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Rapid Method To Characterize Lactococcal Bacteriophage Genomes†

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We present a rapid method to isolate and analyze bacteriophage DNA. Cells are infected and phage replication is allowed to proceed normally for 30 to 60 min. Prior to DNA packaging and cell bursts, the infected cells (1 ml) are harvested and lysed by using a combination of lysozyme and sodium dodecyl sulfate treatments. The total DNA recovered is enriched for phage genomes, and restriction fragments of the phage DNA can be readily visualized on agarose gels. This method was used to grossly compare the genomes of nine lactococcal phages isolated from different cheese plants at different times. The method was also used to visualize the inhibitory effects of pTR2030-induced abortive infection on the replication of phage nck202.31 in its homologous host, *Lactococcus lactis* NCK203.

Bacteriophages have long presented a major economic problem to the cheese industry. Phages are usually detected within a short time after the starter cultures are introduced into the cheese plant. Industrial phages are isolated by propagation on standard indicator bacterial strains, and it is not known whether the phages are related genetically. Standard methods for identification of phage strains rely on morphological examination by electron microscopy and the characterization of phage DNA from purified phage particles. By using these methods, a number of studies have been performed to investigate the relatedness of phage strains isolated against lactococci (2, 3, 8, 9, 13). These studies have provided a greater understanding of the relatedness of lactococcal phage strains. However, these methods are time-consuming and tedious and require expensive equipment. There is also a tremendous need for a rapid and facile method for routine identification of phages and its use to place phages into species and strain groups that may have been characterized previously. Such a method would be particularly useful for examining "novel" phages arising in industrial situations. In this way, it could easily be determined whether phages isolated against the same culture from different geographical locations, or at different times in the same location, are variants of a single phage.

A rapid method has been developed which takes advantage of the internal replication of the phage DNA within sensitive strains. A successful lytic infection requires the proliferation of the phage DNA from one to several hundred copies per infected cell. Sensitive cells are infected and the phage lytic cycle is allowed to proceed normally. At various time points after infection, the total DNA content of the cell is extracted and then analyzed by restriction endonuclease digestion. Phage DNA is easily distinguished from the normal chromosomal content because of its higher relative concentration and subsequent intensity on agarose gels. The ability to follow the replication of the phage DNA within the cell has also allowed the evaluation of the effect of an abortive infection plasmid, pTR2030, on phage DNA replication.

MATERIALS AND METHODS

Bacterial strains, phages, and culture conditions. Bacterial strains and their phages are listed in Table 1. Bacterial strains were propagated at 30°C in M17 broth (17), supplemented with glucose at 0.5% (GM17) when appropriate. All phages were isolated from dairy environments.

Rapid phage identification method. Sensitive cells were freshly grown to an optical density of 0.5 at 600 nm. $CaCl_2$ (final concentration, 10 mM) was added, followed by the addition of the test phage strain at a multiplicity of infection (MOI) of >1.0. Infected cells were incubated at 30°C. At various time points, 1 ml of cells was harvested in an

TABLE 1. Bacterial strains, plasmids, and phages

Strain	Relevant characteristics[a]	Source/reference
Bacteria		
L. lactis		
NCK203	Sensitive host	6
NCK204	NCK203(pTR2030) (Hsp^+)	6
NCK216	NCK203(pTRK70) (M^+/R^-)	6
L. cremoris		
M12	Sensitive host	16
Phages		
ϕm12r	M12 phage, SI	16
ϕot	M12 phage, SI	16
ϕda	M12 phage, U	16
ϕsg1	M12 phage, SI	16
ϕbr	M12 phage, SI	16
ϕ05	M12 phage, SI	16
ϕ31	NCK203 phage, SI	16
ϕ35	NCK203 phage, SI	11
ϕ36	NCK203 phage, SI	11
Plasmid		
pTRK193	pBluescript::3.0-kb *Eco*RI ϕ31 fragment	This study

[a] Hsp, Abortive phage resistance; R, restriction activity; M, modification activity; SI, small isometric headed phage; U, unknown phage morphology.

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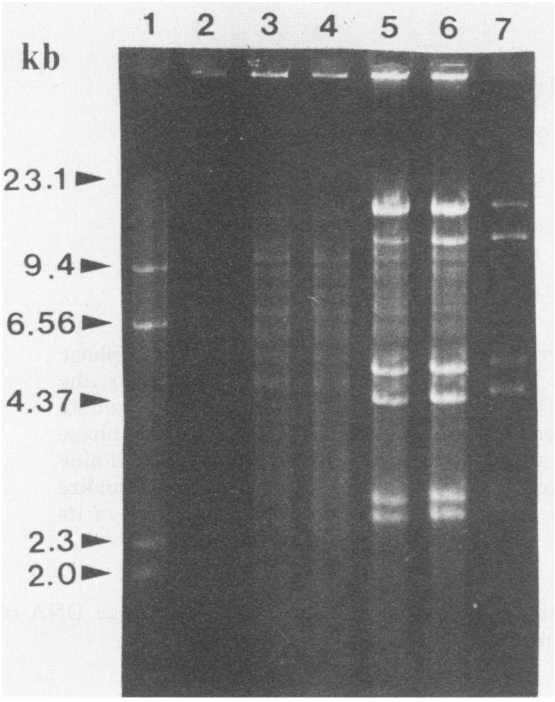

FIG. 1. *Eco*RI digestions of total DNA extracted from NCK203 before and after infection with ϕ31. Lane 1, Lambda *Hin*dIII size standards; lane 2, NCK203 total DNA prior to infection; lane 3, 5 min after infection; lane 4, 20 min after infection; lane 5, 40 min after infection; lane 6, 60 min after infection; lane 7, CsCl-purified ϕ31.

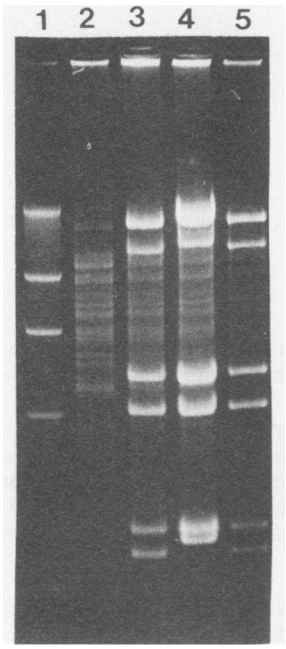

FIG. 2. *Eco*RI-digested total DNA preparations from NCK203 infected with ϕ31, ϕ35, and ϕ36. Lane 1, *Hin*dIII-digested lambda size standards (sizes as shown in Fig. 1); lane 2, uninfected NCK203 total DNA; lane 3, NCK203 infected with ϕ31; lane 4, NCK203 infected with ϕ35; lane 5, NCK203 infected with ϕ36.

Eppendorf centrifuge, and the pellet was frozen rapidly by immersion in ultracold (−70°C) ethanol. After thawing on ice, the pellet was resuspended in 400 μl of ice-cold lysis solution (6.7% sucrose, 50 mM Tris, 1 mM EDTA, pH 8.0). Lysozyme (20 μl of a 10-mg/ml stock solution) was added, and incubation on ice was continued for 20 min. Sodium dodecyl sulfate (SDS; 50 μl of a 10% stock) was added. Proteinase K (20 μl of a 20-mg/ml stock) was added and the lysate was incubated at 65°C for 20 min. The resulting clear suspension was extracted once with TE-saturated phenol (12), followed by a chloroform-isoamyl alcohol (24:1) extraction. One-tenth volume of 3 M sodium acetate and 2 volumes of cold 95% ethanol were added to precipitate the total DNA. The DNA pellet was finally resuspended in 100 μl of distilled H_2O; 5 μl was generally sufficient for restriction analysis.

Restriction endonuclease analysis. Total DNA was digested by using the instructions provided by the manufacturers of the endonucleases (Boehringer Mannheim). Digested DNA fragments were separated on 0.8% (wt/vol) agarose gels in Tris-acetate buffer as described by Maniatis et al. (12) and visualized after staining with ethidium bromide (1 μg/ml).

DNA-DNA hybridization. DNA fragments were transferred to GeneScreen-Plus nylon membranes (Du Pont) by the capillary method of Southern (15) and hybridized under stringent conditions (50% formamide, 25% 20× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% SDS, at 42°C) with ^{32}P-labeled probe DNA (prepared with the multiprime kit; Amersham Corp.). Washes were performed by using the omni-blot apparatus, using 10× SSC–0.01% SDS and 1× SSC–0.1% SDS in succession. Washed membranes were exposed to X-ray film (Kodak XR-Omat) at −70°C with intensifying screens.

Construction of pTRK139. The recombinant plasmid pTRK139 was constructed as follows. Phage nck202.31 (ϕ31) particles were purified in three-step CsCl discontinuous gradients, and the phage DNA was isolated from these purified particles as described previously (11). The extracted DNA was digested with *Eco*RI and shotgun cloned into similarly digested pBluescript (Stratagene) in a standard cloning protocol as described in Maniatis et al. (12). White colonies were chosen and lysed for plasmid content. A recombinant plasmid with a 3.0-kb insert, exactly corresponding to one of the ϕ31 *Eco*RI fragments, was chosen and named pTRK139. Southern hybridization experiments confirmed that the insert was ϕ31 derived.

RESULTS

Rapid identification of phage DNA. *Lactococcus lactis* NCK203 was infected with one of its homologous phages, nck202.31 (ϕ31), and the cells were harvested at various time points. The total DNA content was isolated and digested with the restriction endonuclease *Eco*RI. The resulting fragments were separated by agarose gel electrophoresis (Fig. 1). Lane 2 contains CsCl-purified ϕ31 DNA digested with *Eco*RI. It can clearly be seen that a similar pattern of DNA bands becomes evident in the total DNA preparations after the phage infection has proceeded for 40 min (lane 5). No such pattern is apparent after 5 or 20 min (lanes 3 and 4). In the 60-min sample the bands are also obvious (lane 6), though no increase in the relative amount has taken place. In similar experiments the amount of phage DNA actually decreased at the 60-min time point (data not shown). We conclude that at the earlier time points (5 and 20 min) no

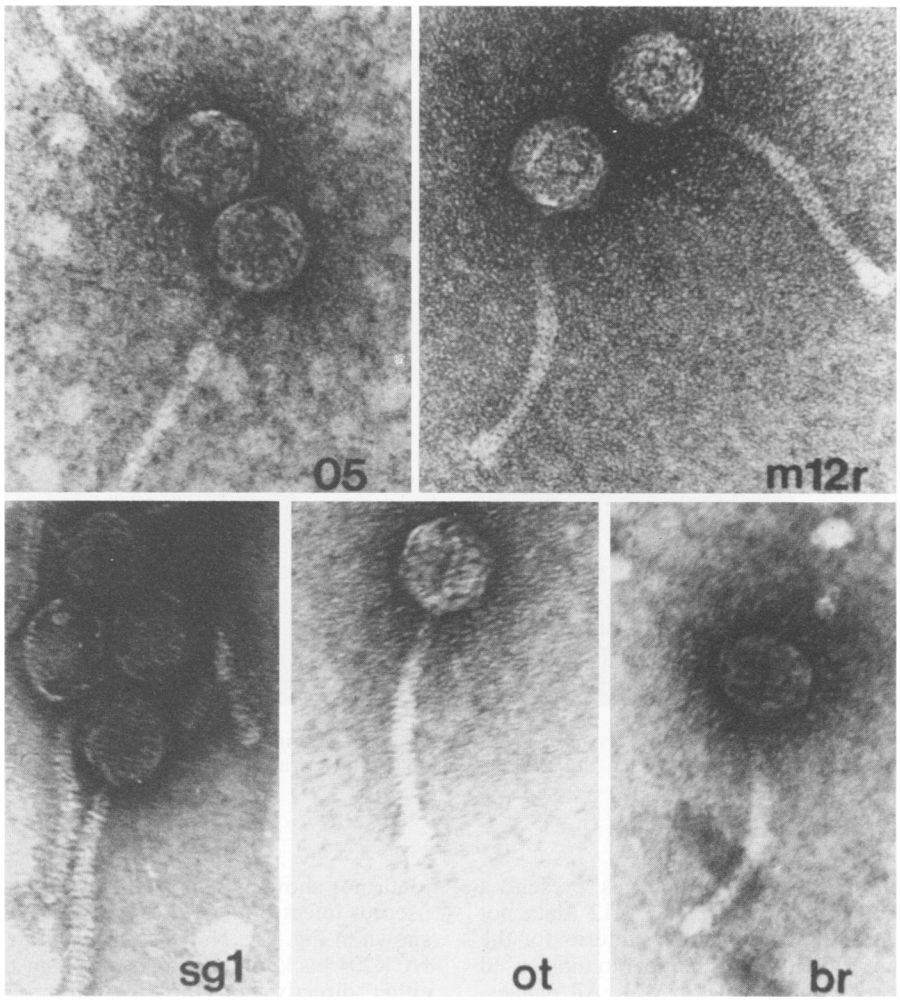

FIG. 3. Electron micrograph of five of the *L. lactis* subsp. *cremoris* M12 phages examined in this study.

DNA replication has taken place, while at 60 min a proportion of the DNA has been packaged and released from the cell. In all subsequent experiments, unless otherwise stated, cells were harvested at 40 min after phage infection. The optimal MOI was also determined. MOIs of >1.0 were found to give the most visible phage bands after DNA isolation. MOIs of <1.0 gave successively lower amounts of visible phage DNA. The number of cells at an optical density of 0.5, when infection is initiated (estimated by the number of colonies on direct plate counts multiplied by the average chain length as determined from light microscopy), was estimated at 4.0×10^8 ml^{-1}. In subsequent experiments phages were added so that the final titer exceeded this value (an MOI of 2.0 was preferred). The lack of dissociation upon heating of fragments known to contain the phage cohesive ends suggests that the phage DNA is in a concatemeric form (data not shown).

Comparison of φ31, φ35, and φ36. *L. lactis* NCK203 was infected with φ35 and φ36 in an attempt to determine the relatedness of these phages to each other and to φ31. All three phages were originally isolated against the NCK203 parental industrial strain and give similar plaque morphologies against NCK203 in standard plaque assays (data not shown). All are small isometric headed particles (11). Total DNAs were digested with *Eco*RI and analyzed (Fig. 2). Phage φ36 gives an identical pattern to that of φ31 and is obviously closely related, if not identical. However, φ35 differs in that the 2.0-kb band is missing and has been replaced by a 2.3-kb fragment. Close examination of the restriction map for φ31 (1) suggests an explanation for this difference. The 2.0-kb fragment is contiguous with a small fragment of 0.3 kb (this 0.3-kb fragment and two other smaller fragments are not visible in Fig. 2). It is likely that a mutation within the *Eco*RI recognition site separating these two fragments resulted in the increase in size of the 2.0-kb fragment in the φ35 genome. The DNAs were also digested with three other enzymes (*Pvu*II, *Eco*RV, and *Hin*dIII; data not shown). In each case the patterns obtained for φ31 and φ36 were identical, while those obtained with φ35 were different.

Relatedness of six *L. lactis* subsp. *cremoris* M12 phages. It was of interest to determine whether the rapid lysis method would work in a background other than NCK203 and also to determine the relatedness of six homologous phages isolated for *L. cremoris* M12 in different dairy environments (16). Electron microscopic examination of five of the six phages demonstrated that they were of the small isometric headed group (Fig. 3), but it was not possible to assess relatedness

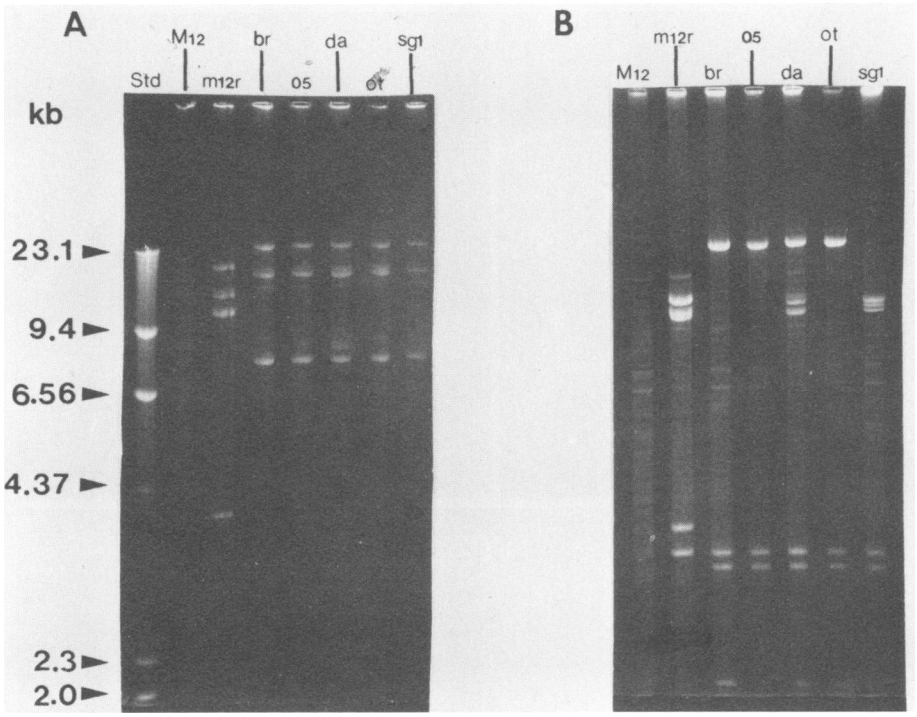

FIG. 4. Restriction endonuclease digestion of *L. cremoris* M12 total DNA and M12 infected with each of six different phages. (A) *Eco*RI fragments; (B) *Hin*dIII fragments. The lane labeled Std contains lambda-*Hin*dIII fragments. The 23-kb fragment is not visible in the photograph, but its position is shown.

by morphological differences. All six phages present a similar plaque morphology on *L. cremoris* M12 (data not shown). A comparison of DNA restriction patterns for the phages is shown in Fig. 4. Conditions identical to those used with the NCK203 system were used for M12. All phages, with the exception of ϕm12r, showed a high degree of similarity with four enzymes tested. The results with two enzymes are shown in Fig. 4. Phages ϕda and ϕsgl showed similar molecular weights for three digestions (*Eco*RI shown in Fig. 4A), but yielded submolar bands upon *Hin*dIII digestion (Fig. 4B). This apparent difference most likely reflects the presence of a *cos* site within the major *Hin*dIII fragment leading to the production of smaller nonstoichometric bands. Based on restriction analysis alone, phage ϕm12r was different from the other five. However, all six phages showed a high level of DNA homology when the ϕbr genome was used as a probe (data not shown). The data indicated that the six phages are closely related and are probably within the same DNA homology group (8) even though restriction analysis did detect differences for the phage strain ϕm12r.

Effect of pTR2030 on phage DNA replication. The conjugative plasmid pTR2030 encodes the abortive infection gene *hsp* in addition to a restriction and modification (R/M) system (6, 7). The manner in which the *hsp* gene product interferes with phage development remains unclear. Using the method described above, we determined the effect of the pTR2030 *hsp* gene on the replication of ϕ31 DNA in NCK204(pTR2030). To eliminate the effect of the R/M system, ϕ31 was first propagated on a host, *L. lactis* NCK216 (6), encoding modifying activity, but no restriction or Hsp^+ activity. In standard plaque assays, it was confirmed that the R/M system no longer affected ϕ31.NCK216 (data not shown). The modified phage, ϕ31.NCK216, was used to infect both NCK203 and NCK204. The results are shown in Fig. 5A. No DNA replication was observed in the NCK204 background, suggesting that the *hsp* gene product either directly or indirectly interferes with phage DNA replication. A second possibility could be that either residual restriction activity or the *hsp* product itself resulted in the destruction of the phage DNA before replication could occur. To test this possibility, the gel was probed with pTRK139, a plasmid containing a cloned fragment of phage ϕ31 (Fig. 5B). Homology to the 3.0-kb *Eco*RI fragment was detected at similar levels in both NCK203 and NCK204 after 5 min. This confirms DNA injection and its subsequent stability and rules out restriction as the reason for the lack of DNA replication in NCK204. The homology to the 3.0-kb ϕ31 fragment persists in NCK204 throughout the 60 min of this study, confirming that DNA degradation does not occur as a result of the action of the *hsp* gene product. In NCK203, the DNA replication can be clearly seen by the higher intensity of the homology to the ϕ31 3.0-kb band at later time points. In all lanes, except when obscured by the signal from the ϕ31 3.0-kb band, a faint signal was observed between pTRK139 and a chromosomal band of 3.2 kb.

DISCUSSION

The development of a rapid and facile method to determine the restriction profile of lactococcal phages should facilitate the study of the relatedness of these ubiquitous particles. This procedure allows a rapid comparison of isolated phages to determine their uniqueness relative to phages already available. Recurrent appearance of the same, or similar, phage against a culture, as found in this study in

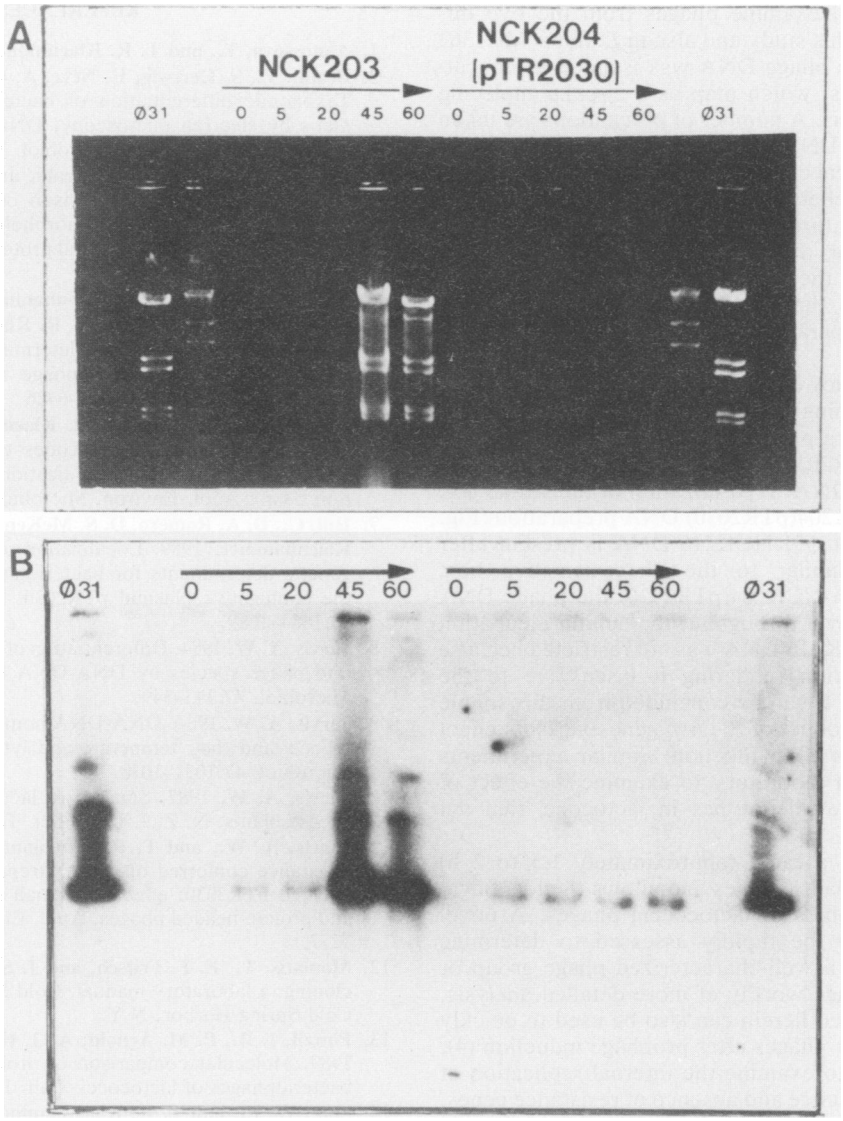

FIG. 5. (A) *Eco*RI digestion of NCK203 and NCK204 total DNA prior to infection with ϕ31.NCK216 (0) and at the indicated intervals (5, 20, 45, 60 min) following infection. Lanes labeled ϕ31 contain *Eco*RI-digested, CsCl-purified DNA. The unmarked lanes contain lambda-*Hin*dIII size standards. (B) Autoradiogram of the gel in panel A after hybridization with ^{32}P-labeled pTRK139, a plasmid containing a 3.0-kb ϕ31 *Eco*RI fragment.

the case of *L. cremoris* M12, may suggest a common source of contamination in a particular cheese plant or incriminate the culture itself as a potential source of the phage. The six *L. cremoris* M12 phages examined in this study were isolated against the same parental culture which was being used in cheese plants in the United States. Three of the phages, ϕ05, ϕda, and ϕsg1, were isolated from the same location, but at different times. All three phages give similar, though not identical, restriction patterns with the four enzymes used. This suggests that the phages are continually undergoing slight changes at a molecular level, but are derived from a similar progenitor. Phage ϕbr was isolated in a separate cheese plant, but again shows considerable similarity to the other phages. The same is true of ϕot, which is also broadly similar to the other phages and identical to ϕbr. These results are in general agreement with those of Jarvis (10), who also found a high degree of relatedness between different phages isolated in different cheese plants which were using the same starter strains. The only phage which is obviously less related to the others is ϕm12r, which was originally isolated from a phage composite. Few of the bands obtained from ϕm12r restriction digests comigrated with those generated from the other five phages. However, hybridization experiments confirmed a high degree of homology among all six phages, suggesting a common progenitor for all M12 phages.

To facilitate the comparison of phages from different laboratories, we suggest the use of at least one common enzyme in these kinds of studies to allow rapid comparison of results. The use of *Eco*RI is suggested, because it recognizes a relatively AT-rich sequence, is salt tolerant, and is widely available.

The method does not require a large degree of "fine tuning" from strain to strain, since we were able to use

identical conditions to examine phages from the two different strains used in this study and also in *L. lactis* MG1363 (data not shown). The phage DNA was isolated as concatameric phage genomes, which map as a circular molecule after restriction analysis. A number of precautions are taken to ensure that the final DNA preparation obtained accurately reflects the physiological condition within the cell at the time of harvesting. Cell pellets are rapidly frozen and held at nonreplicative temperatures for the remainder of the procedure (either below or at 0°C, or at 65°C). Noting the undegraded quality of the final DNA prepared under these conditions, it appears that endogenous nucleases are not effective at these temperatures, and DNA replication does not continue.

This method also allowed us to follow phage DNA replication in cells containing the abortive infection gene *hsp*. The results confirmed a previous report (14) that the phage resistance plasmid pTR2030 does not affect phage adsorption or injection of phage DNA. Hybridization of the 3.0-kb φ31 band in the 5-min NCK204(pTR2030) DNA preparation (Fig. 5B) clearly shows that φ31.NCK216 DNA is present after infection at levels similar to the phage-sensitive host NCK203. However, in NCK204(pTR2030) the phage DNA remains at its initial level throughout the 60-min incubation period. Phage φ31.NCK216 DNA was not restricted because it carried a modification rendering it insensitive to the pTR2030 R/M system. It can be concluded from this simple experiment that the product of the *hsp* gene exerts its effect during the early stages after infection. Similar experiments have been used in our laboratory to examine the effect of other phage resistance phenotypes in lactococci that act postinfection (5).

We have presented a rapid (approximately 1.5 to 2 h) method which uses small volumes and allows the identification of restriction profiles of lactococcal phages. A novel phage strain can now be rapidly assessed to determine whether it belongs to a well-characterized phage group or represents "new" phage worthy of more detailed analysis. The technique described herein can also be used to quickly characterize temperate phages after prophage induction (4). An alternative use is to examine the internal replication of phage DNA in the presence and absence of resistance genes. This allows a more accurate estimation of the time and mode of action of the resistance mechanism under study. The method is not strain specific and can be easily used to study phages homologous for different backgrounds.

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