Lysogenic Strains of Group N Lactic Streptococci

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Received for publication 6 August 1973

A temperate bacteriophage, designated r,t, was inducible from the group N lactic streptococcus, Streptococcus cremoris R1, by ultraviolet irradiation or mitomycin C treatment. Induced lysates produced plaques on lawns of three closely related S. cremoris strains, AM1, SK11, and US1. Strain SK11 was readily lysogenized. S. cremoris AM1 was the most reliable indicator strain, although the age of the culture used for seeding plates was critical. Zones of lysis but no plaque formation were observed on lawns of nine additional S. cremoris strains. Phage r,t could not be detected in filtrates of stationary-phase R1 cultures and was near the limits of detection in logarithmically growing cultures. Phage levels were still very low (1 plaque-forming unit on AM1 per 10 induced cells) in induced lysates of R1 cultures. These low levels of detectable phage may be attributable to an inadequate indicator, lysogenization of the indicator, adsorption of induced phage to cellular debris, concurrent induction of other undetectable phages, or the production of high proportions of defective phages. Electron micrographs of induced R1 lysates revealed a high incidence of incomplete phage particles, fragments, and ghosts.

Lysogeny is known to be widespread amongst streptococci of groups A and C (17). Some lysogenic strains have also been found in groups G (3) and H (11). Very recently lysogeny was demonstrated in strains of group N streptococci designated as Streptococcus lactis (7, 9) and S. diacetilactis (7), although lysogeny was not found in S. cremoris (7). Lysogeny in the S. cremoris strains of group N lactic streptococci, used as starters in cheesemaking, has been suspected for some years (4, 5, 12, 14), but in no case has the presumed lysogeny been rigorously demonstrated. Keogh and Shimmin (6) induced lysis of S. cremoris C 11-56 by ultraviolet (UV) irradiation but were unable to show the presence of a typical temperate phage. Lysates from strain C 11-56 produced clear zones on 9 of 12 strains of S. cremoris when spotted on appropriately seeded plates. Striking features of the lytic spectrum were that strain C 11-56 was itself sensitive to the lysate and that plaque formation was not observed. Electron micrographs showed that the lysate contained phage-like particles, mostly empty heads. The antibacterial activity of lysates was therefore attributed to the presence of an induced defective bacteriophage, or a lethal component of it.

This study (carried out in partial fulfillment of the requirements of the Ph.D. degree in Food Technology, Massey Univ. New Zealand) reports the induction of lysis in cultures of S. cremoris R1 by UV irradiation or by mitomycin C (MC) treatment. These lysates produced zones of lysis on lawns of several S. cremoris strains, and plaque formation occurred on S. cremoris strains AM1, SK11, and US1. Bacteriophage particles were seen in electron micrographs of these lysates, and some characteristics of the inducible phage were investigated using S. cremoris AM1 as the indicator strain.

MATERIALS AND METHODS

Streptococcal strains. All of the cultures used in this study were cheese starter strains of S. lactis and S. cremoris from the collection of the New Zealand Dairy Research Institute.

Bacteriophages. Temperate bacteriophage, designated r,t, was isolated from UV-induced lysates of S. cremoris R1. The virulent bacteriophages, r,v (NZDRI 652), am1 (NZDRI 601), and sk11 (NZDRI 690), were drawn from the New Zealand Dairy Research Institute collection.

Media and growth of cultures. M16 broth and M16 agar were prepared as previously described (8). Streptococci were grown routinely at 22 or 30 °C, without shaking, in M16 broth from a 2% inoculum of an overnight (22 C, 16 h) broth culture.

Optical density. Optical density (OD) of cultures was measured in a Bausch and Lomb Spectronic 20 colorimeter. An OD value of 0.2 at 580 nm represented approximately 10^8 colony-forming units (CFU)/ml.

Colony counts. Samples were first diluted with
chilled 0.14 M NaCl to a final volume of 100 ml and blended at 13,000 rpm for 1 min in an AtoMix blender (Measuring and Scientific Equipment Limited, Crawley, England) to an average concentration of 2.2 to 2.6 coci. Appropriate dilutions were plated on M16 agar by soft agar overlay. Plates were incubated at 30 C for 16 to 24 h.

Assay of phage. Phage was assayed by the soft agar layer method (1) using M16 agar supplemented with calcium borogluconate (Veterinary grade, May and Baker Ltd., Dagenham, England) to a final concentration of 0.005 M (8). Plates were seeded with 0.1 ml of the required overnight (22 C, 16 h) culture. When S. cremoris AM1 was used as an indicator for phage r,t, plates were seeded with 0.1 ml of a 30 C, 24 h culture. The lytic spectrum of undiluted, diluted, and concentrated phage lysates was determined by spotting 0.1-ml quantities of membrane-filtered preparations (HA membrane, 0.45-μm pore size, Millipore Corp., Bedford, Mass.) on plates previously seeded with the selected strains by soft agar overlay. Plates were incubated at 30 C for 16 h.

Induction of lysogens: MC treatment. Selected cultures were grown in M16 broth at 30 C to an OD, of 0.1 when MC (Sigma Chemical Co., St. Louis, Mo.) was added, normally to a final concentration of 1 μg/ml. Incubation was continued at 30 C, and the OD, was followed until completion of lysis.

UV irradiation. Mid-logarithmic-phase cultures in M16 broth at 30 C were harvested by centrifugation (10,000 x g for 10 min), resuspended in 100 ml of chilled phosphate-buffered saline (pH 6.5) and blended in an AtoMix blender as described above. After blending, cells were again centrifuged and resuspended in sufficient buffered saline to give an OD, of 1.0 (5 x 10⁴ CFU/ml). Quantities of 1 ml were UV irradiated in petri dishes (5.5 cm diameter) 29 cm from a Hanovia bactericidal UV lamp. UV irradiation dosages were operationally defined from their lethal effects on a typical group N lactic streptococcus, S. lactis ML10 (NCDO 763; NZRCC 20030) which has a mean CFU of 2.64 coci (15). UV exposures of 5, 10, 15, 20, and 30 s reduced survival of the noninducible strain ML10 at 30, 12, 5, 1.5, and 0.2%, respectively. After UV irradiation, suspected lysogens were diluted 10-fold into M16 broth, incubated at 30 C, and examined at intervals for lysis.

Propagation of phage. Virulent phages were propagated on their homologous hosts in M16 broth, containing 0.005 M calcium borogluconate, by adding phage in a 1:100 ratio (plaque-forming units [PFU] to CFU) to logarithmic cultures (5 x 10⁴ CFU/ml) which were incubated at 25 or 30 C until lysis occurred. Lysates were centrifuged (10,000 x g for 10 min) and filtered through an HA membrane.

MC- or UV-induced lysates of S. cremoris R, were plated on S. cremoris AM1 to obtain phage r,t. Single plaques were picked into 1-ml quantities of M16 broth from which 0.1 ml was inoculated into early logarithmic-phase cultures (about 10⁶ CFU/ml) of S. cremoris AM1. Infected cultures were incubated at 25 C for 16 h when the titers on strain AM1, were 10⁴ to 10⁵ PFU/ml. Attempts to obtain high-titer preparations of phage r,t by conventional methods from these stocks were unsuccessful.

Concentration of phage r,t. A 2,000-ml logarithmic culture (OD, of 0.1) of strain R in M16 broth at 30 C was induced with MC (0.5 μg/ml) at 5 C, 16 h. After lysis was complete, the induced phage was concentrated by the dextran sulfate-polyethylene glycol two-phase separation system as described for coliphage T2 by Albertsson (2). The crude phage concentrate was further purified and concentrated by two cycles of differential centrifugation. Approximately 1 ml of temperate phage r,t at greater than 10⁴ PFU/ml on strain AM1, was obtained per liter of R, lysate.

Electron microscopy. High-titer bacteriophage preparations were diluted 10-fold into 0.1% bovine serum albumin solution and negatively stained with an equal volume of 2% neutralized phosphotungstic acid. Aerosols of stained preparations were sprayed onto carbon film grids and examined using a Phillips EM200 electron microscope.

RESULTS

Search for lysogens. Initially, filtrates from M16 broth cultures of 29 strains of lactic streptococci (22 S. cremoris and 7 S. lactis) were spotted on lawns of these 29 strains. No obvious signs of either phage reactions or of growth inhibition were observed. However, when MC was added to logarithmic (OD, of 0.1) M16 broth cultures at 30 C, at final concentrations of 0.5 and 1.0 μg/ml, overt lysis resulting in complete clearing of the cultures was observed in 4 of 22 S. cremoris and 2 of 7 S. lactis strains tested. In preliminary lytic spectrum determinations, the lysate from S. cremoris R, showed plaque formation on some strains. S. cremoris R, was, therefore, selected for further study.

MC induction of lysis in S. cremoris R,. The effects of MC additions to cultures of S. cremoris R, are shown in Fig. 1. Neither time of addition nor final concentration of MC were particularly critical factors in inducing lysis of cultures. Cultures incubated at 30 C were inducible by a range of MC concentrations from 0.1 to 4.0 μg/ml, provided that cultures were growing logarithmically and contained less than 10⁵ to 2 x 10⁴ CFU/ml. Induction of lysis by MC was most consistent when MC was added at a final concentration of 0.5 to 1.0 μg/ml to logarithmically growing cultures which had reached an OD, of 0.1 (~5 x 10⁷ CFU/ml after blending). Under these conditions there was little effect on growth for some 30 min; then there was a gradual slowing of increase in OD over the next 60 to 90 min, followed by marked and rapid lysis of the culture. Either higher or lower MC concentrations consistently gave less complete lysis of cultures. No visible lysis occurred if MC concentrations were lower than 0.05 or greater than 5.0 μg/ml.

UV induction of lysis in S. cremoris R,. Irradiation for at least 5 s was required to give...
shown either lytic zones or plaques in the spot tests. Plaque formation occurred on only three strains, AM1, an AM1 derivative SK1, and the closely related US1. The zones of lysis observed on the remaining nine strains were not caused by sensitivity of some strains to MC present in the induced lysates, because lysates obtained by UV treatment showed the same combination of plaquing and nonplaquing reactions in the lytic spectrum. The lytic spectrum of a concentrated MC-induced R1 lysate was also tested. The concentration procedures raised PFU on strain AM1 from \( \sim 10^3 \) ml to \( \sim 10^4 \) ml, but no changes were found in the range of strains showing zones of lysis, and there was no increase in the number of strains exhibiting plaque formation.

The nine strains showing a reaction to the lysates, but never exhibiting plaque formation, may have become lysogenized by phage in the lysates. Colonies isolated from lytic zones and turbid areas produced by R1 lysates on all nine strains were tested as suspected lysogens, but the results were either negative or inconclusive.

The phage present in induced R1 lysates was isolated from plaques produced on strain AM1 and propagated on this strain. From Table 1 it can be seen that only two of the nine strains which had shown a positive but nonplaquing

| Strain of S. cremoris | Lytic spectrum |
|-----------------------|----------------|
|                       | R1 culture filtrate | MC-induced R1 lysate | UV-induced R1 lysate | \( r_{AD} \)-AM1 | \( r_{UV} \)-R1 |
| R1                    | -                | -                 | -                 | -              | -          |
| AM1                   | -                | \( 3 \times 10^4 \) | \( 3 \times 10^4 \) | 2.8 \( 10^4 \) | \( 4 \times 10^4 \) |
| AM2                   | -                | -                 | -                 | -              | -          |
| BR1                   | +                | +                 | +                 | -              | -          |
| C19                   | ++               | ++                | ++                | -              | -          |
| HP                    | ++               | ++                | ++                | -              | -          |
| KH                    | +                | +                 | +                 | -              | -          |
| ML1                   | +                | +                 | +                 | -              | -          |
| F1                    | +                | +                 | +                 | -              | -          |
| F2                    | +                | +                 | +                 | -              | -          |
| R4                    | +                | +                 | +                 | -              | -          |
| US1                   | \( 2.3 \times 10^4 \) | \( 2 \times 10^4 \) | \( 2.2 \times 10^4 \) | \( 8 \times 10^4 \) | -          |
| US2                   | \( 1.1 \times 10^6 \) | \( 1.8 \times 10^6 \) | \( 1.1 \times 10^6 \) | \( 3.8 \times 10^6 \) | -          |

* Symbols: +, lysis; ++, pronounced lysis; ±, weak or inconsistent lysis; -, no lysis.
* No lysis was observed on 9 S. lactis and a further 11 S. cremoris strains tested.
* Phage preparation tested.
* Temperate phage from S. cremoris R1, propagated on strain AM1.
* Virulent phage of S. cremoris R1, propagated on strain R1.
* Plaque-forming units per milliliter in preparations which showed plaquing reaction in spot tests.
reaction to $R_t$, lysates were retained in the lytic spectrum after the phage had been propagated on $AM_1$. Plaque formation and relative efficiency of plating (EOP) on strains $AM_1$, $SK_{11}$, and $US_2$ were unchanged.

**Indicator strain.** *S. cremoris* $AM_1$, gave the highest plate counts for the phage present in induced $R_t$ lysates. Strain $AM_1$ was used, therefore, as the indicator for this phage which was designated $r_t$. Initially, the plaque counts of phage $r_t$ on $AM_1$ showed considerable variation. It soon became apparent that the age of the culture used for seeding plates had a marked effect on plating efficiency. Highest EOP on $AM_1$ was found when plates were seeded from cultures grown well into the stationary phase by incubation at 30 C for 24 h. Plaque counts were always 10 to 100 times less if cultures grown at 22 C or still in logarithmic growth were used as host cells for plating $r_t$ phage. Indeed, no plaque formation occurred at any dilution if plates were seeded with early logarithmic $AM_1$ cultures. The age of seed culture also affected EOP of $r_t$ phage on strains $SK_{11}$ and $US_2$, but the effects were not nearly as pronounced as with $AM_1$. These observations suggested that the indicator for $r_t$ phage was itself becoming lysogenized, but specific attempts to demonstrate lysogenization of $AM_1$ by phage $r_t$ were inconclusive.

**Lysogenization of strain $SK_{11}$.** Strain $SK_{11}$, which exhibits better growth characteristics on synthetic media than its parent strain $AM_1$, was tested for lysogenization by phage $r_t$ because EOP on this strain remained lowest regardless of the age of seed culture. Colonies of $SK_{11}$ resistant to lysis by $r_t$ phage were isolated from the turbid areas formed when the phage was spotted on plates seeded with $SK_{11}$. After subcloning three times, the $r_t$ phage-resistant $SK_{11}$ isolates and the appropriate control cultures were treated with MC. The induction of lysis was less obvious in these suspected lysogens than in strain $R_t$, and the level of spontaneously induced phage was higher. Nevertheless, the phage titer in MC-induced cultures increased to greater than $10^9$ PFU/ml on $AM_1$. The increase observed in the untreated resistant isolates was only $10^7$ PFU/ml. Cultures of $SK_{11}$ colonies, sensitive to $r_t$ phage, that were isolated from the same turbid zone as the lysogenized colonies, as well as control stock $SK_{11}$ cultures, sporadically and inconsistently showed lysis when treated with MC. The $r_t$ phage was never isolated from these lysates, and no indicator strain was found. Immunity to the lysogenizing phage was the only difference in phage susceptibility that was observed between strain $SK_{11}$ and the $r_t$ phage-lysogenized isolates. Sensitivity to two serologically unrelated virulent phages, $am_1$ and $sk_{11}$, was unchanged.

**Induction of phage $r_t$.** Once *S. cremoris* $AM_1$ was established as the indicator strain for phage $r_t$, it was possible to reinvestigate the induction of strain $R_t$ and assay for the presence of this temperate phage in induced and noninduced cultures. It can be seen (Fig. 2) that there is a low but consistent rate of spontaneous induction during logarithmic growth of strain $R_t$ in M16 broth. Detectable phage $r_t$ was at a PFU to CFU ratio of $10^{-8}$ until mid-logarithmic growth. This ratio increased slightly as the culture entered late logarithmic growth, and then fell suddenly with the onset of stationary phase. Since the levels of $r_t$ phage detected in noninduced $R_t$ cultures never exceeded $5 \times 10^4$ PFU/ml at any time, and were much lower in stationary-phase cultures, it is not surprising that the lytic spectrum tests on culture filtrates

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**Fig. 2.** The presence of phage $r_t$ in an uninduced culture of *S. cremoris* $R_t$, M16 broth (500 ml) was inoculated (2%) from an overnight culture of strain $R_t$, and incubated at 30 C. Samples (10 ml) were withdrawn at 30-min intervals and colony-forming units per milliliter (curve A) were determined. The balance of the sample was chilled and centrifuged (10,000 x g for 10 min). Supernatants were assayed for phage $r_t$ using *S. cremoris* $AM_1$, as the indicator strain (curve B).
gave negative results (Table 1). The increase in detectable r,t phage in an MC-induced R, culture is shown in Fig. 3. This increase, some 10⁴ PFU/ml over that found in untreated control cultures, coincided with the slowing of OD increase and onset of lysis. The induced culture reached a maximum OD₅₈₀ of 1.7 × 10⁸ CFU/ml (from 6 × 10⁷ CFU/ml at MC addition) before lysis of 90% of the cells. The phage titer in this lysate of 5 × 10⁸ PFU/ml on the indicator strain AM₁, represented a burst of less than 1 detectable phage per 10 induced cells.

**Relationship between virulent and temperate phages of S. cremoris R₁.** The host range of a virulent phage of strain R₁, r,v propagated on R₁, is shown in Table 1. A low incidence of plaque formation on strain R₁ together with the expected virulence on strain R₂ were the only differences in the range of hosts on which the temperate and virulent phages could produce plaques. In routine propagation of phage r,v on R₁, lysis normally occurs when cultures are at late logarithmic growth, the time when spontaneously induced temperate phage is highest (Fig. 2). Plaque formation on AM₁, SK₁₁, and US₁ would, therefore, appear to represent the extent of temperate phage contamination of the virulent phage preparation.

The similarity in host range between the virulent phage (originally isolated from cheese whey) and the induced phage suggested that the former might be a virulent mutant of the temperate phage. An examination of electron micrographs of the two phages showed that they were indeed very similar in appearance and dimensions (Fig. 4A and B). The most striking contrast between the virulent and temperate r, phages was the high amounts of incomplete phage particles, fragments, and ghosts that were seen in temperate phage preparations (Fig. 4C). Extensive searching of grids was necessary in order to find any fragments or ghosts in virulent phage preparations.

**DISCUSSION**

Lysogeny among strains of group N lactic streptococci has been suspected for some years (4, 5, 12, 14), and it is surprising, in view of the increasing commercial significance of *S. cremoris* strains, that this presumed lysogeny has not been confirmed much earlier. Certainly, the simple procedures of looking for phages in culture filtrates and cross-streaking of suspected lysogens have, in the main, been unsuccessful. Where reactions between strains have been observed (14) the possibilities of inhibition of growth by culture products, such as the antibiotic nisin, have not been eliminated. Strains of group N streptococci inducible by the two most frequently used inducing agents, UV irradiation and MC treatment, appear to be relatively widespread. Use of either of these agents caused unambiguous induction of lysis in 4 of 22 *S. cremoris* strains and 2 of 7 *S. lactis* strains that were investigated in this study. Kozak and his colleagues (7) found that 4 of 46 *S. lactis* and 2 of 24 *S. diacetilactis* strains were UV inducible. Induction by UV irradiation of *S. cremoris* strains has also been found by Keogh and Shimmin (6) and by Reiter (13). It may well

![Fig. 3. Phage r,t in an MC-induced culture of S. cremoris R₁. An M16 broth culture of strain R₁, prepared as in Fig. 2, was incubated at 30°C. MC (1 μg/ml) was added when the culture reached an OD₅₈₀ of 0.1 (arrow). Samples were withdrawn at intervals for OD readings (curve A). The balance of each sample was centrifuged and supernatants were assayed for phage r,t as in Fig. 2 (curve B). The OD profile (curve C) and r,t phage levels (curve D) of an untreated control culture are also shown.](http://aem.asm.org/Downloaded from http://aem.asm.org/ on May 5, 2020 by guest)
be that lysogeny, or defective lysogeny, is such a common feature of the group that the absence of indicator strains rather than of lysogens has impeded the characterization of temperate bacteriophages. McKay and Baldwin (9) failed to find an indicator for the phage revealed in

FIG. 4. Phages of S. cremoris R, negatively stained with neutral 2% potassium phosphotungstate. A, Virulent phage r.v. Magnification x180,000. B, Temperate phage r.t. Magnification x180,000. C, Phage, phage fragments, and ghosts in an MC-induced lysate of S. cremoris R. Magnification x85,000. Bar markers represent 100 nm.
electron micrographs of lysates from UV-induced *S. lactis* C2.

Indicator strains, on which plague formation occurred, were found for only one (*S. cremoris* R1) of the six strains that showed overt lysis after UV irradiation or MC treatment. In addition, zones of lysis but no plaque formation were produced on lawns of several strains. Unlike the report of Keogh and Shimmin (6), no lysis was observed on lawns of the strain which gave rise to the lysis. Of the three closely related strains on which R1 lysates produced plaques, *S. cremoris* AM1 was selected as the routine indicator for the phage induced from R1, although strain AM1 is an unusual indicator in several respects. The history of the culture used for seeding plates was found to have a striking effect on the EOP of phage r,t. Highest plating efficiencies were obtained with late stationary-phase cells that had been grown at 30 C. The reasons why these cultures gave highest plate counts are not understood and the finding was unexpected because of the use of early to mid-logarithmic cultures for seeding plates was essential for highest EOP of some group H temperate phages on their indicator *S. sanguis* strain Wicky (11). No plaque formation whatsoever was observed if logarithmically growing AM1 cultures were used in phase r,t assays. It is not surprising, therefore, that AM1 was a poor propagating strain for r,t phage. It seems likely that AM1 may become lysogenized by phage r,t, although direct evidence could not be obtained. However, strain SK11, which consistently showed the lowest EOP for phage r,t (Table 1), did become lysogenized, since r,t phage-resistant colonies from which the phage could be induced by MC treatment were readily isolated from the turbid areas formed on SK1 plates by lysates of strain R1. The sensitivity of the SK11 lysogens to two serologically unrelated virulent phages was unchanged from that of the parent strain. Clearly the resistance of *S. cremoris* R1 to these two virulent phages is not due to the presence of this temperate phage.

An inadequacy of the indicator strain alone seems insufficient reason to account for the generally low level of phage r,t detectable in either induced or noninduced R1 cultures (Fig. 2 and 3). Rapid adsorption of the induced phage to cell debris may cause the apparently low titers of phage, since losses as high as 99.9% of PFU have been attributed to such adsorption in a group H streptococcal temperate phage system (10, 11).

From Table 1 it can be seen that R1 lysates caused lytic reactions but no plaque formation on several strains in addition to those that showed plaques. The possibilities of inhibitory substances or of some modification and restriction phenomenon have not been eliminated entirely. However, it would seem more likely that strain R1 is lysogenized, perhaps defectively, by more than one phage, or, alternatively, that only a small proportion of the induced phage is infective. The high incidence of incomplete phage particles, fragments, and ghosts observed in electron micrographs of R1 lysates (Fig. 4C) is compatible with either possibility. However, all but one of the non-plaques were lost from the lytic spectrum when phage r,t was propagated on AM1 (Table 1). Again, a host range modification of phage r,t by AM1 cannot be eliminated, but it is possible that R1, unlike AM1, carries in addition to phage r,t one or more inducible defective phages similar to those found by Keogh and Shimmin (6) and by Reiter (personal communication), and that R1 lysates must be treated as mixed phage preparations.

Virulent phages have been a major industrial problem in the manufacture of fermented dairy foods for many years (16). Virulent phages of newly isolated strains of lactic streptococci appear rapidly when these strains are used commercially. The possibility that these previously unknown phages arise as virulent mutants of temperate phage may account for their sudden appearance. Neither the electron micrographs (Fig. 4A and B) nor the host range data would be incompatible with this hypothesis in the case of the virulent and temperate phages of *S. cremoris* R1. Experiments to artificially produce mutants of r,t phage that are virulent for *S. cremoris* R1 are in progress.

**ACKNOWLEDGMENTS**  
I wish to thank the Electron Microscopy Unit, Applied Biochemistry Division, D.S.I.R., Palmerston North, New Zealand for carrying out electron microscopy, P. A. Leyland for competent technical assistance, and L. E. Pearce for many helpful discussions.

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