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# Molecular Ecology of *Streptococcus thermophilus* Bacteriophage Infections in a Cheese Factory

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**A mozzarella cheese factory using an undefined, milk-derived *Streptococcus thermophilus* starter system was monitored longitudinally for 2 years to determine whether the diversity of the resident bacteriophage population arose from environmental sources or from genetic changes in the resident phage in the factory. The two hypotheses led to different predictions about the genetic diversity of the phages. With respect to host range, 12 distinct phage types were observed. With two exceptions, phages belonging to different lytic groups showed clearly distinct restriction patterns and multiple isolates of phages showing the same host range exhibited identical or highly related restriction patterns. Sequencing studies in a conserved region of the phage genome revealed no point mutations in multiple isolates of the same phage type, while up to 12% nucleotide sequence diversity was observed between the different phage types. This diversity is as large as that between the most different sequences from phages in our collection. These observations make unlikely a model that postulates a single phage invasion event and diversification of the phage during its residence in the factory. In the second stage of our factory study, a defined starter system was introduced that could not propagate the resident factory phage population. Within a week, three new phage types were observed in the factory while the resident phage population was decreased but not eliminated. Raw milk was the most likely source of these new phages, as phages with identical host ranges and restriction patterns were isolated from raw milk delivered to the factory during the intervention trial. Apparently, all of the genetic diversity observed in the *S. thermophilus* phages isolated during our survey was already created in their natural environment. A better understanding of the raw-milk ecology of *S. thermophilus* phages is thus essential for successful practical phage control.**

Phage attack has always been a major problem in industrial fermentation, especially in the dairy industry (11, 19). Contamination by virulent phages may result in the lysis of starter strains in the vat, causing slow fermentation or even complete starter failure with consequent loss of the product. *Streptococcus thermophilus*, a gram-positive thermophilic lactic acid bacterium, is used as a starter in the yogurt and cheese industry. All of the *S. thermophilus* bacteriophages isolated until now (1, 12, 15, 16, 18, 20; for a review, see reference 4) belong to group B as defined by Bradley ((2); *Siphoviridae*) and share DNA homology. During the last 30 years, about 80 bacteriophage isolates were collected by our company from batches in which fermentation failures occurred with *S. thermophilus* strains used in yogurt and cheese factories situated in various European countries. About 40 yogurt factory-derived phages were classified into two subgroups by serology, host range analysis, and hybridization with subgroup-specific DNA sequences (5), thus documenting rather limited genetic variability. In contrast, cheese-derived phages could not be classified into subgroups: within 41 phages analyzed, 35 distinct restriction patterns and 34 distinct host ranges were detected (5). This apparent variability of *S. thermophilus* phages poses problems for practical phage control, especially for mozzarella fermentation. In fact, due to phage problems alternative lactic acid bacterial starters for mozzarella production in Italy are currently being explored (9).

Ecological studies that explore the origin of phage diversity are lacking. Therefore, we collected data from a single cheese factory during a longitudinal survey to determine whether the diversity of the bacteriophage population in the cheese factory arose from environmental sources or from genetic changes of the resident bacteriophage. A mozzarella factory which used a complex, undefined mixture of starter strains and open, continuous-fermentation tanks was chosen for this study. Such a factory should be very susceptible to invasion by phages from environmental sources. If a single phage invaded the factory and experienced genetic changes during its residence, one would predict a family of closely related phages with similar restriction patterns and one or a few overlapping host ranges. In addition, one would expect an accumulation of point mutations, deletion and recombination events as a consequence of rapid evolution of the phages, and changes in the restriction patterns during the survey period. Alternatively, if the diversity of phages arose before entry into the factory, one would predict a wide range of restriction patterns and clearly distinct host ranges. The factory phages should be genetically as different from each other as any other set of randomly collected phages. The data from the longitudinal survey, presented in the current communication, demonstrate little, if any, evidence in favor of genetic changes of a resident phage during the observation period.

Demonstration of identity between factory and raw-milk phages would be the clearest proof for the origin of phage diversity in the environment. Such proof is, however, difficult to provide, as the phage concentration in raw milk is likely to be very low and one will only see phages for which susceptible cells exist in the starter cultures. In addition, cheese factories tend to be infested with moderate to high titers of phages (5) and will therefore be refractory to invasion by new raw-milk phages. To circumvent these technical difficulties, we conducted an intervention study by replacing the complex, undefined starter system with a defined starter composed of bacterial strains which were unable to propagate the phage population

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resident in the factory. Three possible outcomes were considered. Under the strongly selective pressure of the new hosts, the resident phages could undergo rapid adaptation to the new hosts. This scenario would indicate a remarkable genetic flexibility of the resident phages. Alternatively, the resident phages could be wiped out and replaced by new phages. An ecological survey should then identify the likely source of these new phages (e.g., raw milk, starter cultures, operators, or airborne phage transmission). Finally, if the environmental phage load is low, the factory may run for an extensive time period without phages. The outcome of our intervention trial was the observation of rapid colonization of the factory by new phages which could be traced back to raw milk. This therefore indicates that the diversity of phages observed in a cheese factory reflects the diversity of phages normally found in their natural environment.

## MATERIALS AND METHODS

**Longitudinal survey.** For the longitudinal survey, we chose a mozzarella cheese factory situated in northern Italy. A "madre naturale" starter system was used for cheese fermentation. In this artisan technique, the starter is derived from the natural bacterial flora of local raw-milk samples. Milk is heated in several cycles to select for thermophilic bacteria. The bacterial population is then further selected for proteolytic and acidifying properties. The starter is thus a complex population consisting mainly, but not exclusively, of an undefined mixture of *S. thermophilus* strains. Ten batches of such madre naturale starters, the strain compositions of which were unknown to the cheese maker, were used in rotation, with each batch being used for 1 day of fermentation. The raw-milk source and the processing procedure did not change over the study period. Therefore, phage variability could be studied solely as a function of time.

The study was conducted between December 1993 and February 1996. The factory processed about 500,000 liters of milk daily in a continuous and open fermentation system. Raw milk was introduced and processed in the factory. The milk was heat treated for 15 s at 74°C before transfer to the fermentation vat. Phages were titrated and isolated from cheese whey samples collected 2 h after addition of the starter strains.

**Strain and phage identification.** Individual bacterial colonies from the starter mixture were isolated on M17 agar (Difco) containing 5 g of lactose per liter and propagated at 40°C in M17 broth (23). For the analytical purpose of the longitudinal study, randomly selected colonies of *S. thermophilus* strains composing the madre naturale starter were first classified according to acidification properties.

Filter-sterilized cheese whey samples (0.1 ml) were tested for the presence of bacteriophages in 10 ml of M17 medium inoculated with $10^6$ CFU of colony-purified cells grown in milk. All of the resulting phage isolates were purified by two rounds of single-plaque isolation. Propagation of the phages was then carried out at 40°C in M17 broth supplemented with 5 g of lactose per liter and 0.5 g of $CaCl_2$ per liter.

In a second phase, bacteria not lysed by these phages were identified and cheese whey samples were rescreened for phages attacking these strains. Lytic typing was repeated until we were able to type more than 95% of randomly selected colonies from the starter mixture.

**Intervention study.** Five defined starter cultures, each composed of two *S. thermophilus* strains, were chosen for the intervention trial (February to April 1996). None of the 10 strains, designated N to W, was susceptible to the phages isolated from the factory during the longitudinal survey. Eight of the strains could be infected by phages from our phage collection, but no overlapping susceptibility was observed. Each starter culture was used for 1 day of fermentation and then replaced by the next. For technological reasons (superior fermentation properties of strains from the previous starter population), 2 weeks after the intervention the number of starter mixtures was increased to nine, four of the eight additional strains (strains B, E, G, and K) were susceptible to phages from the longitudinal survey, and for three strains we had no homologous phages in our phage collection. Apart from alterations to the starter system, no other parameter (processing, raw-milk source) was changed. For 2 months, whey samples from the factory were screened daily for the presence of phages.

**Biochemical methods.** Phage purification, phage DNA extraction, restriction enzyme digestion, agarose gel electrophoresis, and Southern and dot blot hybridizations with individual *Pvu*II-*Xba*I restriction fragments labeled with the Random Primed DNA Labeling kit of Boehringer (Mannheim, Germany) were done as described previously (3). Chromosomal DNA was isolated from the starter cells as described by Slos et al. (22). The purified DNA was digested with restriction enzyme *Pst*I, *Eco*RI, or *Hin*dIII, Southern blotted, and probed with plasmid pY30. pY30 consists of a 1.5-kb *Hin*dIII fragment of *Lactobacillus bulgaricus* 23S ribosomal DNA cloned in *Escherichia coli* vector Yrp17 (generous gift of M. Delley, Nestlé).

**PCR.** PCR products were prepared by using synthetic oligonucleotides A and D, described previously (5), or primers 1 and 2 (1, 5′-GGTACTAAATTTAGTA GC-3′; 2, 5′-CCAATAAACTGGCTTATC-3′), purified phage DNA, and Super *Taq* Polymerase (Stehelin, Basel, Switzerland). PCR products were purified by using the Qiaquick Spin PCR purification kit (Qiagen) in accordance with the manufacturer's protocol.

**Sequencing.** PCR products amplified with primers 1 and 2 were sequenced by the Sanger method (21) of dideoxy-mediated chain termination by using the *fmol* DNA Sequencing System of Promega (Madison, Wis.). The sequencing primers were end labeled by using [$\gamma$-$^{33}$P]ATP in accordance with the manufacturer's protocol. Primers 3 (5′-GATACGTCGATATGTCCC-3′) and 4 (5′-TTGGTT AAGTTTCAAGGG-3′ or variants of it) were used for comparative sequencing. The thermal cycler (Perkin Elmer) was programmed for 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min. Sequence analysis was done by using the GCG computer package (Madison, Wis.).

**Nucleotide sequence accession number.** The GenBank accession number of the nucleotide sequence reported here is X77469.

## RESULTS

**Longitudinal survey: host cells.** A total of 13 different *S. thermophilus* strains, A to M, defined by their distinct phage susceptibility, were isolated from the undefined starters used during the longitudinal survey. Determination of the strain composition of the starters led to the observation that different starter cultures possessed common strains, thus compromising the idea of starter rotation. These 13 strains represented about 95% of the randomly selected colonies. The remaining 5% of the colonies could not be further differentiated by phage typing, since they were not attacked by phages from either the cheese whey or our phage collection. Four of the strains represented 85% of the randomly selected colonies (B, 36%; G, 19%; E, 16%; H, 14%). On *Pst*I digestion of genomic DNA, all indicator cells showed distinct ribosomal DNA restriction patterns (ribotypes), with only lytic group E, G, and M indicator cells possessing identical ribotypes. On *Eco*RI digestion, group E indicator cells could be differentiated from group G and M cells (data not shown). About 100 colonies from the different starters were tested for the presence of prophage by the mitomycin C induction test, and no lysogenic starters were identified.

**Phages: biological properties.** Systematic phage counts were done at regular intervals by using group A to M indicator cells defining the different lytic groups. Phages were found in all of the whey samples tested over the 2-year period. Phage titers ranged from $10^4$ to $10^7$ PFU/ml. Any given whey sample contained phages belonging to different lytic groups: the minimum was four different groups, while the maximum was eight. Marked daily variations in phage titers were observed which were due to the starter rotation. Each phage strain was named with a lowercase letter according to the host strain on which it was isolated, which was indicated with an uppercase letter. If more than one type of phage strains were isolated, a number was added as a suffix.

Phages attacking lytic group B and G cells were the most prevalent isolates: 35 and 23% of all isolates belonged, respectively, to these two groups. Phages attacking lytic group A, D, J, and L cells were only occasionally isolated (each group representing less than 5% of all isolates). Phages growing on lytic group C, E, F, H, I, K, and M cells were found at intermediate frequencies. During the 2-year survey period, no significant shifts in the distribution of the phages among the different lytic groups were observed.

During the 2-year period of the longitudinal survey, several hundred phage isolates were plaque purified and their host ranges were investigated by using lysis of bacterial cells in liquid medium as the test. The phages isolated defined the 13 lytic groups A to M of starter strains described above. Plaque assays confirmed that there were, with one exception, no over-

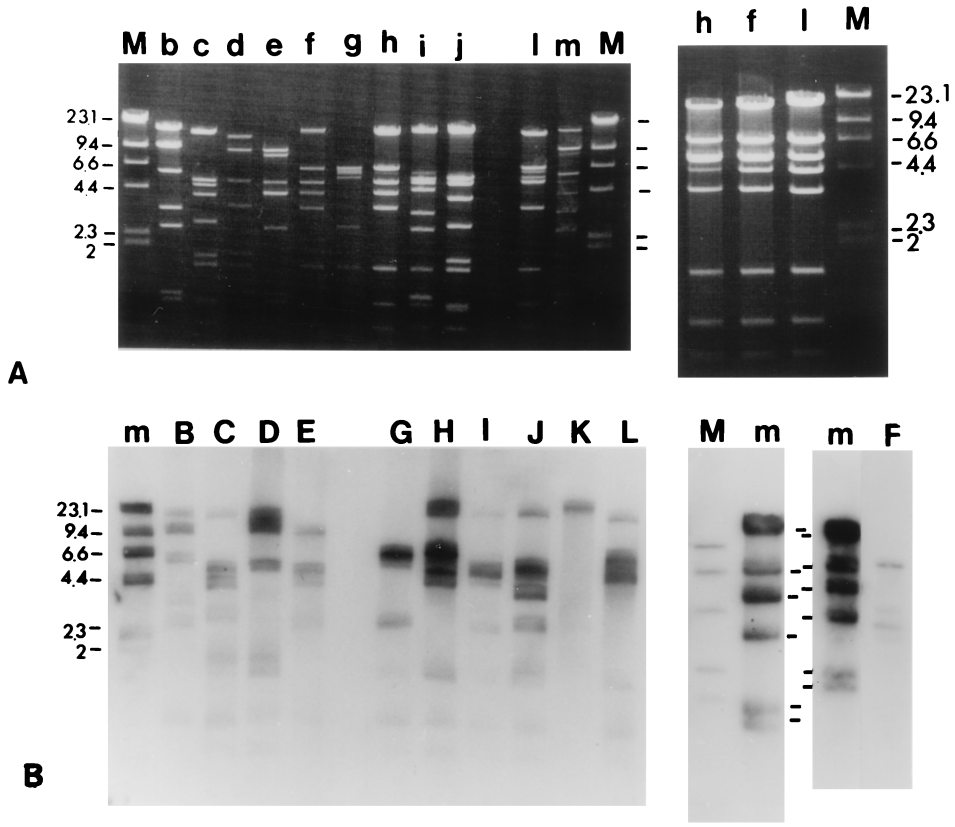


FIG. 1. (A) Agarose gel electrophoresis of *Pvu*II-digested DNAs of phage isolates of the indicated lytic groups. Lanes M, DNA molecular size markers. Molecular sizes are given in kilobases. (B) Southern blot hybridization of an agarose gel with *Pvu*II-digested DNAs of phage isolates attacking host cells of the indicated lytic groups against $^{32}$P-labeled DNA of phage ϕg.

lapping host ranges. The exceptions were phages ϕh and ϕl, which could be grown on three strains (H, L, and our Sfil indicator cells [4]). However, the efficiency of plating was about $10^3$ to $10^4$ times higher on the last propagating host than on the other two strains. As ϕh and ϕl showed similar restriction patterns (Fig. 1A, right half) and as strains H and L possessed distinct ribotype patterns, the observed differences might indicate the presence of a restriction-modification system.

**Molecular analysis of phages.** With the exception of phages ϕc and ϕj and phages ϕh and ϕl, all of the phages showed distinct restriction patterns when tested with the restriction enzymes *Pvu*II (Fig. 1) and *Eco*RI (data not shown). ϕf and ϕh could be distinguished with *Eco*RI but not with *Pvu*II. Independent phage isolates attributed to the same lytic group showed either identical (Fig. 2A) or highly related (Fig. 2B) restriction patterns. For example, four and two variant ϕh and ϕd isolates, respectively, were observed. Each isolate differed from the other in only one (ϕd.1 and ϕd.2) or, maximally, two (ϕh.1 to ϕh.4) fragments. The molecular basis for the variant restriction pattern is unknown. The variant ϕh isolates, for example, were grown on their factory starter H, thus excluding effects of different restriction-modification systems. Major deletion events, as previously observed in *S. thermophilus* ϕSfi21 (6), are also unlikely. A panel of primers was used to amplify DNA regions of these phages by PCR. All of the PCR products obtained for the ϕh variants indicated that they were identical in size (data not shown). In addition, ϕh isolates with different *Pvu*II restriction patterns did not differ when digested with *Eco*RI (data not shown).

All of the phages isolated during the survey belonged to one DNA homology group based on the observation that when ϕg (or, alternatively, ϕb, ϕc, or ϕi) DNA was used as a radiolabeled probe in Southern blot hybridization, many *Pvu*II restriction fragments of phages belonging to other lytic groups gave a hybridization signal (Fig. 1). All hybridization experiments were done under high-stringency conditions (3). A high degree of overall DNA homology has already been described for *S.*

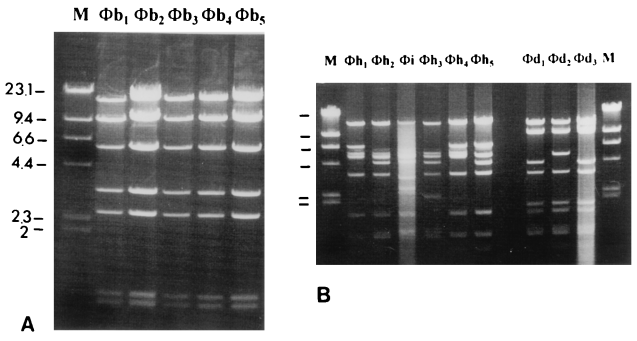


FIG. 2. Agarose gel electrophoresis of *Pvu*II-digested DNAs of independent phage isolates attacking lytic groups B (ϕb1 to ϕb5), H (ϕh1 to ϕh5), and D (ϕd1 to ϕd3) recovered during the 2-year survey period. A phage isolate attacking group I host cells was included for comparison. Lanes M, DNA molecular size markers. Molecular sizes are given in kilobases. Identical (A) and highly related (B) restriction patterns are shown.

TABLE 1. Cross-hybridization of DNAs from *S. thermophilus* phages of the ecological survey with radiolabeled individual *Pvu*II-*Xba*I restriction fragments of temperate ϕSFi21 as revealed by dot blot hybridization[a]

| Phage | Degree of cross-hybridization[b] with fragment: | | | | | |
|---|---|---|---|---|---|---|
| | PX2 | PX5 | PX4 | PX3 | PX6 | PX1 |
| ϕa | ++ | | ++ | + | + | ++ |
| ϕb | ++ | ++ | ++ | + | | ++ |
| ϕc | ++ | + | | | | ++ |
| ϕd | ++ | ++ | + | | | ++ |
| ϕe | ++ | ++ | ++ | + | + | ++ |
| ϕf | ++ | ++ | + | | | ++ |
| ϕg | ++ | ++ | + | | | |
| ϕh | ++ | + | + | | | ++ |
| ϕi | ++ | ++ | ++ | + | + | ++ |
| ϕj | ++ | + | + | + | | ++ |
| ϕk | ++ | + | + | | | |
| ϕl | ++ | ++ | ++ | + | + | ++ |
| ϕm | ++ | + | ++ | + | | ++ |

[a] The phages investigated are listed in the first column. The second to seventh columns give the hybridization with the indicated labeled *Pvu*II-*Xba*I restriction fragments of our prototype ϕSFi21 DNA; the fragments are ordered according to their positions on the restriction map of ϕSFi21 (3). Fragments PX7 and PX8 were not used due to their small size. A sequencing project has identified the following functions of the ϕSFi21 fragments: PX1, DNA replication module (10); PX6, antirepression (7); PX3, lysogeny module, lysis (8); PX4, tail (?) morphogenesis; PX5, head (?) morphogenesis; PX2, *cos* (cohesive end) site, DNA packaging (?).

[b] There was moderate (+) or strong (++), cross-hybridization with the indicated probes under stringent hybridization conditions on dot blots.

*thermophilus* phages from our collection (4). To obtain better resolution of their genetic relationship, we used individually labeled *Pvu*II-*Xba*I (PX) restriction fragments from ϕSFi21 in dot blot hybridization assays against the phages from the survey (3). All of the factory phages except ϕg and ϕk hybridized with fragment PX1, all hybridized with fragment PX2, and most hybridized with fragments PX4 and PX5, while only moderate levels of cross-hybridization were seen with fragments PX3 and PX6 (Table 1).

Previously, we described a conserved DNA module (5) localized in fragment PX1 from ϕSFi21. Sequence analysis revealed a DNA replication module in this region (10). Within this DNA module, we targeted a 564-nucleotide-long region for PCR and comparative sequencing. This region was localized in an open reading frame coding for a putative DNA primase (10).

Alignment of these sequences demonstrated a series of graded relatedness (Fig. 3). Maximal sequence diversity was 65 nucleotide differences (12%), but many phage isolates showed much fewer or no sequence differences over this region. Phages ϕh and ϕl gave identical sequences which differed at only one base position from the prototype ϕSFi21 DNA sequence. ϕa, ϕd, and ϕf differed at 15 to 17 positions from the prototype sequence, while they differed from each other only at two positions. Identical sequences were found for ϕc and ϕj, which differed at 56 positions from the prototype sequence. ϕe and ϕm differed from the reference DNA at 63 and 62 nucleotide positions, respectively, while they differed from each other at only 3 positions. PCR products from nine independent ϕb isolates, which were the predominant phages during the survey, yielded identical nucleotide sequences. DNAs from phages in our collection isolated over more than 30 years from different European countries (4) covered the same range of diversity as the phages recovered during 2 years from a single factory (data not shown).

**Intervention study.** Four days before the introduction of the new starter rotation system composed of defined *S. thermophilus* strains (see Materials and Methods), we investigated the phage population in the factory to establish the baseline level of phage contamination. Phages attacking starter strains A, B, C, E, G, H, I, L, and M were detected, with the highest titers being obtained for phages attacking starter strain G (maximal titer, $10^5$ PFU/ml; Fig. 4). The whey samples contained two to seven distinct types of phages with a minimal phage titer of $10^3$ PFU/ml. During the first 2 weeks, only starters which were unable to propagate the resident phage population were used. As a consequence, the resident phage contamination level fell: the maximal phage titer was $10^3$ PFU/ml, and 18 of 26 whey samples had no detectable phages. This is in stark contrast to the situation of the 2-year survey period during which a phage-free whey sample was never observed. However, only 5 days after the introduction of the new starter system, two "new" phage types were observed (ϕn and ϕp; Fig. 4). By new we mean that phages of this host range were not detected in whey samples taken 1 week, 1 year, and 2 years before the intervention. Seven days after the introduction of the new starter system, a third new phage type was detected (ϕo). The phage titer rose quickly: a titer of $10^7$ PFU of ϕp per ml was observed on day 10 (Fig. 4).

In the second phase of the intervention trial, eight further starter strains were added to the new starter system. This was done for technological reasons. Four of these strains were susceptible to attack by four distinct phage types (ϕb, ϕe, ϕg, and ϕk) of a phage population previously resident in the factory. This gave us the opportunity to test for recolonization of the factory by what remained of the previous phage population. During this second phase, we observed regularly and at high titers two of the new phage types, ϕo and ϕp, which apparently got established in the factory (Fig. 4). ϕn was observed for only 1 week. No other new phages were isolated during the 50-day follow-up. All four strains susceptible to the previous resident phage population once again experienced phage infections. Phages attacking starter G were sampled before the intervention (day −3) and at three time points (days 18, 31, and 59) after the reintroduction of starter G into the factory. The ϕg isolates taken before and after the intervention did not differ in their *Eco*RI, *Xba*I, and *Hin*dIII restriction patterns (Fig. 5A).

**Origin of the new phage types.** The appearance of the new phage types gave us the opportunity to trace the origin of these phages. The restriction profile of up to six independent isolates of each of the new phage types (ϕn, ϕo, and ϕp) was analyzed. Each type presented a unique restriction pattern (Fig. 5B). The restriction patterns of the new phages showed no relatedness with each other or those of the previous resident bacteriophage population. The PCR fragments of the putative DNA primase genes of the new phages were sequenced. They differed from each other and from ϕm at a maximum of two nucleotide positions (Fig. 3). We postulated that the raw milk delivered to the factory during the intervention trial is the most likely source of these new phage types, since we have detected small amounts of phages in several raw-milk samples. Phages attacking starter cells belonging to lytic groups A to M were found in raw milk on only two occasions (10 and 100 PFU/ml against starters B and M, respectively). However, the raw-milk ϕm isolates differed in restriction pattern (Fig. 5B) and sequence analysis (data not shown) from the resident ϕm. In contrast, the phages attacking strains N, O, and P, which were detected with titers between 10 and 130 PFU/ml in raw-milk samples, possessed restriction patterns identical to those of new factory phages ϕn, ϕo, and ϕp (Fig. 5B). Phages from our phage

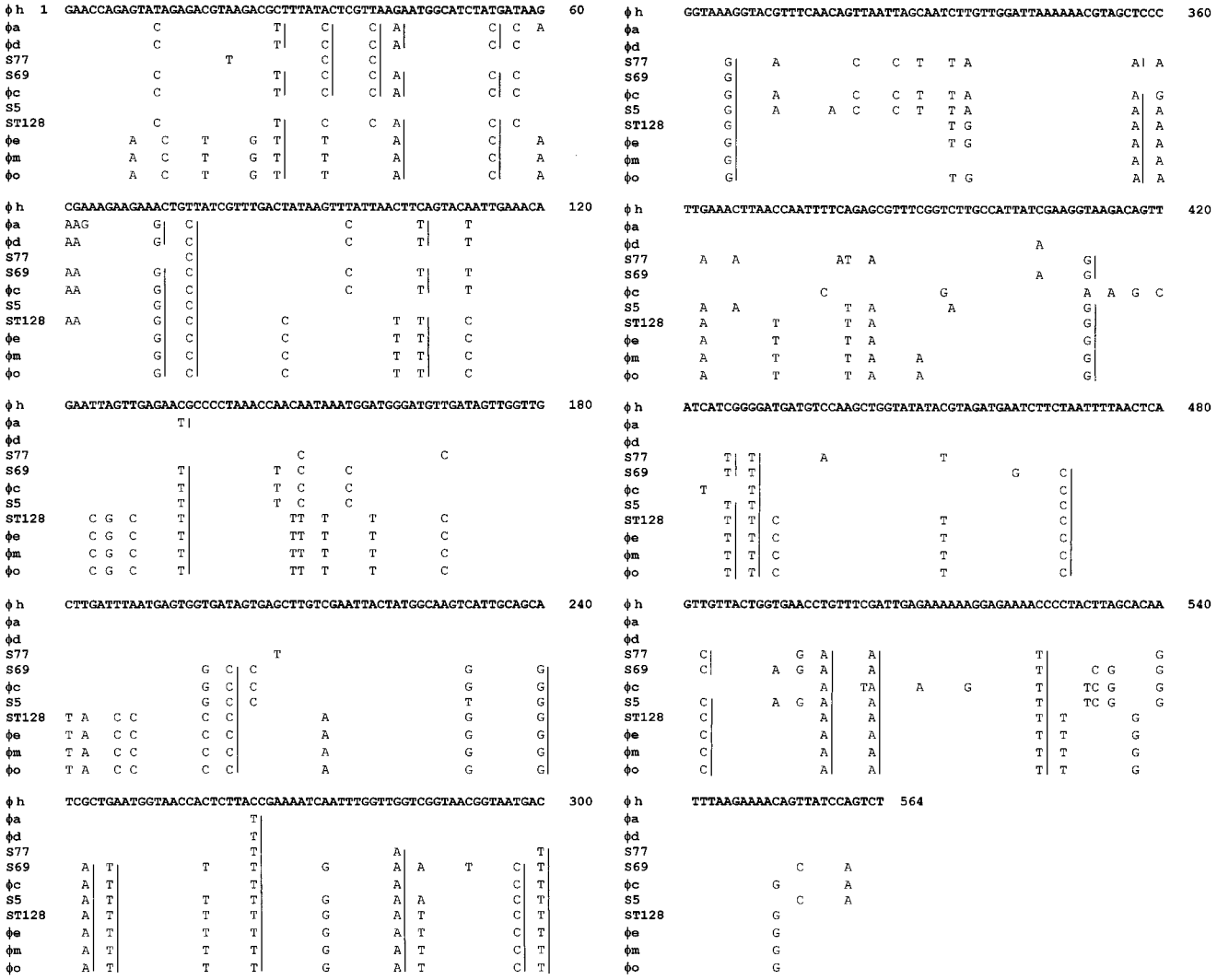

FIG. 3. Alignment of 564-nucleotide-long sequences from open reading frame 2 of the conserved *S. thermophilus* phage DNA module (6, 10) for phages ϕh (first line), ϕa, ϕd, ϕ*S77* (phages from our collection [in italics] are given with their code numbers [5]), ϕ*S69*, ϕc, ϕ*S5*, ϕ*ST128*, ϕe, and ϕm (lines 2 to 10). The phages were chosen to be representative of each branch of the tree obtained with the CLUSTAL W program when sequences from all of the factory phages and 20 representative phages from our collection were compared. Only variant nucleotides were noted. ϕh differs from the published prototype sequence (6) at only one position (underlined; the prototype has a T). Differences from the prototype sequence which were identical in six or more phages are marked by vertical lines.

collection, which were isolated from a different cheese factory in northern Italy, that attacked strains O and P showed a restriction pattern distinct from those of the raw-milk and factory phages (Fig. 5B). Interestingly, one raw-milk ϕm isolate, but not the resident factory ϕm isolate, possessed a restriction pattern that was very similar, although not identical, to that of the new factory ϕn isolate (Fig. 5B). All ϕn isolates also yielded plaques on host strain M (efficiency of plating, $10^{-3}$), while the different ϕm isolates showed an efficiency of plating of less than $10^{-5}$ on host strain N. *S. thermophilus* strains susceptible to ϕn were detected in raw milk, while we could not detect the host cells for ϕo and ϕp in raw milk during the intervention trial.

## DISCUSSION

In general, *S. thermophilus* phages isolated from cheese factories show much greater diversity than those from yogurt fermentation. An explanation for this may lie in the differences of starter strains and fermentation conditions. Since phage propagation is dependent on the presence of suitable host bacteria and yogurt factories use a small number of well-defined starter cultures, a greater variety of phage types is expected in cheese factories, using mainly undefined and probably complex cultures, than in yogurt factories. In addition, due to different raw-milk heat treatment regimens and differences in factory design and product processing (closed versus open fermentation vats), yogurt factories are much less susceptible to invasion by phages from environmental sources than are cheese factories. The most critical process in cheese making is whey separation, which inevitably leads to aerosol-borne phages in the factory. As the production line in some cheese factories is open, it allows the possibility of long-term maintenance of phages in the factory.

The aim of the present study was to settle the question of whether the diversity of phages obtained from a cheese factory reflects the natural diversity present in the environment or phage diversification in the factory. For this purpose, a two-

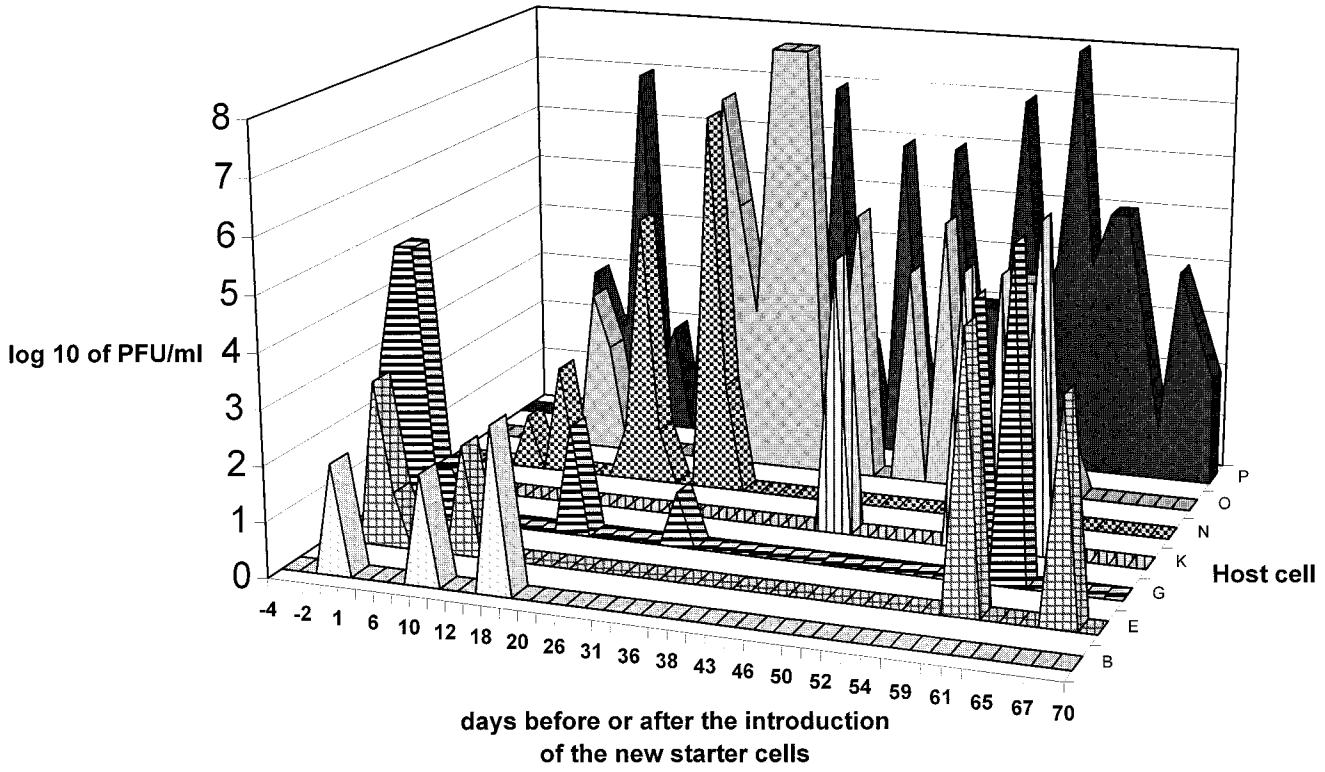


FIG. 4. Cheese whey phage titers (*y* axis; expressed as $\log_{10}$ PFU per milliliter) observed on the starter strains indicated on the *z* axis on a given day (*x* axis) before (negative numbers) or after the introduction of new starter strains.

stage ecological study was conducted. In the first stage, we monitored a cheese factory using an undefined starter system over a 2-year period. Based on host range analysis, 12 distinct phage types were observed. This large variety of phage types confirmed our initial observation of the diversity of phages detectable in cheese factories. According to Southern and dot blot hybridization studies, all of the factory phages were closely related. This observation does not, however, prove a single phage invasion event in the factory, since all of the *S. thermophilus* phages investigated until now and originating from different countries have shown extensive cross-hybridization (1, 3, 5, 12, 18, 20).

With two exceptions, phages defining different lytic groups showed clearly distinct restriction patterns while multiple isolates of phages attacking the same host, sampled over the 2-year observation period, showed identical or highly related restriction patterns. Four variants of φh were observed. Even if the differences were relatively minor, in terms of genetic evolution the observation might be significant. The molecular basis for the differences between phages showing highly related restriction patterns is unknown. Similar differences between closely related raw-milk phages were found. Variant phages have been previously observed for our prototype *S. thermophilus* phage φSfi21 during serial passages in the laboratory. In that case, the differences in the restriction patterns reflected site-specific deletions in three regions of the phage genome (7).

Sequence analysis in a conserved region of the genome from multiple isolates of the same phage type revealed no point mutations. In contrast, up to 12% nucleotide sequence diversity was observed between different phage types. This diversity was as great as that between the most different phages from our collection.

The observations of our survey do not support a model that postulates a single phage invasion event and subsequent diversification of the phage during its residence in the factory. On the one hand, such a rapid evolution of phages in a cheese factory requires a very high mutation rate in *S. thermophilus* phages, and on the other, a strong selection imposed by the starter system is required for new phage types to become detectable. It is not apparent how an undefined starter system like that used in our longitudinal survey could provide this selection.

In the second part of our factory study, intervention with defined starter cultures exerted strong selective pressure on

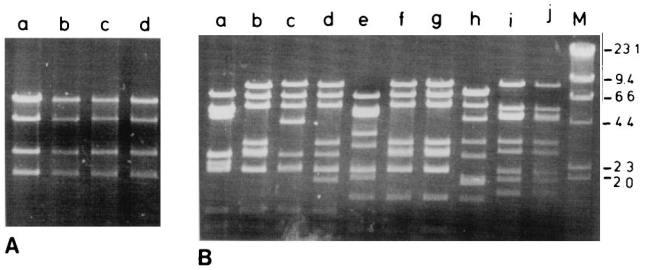


FIG. 5. Restriction analysis of DNAs from phages isolated during the intervention trial. (A) Agarose gel electrophoresis of *Xba*I-digested DNAs of independent φg isolates taken 3 days before (lane a) and 18, 31, and 59 days after (lanes b to d) the intervention. (B) Agarose gel electrophoresis of *Hin*dIII-digested DNAs of φm samples from cheese whey before the intervention (lane a) and from raw milk (lanes b to d); φn from our collection (lane e), raw milk (lane f), and cheese whey (lane g) after the intervention; and φp from our collection (lane h), raw milk (lane i), and cheese whey (lane j) after the intervention. Lane M, DNA molecular size markers. The molecular sizes of *Hin*dIII-digested λ DNA are given in kilobases.

the factory phage population: the new starter cultures could not propagate the resident phages. Therefore, any phages isolated on the new host cells must be either host range mutants of the resident phages or else new phages. Interestingly, the phage population changed quickly following the intervention. Within 5 to 7 days, three apparently new phages were isolated that attacked 3 of the 10 new starter strains. Similar data have been reported in ecological surveys of cheese factories using lactococcal starters (17). Phages ϕo and ϕp quickly became established in the factory. The restriction patterns of the new phages indicated that they were not derivatives of the previous resident phages. For all three new phages, raw milk was the most likely source, as phages with identical host ranges and restriction patterns were found in the raw milk delivered to the factory during the intervention trial. Phages in raw milk were apparently also the source for phage problems in cheese factories using lactococcal starters (13, 14, 17), demonstrating the general applicability of our results for cheese factories. The reports on lactococcal phages differ, however, in some important aspects from our data. In these reports, no proof of molecular identity between raw-milk and factory phages was provided. In addition, in two reports raw-milk lactococcal phages appeared to represent induced prophages from lysogenic strains (13, 14). Lysogenic *S. thermophilus* starters are relatively rare (5) and were not detected at all in our factory survey. In New Zealand cheese factories, the whey was sprayed on pastures where the dairy herds that provided the raw milk for the factory grazed (17). This procedure could contaminate the raw milk with factory phages. In our factory, the whey was concentrated in the factory and sent abroad for further processing, thus excluding recycling of the factory phage via raw milk.

Theoretically, the intervention should wipe our the resident phages from the factory unless they are able to mutate to new host ranges. The resident phage population was, however, not eliminated after 10 days of use of nonsusceptible starters. Reintroduction of susceptible strains led to the rapid reappearance of the resident phage, indicating that phages can be maintained in the factory for at least 3 weeks in the absence of their host strain in the starter system.

In summary, data from our surveys identified the environment, specifically, raw milk, as the source of phages which contaminated the factory. We have no evidence for genetic changes in the factory phages during the survey period. The absence of recombination within the factory phage population appears surprising in view of laboratory experiments which reported frequent recombinations between phages after double infections of starter cells (12). This may be due to the lack of overlapping host ranges, resulting in a kind of reproductive isolation which could be very important in the design of starter systems in the cheese industry. Apparently, all of the genetic diversity observed in *S. thermophilus* phages during our survey was already present in the environment outside of the factory. Therefore, detailed knowledge of the genetic diversity of *S. thermophilus* phages and their hosts within their natural environment is essential for the success of any phage control measures in cheese factories.

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