated from cheese whey obtained from Argentinian dairy plants, are described. According to the nomenclature of Ackerman et al. (4), the phages were designated Stl 1, Stl 3, and Stl 5, respectively. Their interaction with host cells was studied by electron microscopy.

The bacterial strains used were: *Streptococcus lactis* subsp. *lactis* C2, *Streptococcus lactis* subsp. *cremoris* B1, and *Streptococcus lactis* subsp. *diacetilactis* DRD1, provided by L. McKay. *S. lactis* subsp. *lactis* SL1 was from Visby (Germany), *S. lactis* subsp. *diacetilactis* SD1, *S. lactis* subsp. *lactis* SL2, *S. lactis* subsp. *cremoris* SC1, and *S. lactis* subsp. *cremoris* SC3 were from C. Hansen (Denmark). Other strains used in the present study were *Lactobacillus casei*, *Lactobacillus brevis*, *Streptococcus faecalis* SF19, *Bacillus subtilis*, *Sarcina lutea*, *Staphylococcus aureus*, and *Escherichia coli*, from the collection kept in the Food Microbiology Laboratory of the University of Buenos Aires.

Tryptone soy medium (TS) from Britania (Buenos Aires, Argentina) or TS supplemented with 0.3% yeast extract (TSY) was used to grow *S. lactis* subsp. *lactis* C2 and the other bacterial strains. M broth (13) contained (in grams per liter): tryptone, 20; glucose 5; NaCl, 4; sodium acetate, 1.5; $CaCl_2 \cdot H_2O$, 0.15; $MgSO_4 \cdot 7H_2O$, 0.2; and $MnSO_4$, 0.05; adjusted to pH 7.0 before being autoclaved at 121°C for 15 min. Skim milk was prepared from Nestlé dry milk (120 g/liter) and heated at 100°C for 30 min. Different cheese whey samples, obtained from presumably phage-contaminated commercial dairy fermentation processes, were centrifuged at 3,000 rpm and filtered through a Millipore membrane (0.45-μm pore size). Several strains of lactic streptococci were then inoculated into the heated skim milk, with and without the filtrates. Phage isolations were attempted from noncoagulated cultures of strain C2. Propagation was made on the sensitive strain of *S. lactis* subsp. *lactis* C2 growing in M broth. Agar plates of this medium were used for plaque isolation, and the three phages were purified by replaquing. *S. lactis* subsp. *lactis* C2 was the only strain sensitive to phages Stl 1, Stl 3, and Stl 5. Further studies with these phages were made by spotting test (7) on M agar plates inoculated with *S. lactis* subsp. *cremoris* SC1, SC3, or B1, *S. lactis* subsp. *diacetilactis* SD1 or DRC1, *S. lactis* subsp. *faecalis* SF19, and *S. lactis* subsp. *lactis* C2, SL1, or SL2. The results supported the specificity of the phages for *S. lactic* subsp. *lactis* C2 among the tested strains. *L. brevis*, *L. casei*, *S. aureus*, *B. subtilis*, *S. lutea*, and *E. coli* did not show any evidence of bacterial lysis.

On M agar seeded with *S. lactis* subsp. *lactis* C2, the diameters of Stl 1, Stl 3, and Stl 5 plaques were 1 to 2, 2.0 to 2.5, and 3 to 4 mm, respectively. In all cases, the plaque size on M agar was larger than on TS or TSY medium.

S. lactis subsp. *lactis* C2 was grown for 18 h in skim milk at 30°C and used to inoculate M broth. After growth, it was inoculated by a second transfer (2.5% inoculum) into fresh M medium. After 2 h, phage was added at a multiplicity of infection of about 1, and the incubation continued until lysis occurred. To eliminate debris, the clear lysates (10^8 to 10^9 PFU/ml) were filtered through the Millipore membranes and stored at 4°C. Phage titers were determined on M medium by the double-layer agar method (5).

To determine the morphological features of the phages, they were concentrated and resuspended in 0.1 M ammonium acetate, as described by Accolas and Spillmann (1). Negative staining was performed with a saturated solution of uranyl acetate in water. A small drop of phage suspension was touched by the surface of carbon-coated Formvar film covering the electron microscope grid. Excess material was removed by absorption with filter paper, and the grid was

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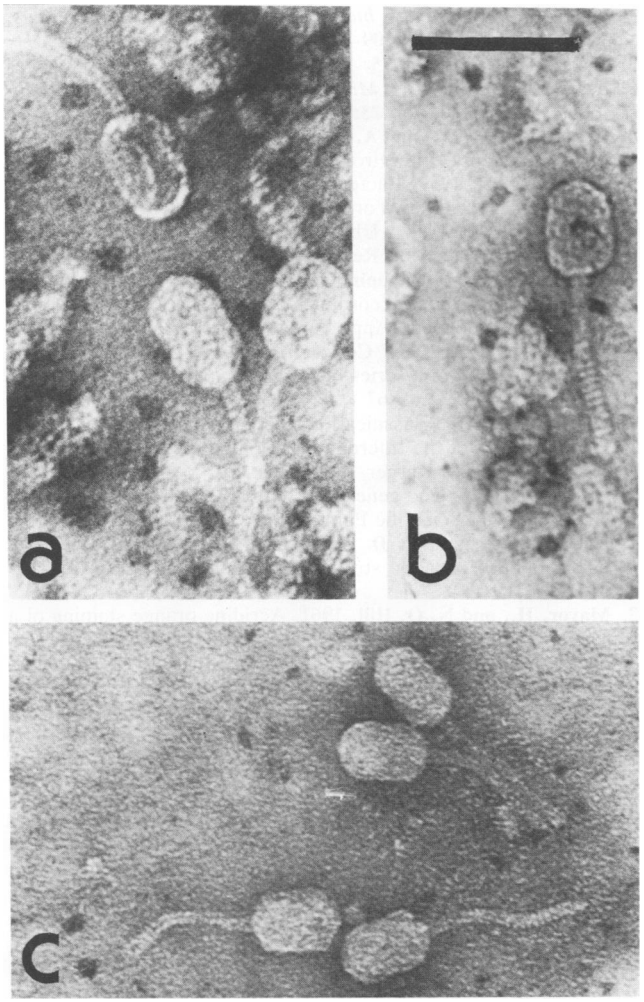

FIG. 1. Electron micrographs showing the phage morphology of Stl 1 (a), Stl 3 (b), and Stl 5 (c). Bar, 0.1 μm.

briefly floated on uranyl acetate for 20 s, removed, and touched to a filter paper surface. The grid was examined in a Siemens Elmiskop I electron microscope at original magnifications of ×40,000 and ×72,000. Magnifications were checked with a carbon grating replica (54,864 lines per inch). Some specimens were negatively stained with 2% phosphotungstic acid in aqueous solution brought to pH 7 with NaOH. The phage sizes were determined from the average of 7 to 10 independent measurements.

The three phages, Stl 1, Stl 3, and Stl 5, were found to be similar in shape. Electron microscopy revealed that the phages possessed prolate heads of hexagonal shape (Fig. 1). The estimated head sizes were: 69 by 45 nm for Stl 1, 58 by 40 nm for Stl 3, and 52 by 36 nm for Stl 5. The tails measured about 102 to 110 by 7 to 10 nm and were flexible and regularly cross-striated without sheaths, collars, base plates, or terminal fibers. The periodic striations had spacings of 4.0 to 4.2 nm. These phages resemble those included in the morphological group B of Bradley (6).

Phages concentrated by the procedure described by McKay et al. (13) were used to determine the type of nucleic acid by acridine orange staining of phage droplets on cover slips, as described by Mayor and Hill (12). The preparations were observed under UV light with a Zeiss fluorescence microscope. The typical green fluorescence observed by UV microscope was attributed to the presence of double-stranded DNA. To carry out the host-phage interaction experiments, the clear lysate of Stl 5 (10^9 PFU/ml) was concentrated as follows: 10 ml of lysate plus 26 ml of sterile water was centrifuged in a Beckman Spinco L2-65B centrifuge for 2 h at 24,000 rpm, with an SW27 rotor. The supernatant was carefully discarded, and 1 ml of cold M medium was added to the pellet. The pellet was left overnight at 4°C before being dispersed with a Pasteur pipette.

In a small tube, 0.2 ml of Stl 5 phage suspension, 0.02 ml of 0.2 M $CaCl_2 \cdot 6H_2O$, and 0.2 ml of a log-phase culture of *S. lactis* subsp. *lactis* C2 diluted to 4% with M medium were incubated at 25°C for 10 min. The preparations were stained with uranyl acetate and observed by electron microscopy.

The first step in phage infection is adsorption onto the bacterial surface. The micrographs (Fig. 2) revealed phage Stl 5 interacting with the *S. lactis* cell wall with the free end of its tail (Fig. 2a, details at ×240,000). To understand the true nature of the infection process, a similar preparation was incubated at 25°C for 80 min, stained as above, and subjected to electron microscopy observation. The empty head of phage Stl 5 was observed to be slightly collapsed, and the tail was shortened, with a visible hollow axial tube (Fig. 2b).

Since the discovery of specific phages for lactic acid bacteria, the interest in their detection, classification, and control has increased. This knowledge is particularly important for the dairy industry because phages may disturb or retard growth of the lactic acid bacteria used for production of cheese and fermented milks. Furthermore, the isolated phages may be used to isolate strains resistant to their virulent action (15).

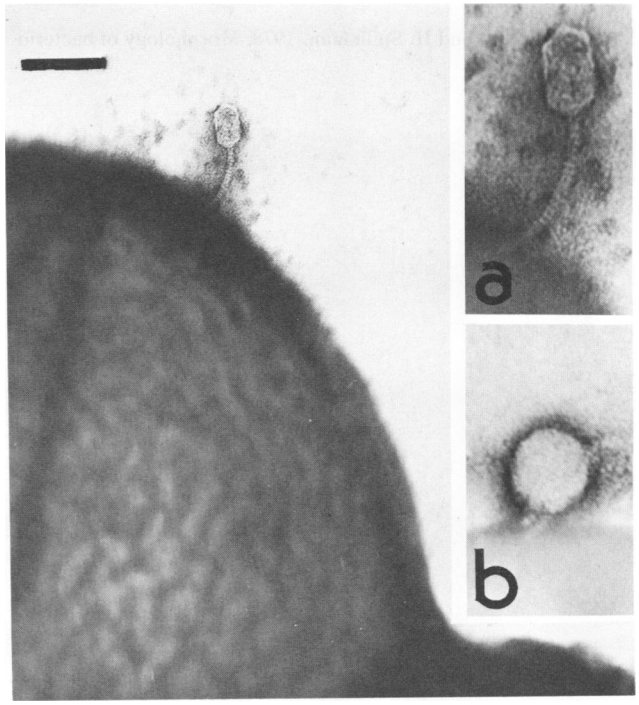

FIG. 2. Electron micrographs of phage Stl 5 adsorbed on the *S. lactis* subsp. *lactis* C2 surface (×120,000). Bar, 0.1 μm. (a) After 10 min of incubation at 25°C (×246,000). (b) After 80 min of incubation at 25°C (×254,000).

Three double-stranded DNA phages which lyse *S. lactis* subsp. *lactis* C2 were isolated from cheese whey from Argentine factories. Although their head shape may not be established with certainty from micrographs, it is assumed to be polyhedral, probably an icosahedral, tridimensional structure. Given their general features, they belong to group B of the Bradley classification system (6) or the B2 group of Ackermann (3), and they could also be included in the Styloviridae group of Fenner (9). Given their size and form, they have similarities and differences with respect to other phages previously described (1, 3, 11, 14, 15, 17) for *S. lactis*. As with other phages specific for *Streptococcus* (1) and *Lactobacillus* (2) species, the tails showed periodic cross-striations, without a visible axial hollow tube. This is an indication that in the free state nucleic acids occupy the heads and also the tails of the phages. Host-phage interactions were made evident by electron microscopy. Phage Stl 5 was adsorbed onto the bacterial surface, probably on specific receptor sites, as was described for other bacteriophages (10). After injection of the DNA into the cell, the head lost its original hexagonal conformation and appeared empty and slightly collapsed. The visibly shortened tail showed an internal axial channel, through which the genetic material was presumably injected. After a period of time, complete lysis of the *S. lactis* subsp. *lactis* C2 culture occurred.

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