STUDIES IN LYSOGENY

ELIMINATION OF PROPHAGES FROM A LYSOGENIC STRAIN OF *STREPTOCOCCUS CREMORIS*: "CURING" AND LYSOGENIC CONVERSION

A thesis presented in fulfilment of the requirements for the degree of Master of Science in Genetics at Massey University, New Zealand.

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ABSTRACT

The naturally lysogenic strain Streptococcus cremoris R_1, yielded after ultraviolet light and Mitomycin C induction, phages of two types. A collared form, r_1t_1 with a buoyant density 1.50 g/cm3 which differed morphologically and serologically from the collarless type r_1t_2 (buoyant density 1.47 g/cm3). These two phage appear to differ also in their lysogenic immunity specificity.

Elimination of these two prophages from their host was accomplished by UV irradiation of a logarithmic culture after reduction of the long chains to mono-or diplococci by blending, and plating on a medium free from Ca^{2+}. A screening procedure for the isolation of the newly cured strain has also been devised which permits examination of as many as 24 cultures at a time.

Morphologically and physiologically the lysogenic strain was not different from its cured derivative. They both formed long chains in broth media, they produced lactic acid at the same rate, fermented lactose, glucose, galactose, but not sucrose and maltose, they did not hydrolyse arginine and they did not grow at 40°C. That is, the prophage(s) themselves appeared to be playing no part in determining the bacterial phenotype.

Microbiologically, no difference in virulent phages sensitivity between the two strains was observed and no host induced modification/restriction was observed to be due to the presence of the prophage. However when temperate phages liberated from 22 strains of S. cremoris and 2 strains of S. lactis were spotted on lawns of both the R_1 and its cured derivative it was found that the presence of the R_1 prophages conferred immunity against phages liberated by 13 out of 24 strains examined. Relysogenization of the cured R1 strain with one of the two R_1 phages conferred immunity to the phages liberated by only a few of the other strains. Comparison of the morphology of the various phages involved, showed that the collared phage liberated from R_1 conferred immunity to collared

temperate phages liberated by the other strains examined, and were heteroimmune to the collarless temperate phage.

During the course of this project two unusual mutants were isolated which had in common the fact that they did not form chains. One of these was physiologically quite different from the parental strain.

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CHAPTER ONE: INTRODUCTION

1. Introduction to Lysogeny

1.1. Terminology

The bacteriophages form a diverse collection of viruses that multiply in bacterial cells. They persist in one of three general ways in nature: as prophages of temperate viruses integrated into the bacterial genome (or the closely associated membrane-bound DNA replicatory system (Gilbert and Dressler, 1968); or as persistent viral infections (carrier state, pseudolysogeny) in which they multiply lytically in a fraction of the bacterial population; or as standard virulent phages. Either of the first two of these conditions may bring about real and/or apparant changes in characteristic properties of the bacterial population. Such changes are called phage conversions and may be due to the expression of prophage genes, of vegetative viral genes or to modifications which lead to the expression of cryptic bacterial genes.

Temperate bacteriophages multiply in the host cells in either of two ways. In productive or lytic growth the injected DNA molecule directs the synthesis of numerous gene products, which promote autonomous replication of the phage DNA and its packaging into phage particles. These events occur within 50 minutes at 37C in the case of phage λ and Escherichia coli.

Temperate bacteriophage DNA can also replicate as part of the bacterial chromosome. To do so, the injected DNA must first direct the synthesis of gene products that promote insertion of the phage chromosome into the DNA of the host, and then express other genes that act promptly to repress autonomous DNA replication and most phage functions. When these processes mesh properly, the phage DNA is inserted into the bacterial chromosome at a characteristic site and is called a prophage. Figure 1 diagrams the insertion and excision of prophage.

Lysogeny is the perpetuation of prophages as part of the bacterial replicating system. When a bacterium incorporates the genome of a temperate bacteriophage into its chromosome or into the closely associated replicating machinery of the bacterial

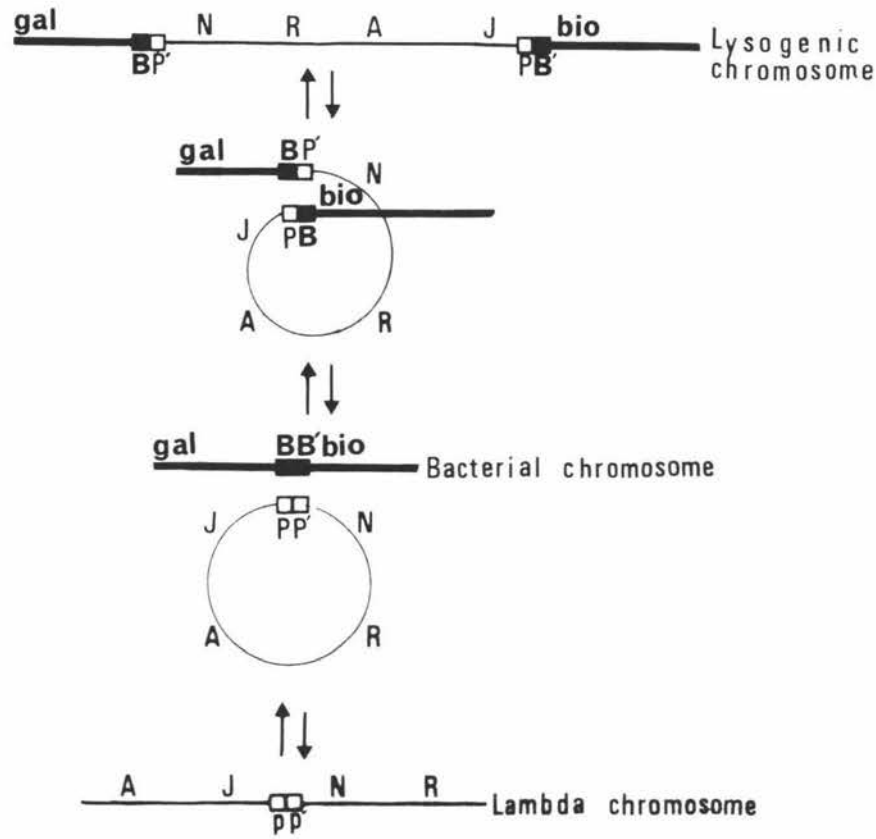

Figure 1: Prophage insertion and excision. The light lines represent the phage and prophage chromosomes and the heavy lines the bacterial chromosome. The rectangles represent the attachment sites, A, J, N and R are phage genes. P.P' and B.B' are respectively the phage and bacterial sites of insertional recombination. Bacterial genetic symbols are: *gal*, a cluster of three genes determining enzymes of galactose catabolism; *bio*, a cluster of at least five genes determining enzymes of biotin biosynthesis (from Campbell, 1962).

membrane system it becomes lysogenic i.e. it carries a phage genome as a prophage.

Lysogeny signifies the hereditary power to produce phage particles. A lysogenic bacterium or lysogen, is one that regularly transmits to its progeny the power to produce phage particles (Jacob et al., 1953). The word prophage signifies the hereditary structure present in a lysogen and absent in the corresponding nonlysogenic bacterium.

The proportion of cells infected with temperate phage that become lysogenic (i.e. the frequency of lyrogenization) may be influenced by environmental factors such as the multiplicity of infection (Boyd, 1951), temperature (Lieb, 1953; Bertani and Nice, 1954), the physiological state of the bacteria, anaerobiosis, starvation and treatment with various chemical substances (Lwoff et al, 1954). The frequency of lysogenization is also dependent on the genotypes of the phage and the host.

Prophage contain all the genetic information of the phage itself, but artificial lysis of lysogenic cells reveals no infectious phage within them. Thus, a prophage is a latent non-infectious form of the virus. In the change from the nonlysogenic to the lysogenic state every bacterium becomes converted to the capacity to liberate phage and to an immunity against lytic infection of that phage. The aquisition of these two properties is the sine qua non of all lysogenic conversions (Arden, 1974). In addition many temperate phages carry genes which effect the synthesis of what are clearly bacterial products, such as bacterial toxins. A common type of lysogenic conversions is the modification of surface antigens of the host cell.

Lysogenic conversions often involve exclusion of superinfecting phages. Usually exclusion acts after phage adsorption and usually involves breakdown of the superinfecting phage DNA. Lysogens of some temperate phages like the noninducible coliphage P_2 and the inducible Salmonella phage P22, exclude many other phages. It thus confers protection against phage infection in general and also supplements the role of immunity. Another way by which lysogenic conversions can act to protect the lysogen from further phage infection is shown by the coliphage P_1, whose prophage

confers a new restriction and modification phenotype on the host. Thus phages grown on hosts lacking P_1 cannot successfully infect a P_1 lysogen (Lederberg, 1957).

Finally, lysogeny often makes bacteria resistant to phages and this is perhaps the major way in which lysogeny serves host purposes.

1.2 Inducibility of lysogenic bacteria

Although lysogeny was discovered in 1921, the subject remained controversial for many years, until 1950 when Lwoff and Gutmann reported their pedigree studies with single-cell isolates of Bacillus megaterium which remained lysogenic through nineteen generations and without the intervention of phage particles. The discovery by Lwoff, Siminovitch and Kjelgaard (1950), that exposure of lysogenic B. megaterium to small doses of ultraviolet light was followed by the production of phage by almost every cell in the population has provided a tool for probing the molecular basis of lysogeny and for characterizing lysogenic populations.

Twenty six years after the discovery of lysogenic induction, the molecular mechanism still remains obscure. Yet the intervening years have brought considerable understanding of this "Manichean situation" in which .....the good changed into bad as a temperate phage was induced. As a result of the work of many people the purely concephal "prophage" is now well defined genetically and physically in several host-bacteriophage systems, but the molecular details of the transition to the vegetative state continues to be enigmatic.

It has been shown that other agents may also lead to induction, such as exposure to the antiobiotic Mitomycin C (Otsuji et al, 1959), fluoropyrimidines or shifts in temperature (Lied, 1966).

There is however, considerable variation in the degree of induction in different bacterial systems and even in lysogenic bacteria of the same species. Wild type temperate phages fall into three classes with regard to induction:

a. those that are readily inducible on exposure to UVL such as coliphage lambda;
b. those which show a slight increase in phage following irradiation as is the case of coliphage P_1 and

c. those which are not induced at all, as is the case of coliphage P2.

1.3 Curing of Lysogenic bacteria

Prophages may be viewed as the true wild types of temperate phage genomes. The stability of these wild types in nature is an impressive outcome of natural selection. Thus, while it is easy to cure pseudolysogenic bacteria of phage, it is often difficult to cure lysogenic bacteria of their prophages.

In the case of phage elements of the immunity region maintain the lysogenic state and genes situated in other regions of the lambda chromosome act at one or another stage in productive growth. These two sets of genes are mutually exclusive in function forming the basis of a bi-stable switch: the product of the cI gene represses genes controlling productive growth, and the cro gene product repressing the genes in the immunity region. The stability of the derepressed phase of the bi-stable system serves to permit efficient productive growth, unimpeded by continued synthesis of repressor. The stability of the immune phase serves to permit efficient lysogenic growth Sometimes the repressor of the cI gene can be inactivated reversibly. Then if the timing is right the prophage is excised, repression is established again, and the repressed phage DNA fails to replicate as the cells multiply. The nonlysogenic bacterial progeny are said to be "cured".

Three methods have commonly been used for the curing of lysogenic bacteria. The first is to expose the cells to high doses of irradiation. The second is to heat the cells, which carry a temperature sensitive repressor, for a short time, which is insufficient to induce phage production, and which can lead to a high survival of cured cells (Weisberg and Callant, 1966). The third is to infect them with a weakly virulent or heteroimmune relative of the prophage (Campbell, 1966). The integrase of the superinfecting phage can detach the resident prophage. Prophage is site specific and only heteroimmune phages attaching at the same chromosomal site as the prophage will cure.

2. Introduction to group N streptococci.

2.1 Taxonomy and classification

It was a great advantage to find that streptococci could be classified into consistent serological groups by means of group specific substances extracted from the streptococci and tested in precipiting tests with the corresponding streptococcal group specific antisera (Table I). Identification of the individual species is based on serological and physiological tests. Species lacking a recognizable group antigen (e.g. S. mitis. S. thermophilus) are identified by physiological tests. A strain that lacks a group antigen can often be placed in a serological group by its physiological characteristics. Identification by both physiological and serological procedures offers the most definitive approach.

Group N streptococci were previously studied extensively because of their significance in the Dairy industry where they are used as 'starters' in the manufacture of cheese. Many cultural and biochemical tests were used to classify those streptococci that were closely related to each other and were different from strains pathogenic to man and animals.

Two main species are still recognized in the 8th edition of Bergey's manual for the group N streptococci Streptococcus cremoris and S. lactis. Table II shows the physiological differentiation of these two species.

The vast amount of information available concerning the basic bacteriology and immunology of streptococci contrasts strongly with the paucity of our knowledge of streptococcal genetics. However there is little doubt that genetic experiments will provide data of taxonomic importance and will help us to understand more fully the behaviour of the specific starter strains both in isolation and in defined mixed systems, because transduction through temperate phages and even transformation through autolysis of cells may take place in nature. (Reiter and Møller-Madsen; 1963).

TABLE I Group Classification of Streptococci

Group	Hemolysis*	Usual habitat	Group antigen
A	+	Man	Rhamnose-N-acetyl-glucosoumine polysaccharide
B	+	Cattle	Rhammose-glucosamine polysaccharide
C	±	Many animals, Man	Rhamnose-N-acetyl-galactosamine polysaccharide
D	±	Dairy products, Man, animals	Glycerol teichoic acid containing D-alanine and glucose
E	+	Milk, Swine	Rhamnose polysaccharide
F	+	Man	Rh & glucopyranosy 1,-N-acetyl-galacto samine tetrasaccharide
G	+	Man, Dog	Rh-galactosamine polysaccharide
H	±	Man	Rh polysaccharide
K	±	Man	Rhpolysaccharide
M	+	Dogs	
N	-	Dairy products	Glyserol teichoic acid containing D-alanine & gal-P
O	±	Man	

* + all strains hemolytic; ± some strains hemolytic others non hemolytic

\- all strains nonhemolytic

TABLE II Physiological differentiation of S. lactis and S. cremoris

SPECIES	NH_3 from Anginine	Growth at 40°C	Growth with 4% NaCl	Growth at pH = 9.2	Acid from Maltose	Acid from Sucrose	Growth in 0.3% Methylene blue
S. lactis	+	+	+	+	+	±	+
S. cremoris	-	-	-	-	±	(-) rarely	-

2.2. Lactic Streptococcal Bacteriophages

2.2.1 Structure and Morphology

Inconsistent acid production and complete starter failure of the group N streptococci continues to be an industrial problem resulting in substantial economic losses to the manufacturer of cultured dairy products. Erratic acid production may be caused by a variety of factors, the most frequent being bacteriophage infection.

Bacteriophages were first suspected of having serious economic and public health implications for the food industry after 1935 when Whitehead and Cox reported that bacteriophages was the cause of insufficient acid production. However until recently little was known of the actual morphology of lactic streptococcal bacteriophages, although the earlier electron photomicrographs were published by Parmelee et al in 1949.

Electron microscopic studies showed that lactic streptococcal phages can be grouped into two general prototypes. Each of these two prototypes contain phages similar in general ultrastructure, varying only in size of the component structures, presence or absence of collar and the morphology of the tail base plate.

Phages belonging to the first prototype have an isometric head which appears to be octahetral in shape and with size ranging from 52nm to 62nm across with a non contractible tail measuring from 113nm to 153nm (Terzaghi, 1976). Both virulent and temperate variant phages were found for this prototype.

Phages belonging to the second prototype have a prolate polyhedral head which again varies in size from 44 x 56 nm to 47 x 63 nm. Tails are non-contractile, measuring from 82nm to 95nm. Collars were also reported for some of those phages as well as for some isometric ones. No temperate phages were found to belong to this prototype.

The genetic information of all lactic streptococcal bacteriophages described to date is contained in double stranded DNA which is linear (and not perpetuated) and with Guanine-Cytocine contents ranging from 32.7% to 40% (Nyiendo, 1974). The average genome size ranges from 5.9 x 10^6 Daltons (the smallest found) to 24.2 x 10^6 Daltons. The buoyant density

of these phages covers a wide range from 1.428 to 1.531 g/cm^3 (Nyiendo, 1974).

2.2.2 Incidence of lysogeny in group N streptococci

With this brief discussion on the morphology and structure of several types of streptococcal bacteriophages in mind the influence of these bacteriophages on streptococcal functions now will be reviewed.

Several relevant questions concerning the role of these bacteriophages immediately comes to mind. The first is whether lysogeny is common among group N streptococci;the second is what evidence do we have that these bacteriophages play a role in alteration in known constituents of the cellular structure; finally what evidence do we have that the presence of the prophage confers protection against phage infection beyond the presumed lysogenic immunity and a new restriction and modification specificity.

With respect to the first question, only recently a number of investigators have shown that lysogeny is indeed common in group N streptococci. A report dealing with lysogeny in these organisms appeared in 1949, i.e. 28 years after the discovery of the phenomenon although Whitehead and Cox clearly stated in early 1935 that "from a practical point of view it is necessary to find some method of eliminating the phage or of using (as a starter in cheese-making) an organism immune to its action. The isolation of phage immune varieties seems to offer the greater promise of success....." Since Whitehead and Cox first discussed the significance of using the phage immune strains only recently have attempts been made on this direction. All other attemps at phage control were along the line of eliminating the phage either by using asceptic propagation of single-strain starters, by removing the calcium from the starter milk or by using mixed starter cultures in which the strains appeared to serve as host to no phage in common.

Deluged on all sides for over twenty years by a wealth of information concerning the important role bacteriophages play in the structure, genetic make up, and cellular function of many bacterial species investigators .concerned with the biology of

the streptococci could not help but wonder about the role streptococcal bacteriophages might play in phage host relationships in this group of organisms. Yet, if one turns to the literature for reports concerning these streptococcal phage-host relationships, it is surprising to note that until recently only a few scattered reports of this interaction have appeared. As usual, there are reasons for this discrepancy, and the knowledge that streptococci require complex media for optimal growth, grow in chains rather than as single cells, excrete a prodigious number of extracellular products and give poor lowns on overlay plates (Zabriskie et al, 1972), certainly did not encourage the investigator interested in basic phage-host relationships. However the importance of the lactic streptococci to the dairy industry, and their close relationship to pathogenic streptococci suggest that the time is ripe for subjecting this group of organisms to closer genetic examination. This was recognised by W.T. Dobrzanski who clearly stated in the 1972 conference held in Minnesota : "We have been surprised that we could not find in the literature a report concerning lysogeny in lactic streptococci". So he examined the problem and found 10% of S. lactis strains to be lysogenic. These results were confirmed by Kozak et al (1973). Clear demonstration of lysogeny in S. cremoris was first given by Lowrie (1974) by the induction of strain R_1. The current work of Terzaghi (ms. in preparation) suggests that most if not all lactic streptococci used in dairy fermentations in New Zealand are lysogenic for a variety of phages.

With the knowledge that lysogeny is a common phenomenon among lactic streptococci investigators tried to cure the lysogenic cells of their prophage in order to investigate the influence of the resident phage on certain properties of the host cells but all attempts in this direction were unsuccessful (Lawrence et al 1975). Zabriskie concluded that lysogeny is a very stable phenomenon in streptococci and that since they are chain-forming organisms, the isolation of a cured coccus in the chain might be very difficult (Zabriskie et at, 1972).

The objective of this research project was to investigate the effects of lysogeny (i.e. lysogenic conversions) on a lysogenic strain of Streptococus cremoris. It describes the isolation of cells that have lost their prophage and a comparison of the properties of this strain with its naturally lysogenic parent.

CHAPTER TWO MATERIALS

1. Organisms used

1.1. Bacterial strains

Nine bacterial strains of Streptococcus cremoris were available from the culture collection maintained in the Dairy Research Institute, Palmerston North. These included S. cremoris R_1, AM_1, AM_2, P_2, 158, 104, 368, SK_{11} and 166.

Strain R_1, which was later selected for further studies is a rather slow acid producer imported to New Zealand in 1917 and used extensively by Whitehead and Bush. It is sensitive to the attack of a series of virulent phages, and it is a known lysogen (Lowrie, 1974). Its lysate has indicator strains AM_1 and its derivative strain 368.

1.2 Bacteriophages

The bacteriophages used in this study are listed in table III.

Table III Bacteriophages used

Phage	Description	Source
879/368	Virulent; forms clear plaques on 368	Supplied from DRI
690/SK_{11}	Virulent; forms clear plaques on SK_{11}; prolate Head size 62x40nm. Tail 100nm	"
652/166	Virulent; forms clear plaques on 166 and R_1	"
949/AM_2	Virulent; forms clear plaques on AM_2. Isometric; Head 77-83nm. Tail 455nm	"
799/368	Virulent; forms clear plaques on 368. Isometric, head 57nm; Tail 132. Collar	"
949/402	Virulent; forms clear plaques on 402. Isometric	"

Note: The characteristics of the virulent bacteriophages were obtained from B.E. Terzaghi (personal communication) In the phage labels the first number is simply the DRI isolate number, and the second number is the host bacteral strain upon which it was last propagated.

2. Media and other solutions

2.1 Growth Medium: M17

M17 broth (Terzaghi and Sandine,, 1975) was prepared by adding per liter of distilled water:

Polypeptone (BBL, Cockeysville, M d.)	5.0g
Phytone peptone (BBL),	5.0g
Beef extract (BBL),	5.0g
Yeast extract (BBL),	2.5g
Ascorbic acid (Sigma Chemical Co.,)	0.5g
β -disodium glycerophosphate (Sigma Chem.Co.,)	19.0g
Lactose (May & Baker Ltd., England)	5.0g
1 M $MgSO_4$. $7H_2O$ (May & Baker Ltd. England)	1.0ml
1 M $CaCl_2$ $\cdot 6H_2O$	10.0ml

The pH was adjusted to 7.1 with 2N NaOH and autoclaved at 121oC for 15 minutes.

Bottom M17 agar used for assay of bacterial colonies or phage plaques is prepared by adding 12.0g of Davis agar (Davis Gelatine Ltd., Christchurch, N.Z.) to 940ml of glass-distilled water and heating the mixture to boiling to dissolve the agar. The remaining ingredients, except lactose, Mg $SO_4.7H_2O$ and $CaCl_2.6\ H_2O$ are added to the dissolved agar and the mixture is autoclaved at 121C for 15 minutes. After cooling to 55oC, 25ml of sterile 20% lactose to which has been added 10.0ml of sterile 1.0M $CaCl_2$ and 1.0ml of $MgSO_4$ is gently added to the melted agar basal medium. After mixing carefully 15- to 18-ml quantities are added to sterile petri plates. Top overlay agar is prepared by adding 4.5g of Davis agar to 1 lt of distilled water and heating until the agar is dissolved. The remaining broth ingredients are then added and the medium is dispensed (50 ml quantities) into prescription bottles and autoclaved (121°C, 15 min).

2.2. Indicator media

M17 medium is inappropriate for metabolic and other studies since it already contains fermentable carbohydrate as well as an unknown concentration of arginine.

The following indicator media were used during the metabolic and other studies of lactic streptocci.

2.2.1 GMA agar medium for the differentiation of 'fast' and 'slow' coagulating cells in lactic streptococcal cultures.

This is a glycerophosphate buffered milk-based agar medium which has been developed by Limsowtin and Terzaghi (1976) and contains per liter of water : non-fat spray dried, heat stable milk powder, 100g; β-disodium-glycerophosphate. $5\frac{1}{2}$ H_2O (grade II, Sigma Chemical Co) 19g; Davis agar, 8g. The three components were dissolved separately in 450, 100 and 450ml of deionized water respectively and autoclaved at 121°C for 20 min. After cooling to 55°C, the components were thoroughly mixed together and dispensed to plates in 18ml aliquots.

To examine for growth and differentiation on GMA, 0.1ml of the appropriate dilution of the culture were spread on GMA plates and then incubated at 22°C. Fast coagulating cultures give large colonies frequently of mucoid appearance. Slow coagulating cultures were obtained from the small translucent flat colonies.

2.2.2. Lactic agar medium for the examination of sugar fermentation.

This was prepared as described by Elliker et al (1955): to a liter of water add : Tryptone 20g; yeast extract 5g; gelatine 2.5g; sodium acetate 1.5g; ascorbic acid 0.5g; agar 15g; selected sugar 10g.

Lactic agar containing 10g lactose as the primary carbon and energy source supplemented with 0.04g bromocrecol purple (BCP) serves as an indicator medium for the fermentation of lactose : lac$^+$ forms yellow colonies and lac$^-$ forms white colonies.

Lactic broth : as for lactic agar medium but without agar.

2.2.3. BCP agar medium for the detection of the hydrolysis of arginine.

This medium which was devised by Reddy et al (1969) contained per liter 5.0g tryptone 5.0g yeast extract, 4.0g L-arginine hydrochloride, 4.0g $K_2H\ PO_4$, 3.0g $CaCO_3$ 6.0g carboxy-methyl cellulose (CMC) and 15.0g agar. Before

pouring plates, 5ml of sterile 11% reconstituted non-fat dry milk and 2.0ml of sterile 0.1% bromocresol purple (BCP) in distilled water were added to 100ml of agar. The amount of agar necessary to make up to 1.0 liter of the medium was suspended in 500ml of distilled water and steamed until dissolved. In another glass beaker containing 500ml of distilled water, 6.0g of CMC was suspended and heated in a boiling water bath until the opacity disappeared. The two portions were mixed together in a stainless steel container with the required quantities of tryptone, yeast extract, arginine, K_2HPO_4 and $CaCO_3$, covered with aluminium foil, and steamed for 10min. The pH of the medium after steaming should be 6.8 ± 0.1 The agar was then dispensed into bottles in 100ml quantities and sterilized at 121°C for 15 min.

Agar plates were prepared as follows. After adding the milk and indicator to the melted medium, the bottle contents were mixed thoroughly, avoiding incorporation of air, and poured into previously chilled petri plates to obtain a layer 4 to 5mm thick. After the medium solidified, the plates were dried for 18 to 24 hours in an incubator at 37C.

0.1ml samples of the appropriate dilutions of the cultures to be examined for the hydrolysis of arginine were spread evenly with a bent glass rod on the surface of the agar layer and incubated at 30°C for 24-48 hours.

Yellow colonies : No hydrolysis of arginine
White colonies : NH_3 from arginine

3. Other solutions

3.1 Phosphate buffer : to 1.0 liter, 0.05M $Na2HPO_4$, 0.025 M KH_2PO_4 pH = 7.2 Add 4.0g NaCl. Before use add 1ml 1M $MgSO_4$ 7 H_2O per liter

3.2 Tris buffer : in a total of 1.0 liter, 250ml 0.2M tris (hydroxy methyl) aminomethane, 221 ml 0.2 M HCl. pH = 7.2 Add 11.6 g NaCl

CHAPTER THREE: METHODS

1. Bacterial growth conditions

Cells were grown without shaking in M17 broth from an 1% inoculum of an overnight (22C, 16 h) culture at 30C for 3½ hours unless elsewhere stated. Growth was recorded as optical density (OD) using a Bausch & lomb spectronic 20 at 600nm, or by direct microscopic count with a Petroff-Hausser bacteria counter in a phase contrast microscope. Alternatively, samples were plated for colony forming units on the appropriate agar medium, by the soft agar method.

2. Propagation of the phage

For propagation in broth, an overnight culture of the appropriate strain of S. cremoris is diluted 1:100 in 100ml M17 supplemented with 1ml $CaCl_2$. A single phage plaque is then added to the broth culture of the propagating strain and the mixture is incubated for 6 hours at 30C. The lysate obtained is then centrifuged at 8,000 rpm for 15 minutes to remove cells and debris. The supernatant fluid is decanted and filtered. The lysate was then titrated on the propagating strain by the soft agar overlay method and stored at 4C as the stock suspension.

For propagation in a soft-agar overlay (Adams, 1959) the propagating strain was grown in M17 broth overnight. A phage suspension was diluted to 0.5×10^6 to 1.0×10^6 plaque forming units (PFU)/ml, and 0.1ml of this dilution was added to tubes containing 0.2ml bacterial broth culture supplemented with one drop of 1.0M $CaCl_2$, in a water bath at 30C. After 10 minutes at 30°C to allow the phage to adsorb 2.5ml molten soft-agar was added and the mixture was poured over the surface of an M17 plate. The agar overlays were allowed to solidify, and the plates were then incubated at 30oC. After overnight incubation, semiconfluent to confluent lysis was observed. The soft-agar layers were scraped into centrifuge tubes by using a sterile glass rod. Two milliliters of M17 medium per plate was added, and the tubes were placed on a vortex mixer to disintegrate the bits of soft agar. The lysates were centrifuged at 8000 rpm for 15 minutes and filtered through membrane filters (Millipore corp., filter

0.45 m pore size). These lysates were then titrated on the propagating strain and kept at 4oC.

3. Phage assays

Phage assays were carried out by the usual agar layer method of Adams (1959). 2.5ml of melted M17 soft agar (45oC) seeded with 0.2ml cells of an O.N. culture, 0.1ml of phage dilution and 1 drop of 1M $CaCl_2$ was poured on M17 plates and incubated overnight at 30C. All the phage dilutions were made in 10% M17 solution (10ml broth in 90ml sterile distilled water).

4. Phage adsorption

The bacterial overnight cultures were standardized spectrophotometrically so that they contained $4x10^8$ chains per ml. 0.5ml of the O.N. culture, containing $2x10^8$ chains/ml were infected at a multiplicity of less than 0.01 and allowed to adsorb for 10 min at 30oC. The adsorption process was followed by determining the residual unadsorbed phage in supernatants from samples of the incubation mixture diluted 10-fold and centrifuged for 10 min at 8,000 rpm. Control samples consisting of phage only were run in parallel in order to provide the phage concentration at zero time and the proportion of phage which was unadsorbed.

5. Sensitivity of the lysogenic strains to different inducing agents.

A lysogenic culture subjected to different inducing agents may produce cured colonies which can be detected as nonimmune survivors (Sly et al, 1968). Having this in mind I first examined the response of the lysogenic strains on different inducing agents.

5.1 Ultraviolet sensitivity

The ultraviolet sensitivity of the cultures of the different strains was examined by growing cells in 10ml M17 broth medium to a concentration of 4×10^8 chains/ml centrifuged and resuspended in 100ml 1% chilled peptone and finally blended at full speed (13,000 rpm) for 2 min in an AtoMix blender (Martley 1972). This treatment reduced the chain length of the strains

to an average of 2 to 6 cells per chain as is shown in plates 1 and 2. After blending the cells were again centrifuged and resuspended in chilled phosphate buffer, supplemented with 0.03M $MgSO_4$, to a concentration of 2 x 10^8 chains /ml. The cell suspension was pipetted into a flat-bottomed glass petri-dish (9cm diameter) and irradiated with a 15.W Phillips TUV P/40 bactericidal lamp of wavelength 253.7nm; radiation intensity is such that one T2 hit is delivered in 0.7 sec. for various time intervals. The suspension was stirred during the exposure to ensure that all the cells were subjected to the action of the rays, as these only penetrate a short distance into the suspension. At each time interval a sample was taken and plated at the appropriate dilution on an M17 agar plate. To avoid photoreactivation all the experiments were done in a room illuminated with a 'Pure-Yellow' bulb which emits only above 5,000 Å.

5.2 Sensitivity to Mitomycin C

Cells were grown in M17 broth medium to a concentration of 4 x 10^8 chains/ml; then they were centrifuged, blended, resuspended in phosphate buffer to a concentration of 4 x 10^8 cells/ml and spot tested on a series of M17 plates supplemented with various concentrations of MC (Mitomycin was from Sigma Chemical Company, St Louis, Mo.) Stock solutions were made in sterile distilled water at a concentration of 0.1 mg/ml and stored in the dark at 4°C). After overnight incubation of the plates at 30°C to allow for growth and induction, the lysogenic cells produce partially lysed, or less frequently, mottled colonies on the MC plates.

5.3 Temperature sensitivity

Logarithmic cultures of each strain were centrifuged and blended as described before, diluted 10-fold into 10ml fresh medium in a 43°C water bath. At time intervals of 0', 5', ...,30' samples were taken, diluted and plated on M17 plates, and incubated at 30°C.

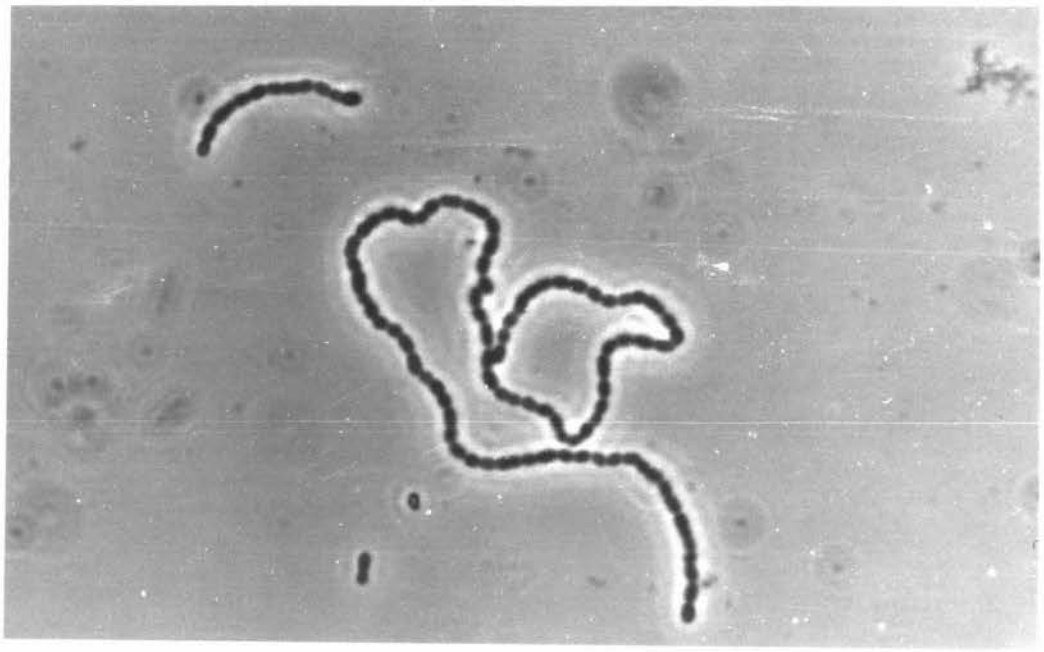

Plate 1. Streptococcus cremoris R_1 before blending

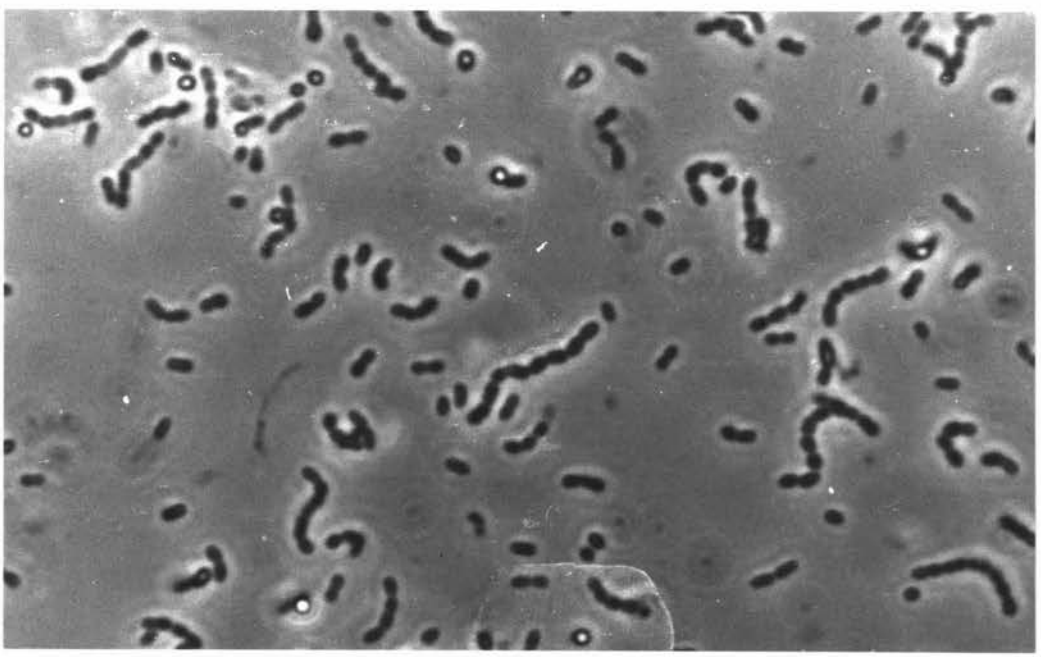

Plate 2. S. cremoris R_1 after blending

6. Induction of Lysogenic Strains

6.1 Ultraviolet Induction

The cells were grown in M17 broth medium to a concentration of 4 x 10^8 chain/ml; then they were centrifuged and resuspended in 1% chilled peptone, blended as described before, centrifuged and resuspended in phosphate buffer and irradiated for 10 seconds which was found to be the dose appropriate for induction of almost every cell of the population. As before, care was taken to prevent photoreactivation. Then the irradiated cells were supplemented with M17 nutrient broth and $CaCl_2$ to a final concentration of 0.08M, and incubated at 30oC for 4-5 hours. At intervals of 30 minutes the turbidity was measured in a Bausch and Lomb spectronic 20 at 600nm

6.2 Mitomycin C induction

For tests of bacteriophage induction using MC as the inducing agent (Levine, 1961) streptococci were first grown from a 1% inoculum of an overnight culture for 3½ hours in a water bath at 30°C. These log-phase lysogenic cells were exposed to different concentrations of MC in broth. After 5, 10 and 15 minutes of incubation at 30oC the cultures were washed, resuspended in fresh M17 medium and incubated at 30oC for 4 hours. The turbidity was followed at intervals of ½ hour until completion of lysis or until the culture entered the stationary phase of growth.

6.3 Heat induction

Log-phase cells were exposed to a high temperature of 43oC in a water bath for 12 minutes and then incubated again at 30oC for 5 hours until lysis occurred or until the culture entered the stationary phase of growth. This was followed spectrophotometrically at 30 minutes intervals.

7. Isolation of 'Cured' Strains of S. cremoris

Lysogenic strains are known to revert to the non lysogenic (cured) state at a low rate. With some lysogenic

bacteria irradiation with ultraviolet light or treatment with other inducing agents seems to produce cured colonies, which can be detected as nonimmune survivors (Sly et al, 1968).

Attempts were made to obtain 'cured' derivatives of different strains of S. cremoris. Such strains are expected to be : (a) sensitive to the U.V. induced lysates produced by the parent strain, (b) unable to lyse after irradiation with a small dose of U.V. light. Several methods were used in order to obtain a derivative which had lost the resident prophage.

A technique similar to Stolp's (1957), was first used. The cells in the exponetial phase of growth were centrifuged, resuspended in phosphate buffer,irradiated with ultraviolet light to a survival of 10^{-5}, and plated on hard M17 agar and incubated at 30oC. When colonies began to be visible, the plates were sprayed with a UV induced lysate of the parent strain. After further incubation, the colonies containing sectors of bacteria, which had lost the resident prophage and were thus sensitive to the phage sprayed, were expected to be partially lysed. Among 400 colonies examined, no such nibbled colonies were observed.

Another method used was a modification of the rapid plate method for the isolation of lysogenic bacteria described by A. Siddiqui et al (1974). Cells in the exponential phase of growth were blended, and irradiated as described before to a survival of 10^{-5}, and plated on M17 agar plates. After overnight incubation at 30oC colonies were replicated by means of sterile toothpicks to a master plate containing nutrients and agar and also to a second plate of the same medium supplemented with 0.14μg of the inducing agent mitomycin C (MC) per ml. After overnight incubation of the plates to allow for growth and induction, the lysogenic cells were expected to produce partially lysed colonies on the MC plate. Colonies produced by cured cells were expected to be unaffected. Such colonies were picked and examined for

lysogenicity. Exhaustive attempts to isolate in this way a cured strain of S. cremoris 104, R_1, P_2, AM_1, and AM_2, were unsuccessful.

The third method used was based on the assumption that a pulse-heat treatment would allow a brief derepression (Guarneros and Echols 1970), resulting in effective prophage excision. The cells of strain R_1 were grown in M17 broth without aeration at 30oC to mid log phase, spun and resuspended in 0.1% chilled peptone, blended as described before and plated undiluted (0.1ml) on M17 plates with soft agar and incubated at 44oC for 24 hours. Another plate was incubated at 30oC. The surviving colonies were picked and examined for immunity to the lysate from the parent strain R_1 as well as for indùcibility of the prophage after UV irradiation. These survivors will be reported as R_1t.

A large number of experiments using the methods described above, were unsuccessful. I then decided to try and cure the cells by using high doses of ultraviolet irradiation after taking some precautions. Briefly, the general procedure used is as follows:

> Lysogens of strain R_1 were grown in M17 broth at 30°C to approximately $2x10^8$ chains/ml, were washed twice to remove the free phage present at a low frequency in the lysogenic cultures, blended to reduce the chain length, washed again and then resuspended in phosphate buffer and irradiated to approximately 10^{-5} survival of colony forming units and then plated on M17 plates. The plating was done by the soft agar overlay method as described before except that the Ca^{2+} was omitted from the soft agar in order to avoid readsorption of phage with subsequent killing or relysogenisation of the newly 'cured' cells.

The precautions taken by removing most of the free phage from the culture and blending the cells before irradiation together with plating the irradiated cells in media without Ca^{2+} prove to be very important for the

production of cured cells in a culture of streptococcus.

After an O.N. incubation at 30°C individual colonies were picked into 0.1ml aliquots of M17 broth distributed to the wells of microtiter dishes (Linbro Multi - dish Disposo - trays) and grown for 3 hours at 30°C.0.05ml from each culture was then spotted with a wire loop on a master plate and to the remaining 0.05ml 0.2ml phosphate buffer was added. The cultures were irradiated for 10 seconds and then incubated at 30°C for 3 hours (until lysis occurs). Each of the 100 cultures was then spotted on a lawn of sensitive indicator bacteria (supplemented with Ca^{2+}) incubated for 16 hours at 30°C and then examined for lysis. Those cultures that did not lyse were streaked and colonies reisolated twice and checked for curing. The examination included UV induction, sensitivity to the phage produced by the parent strain and examination of the UV induced lysate of the culture in the Electron Microscope.

8. Isolation of a Relysogenised Strain

A 0.2ml sample of a log-phase culture of the 'cured' strain of R_1 (designated for the purpose of this study as R_1 cured) was plated in a soft agar layer on M17 agar, with added Ca^{2+}. Temperate phage produced by induction of R_1 was spotted on the plate seeded with R_1 cured and incubated for 2 hours at 22°C and then overnight at 30°C. Bacteria from turbid spots were streaked out and the resulting isolated colonies were purified by restreaking, and tested for immunity to the R_1 resident phage as follows: 0.2ml of a log phase culture was plated in soft agar supplemented with 1 drop of 1.0M $CaCl_2$; droplets of the R_1 resident phage grown on the R_1 cured strain were deposited on the lawn and incubated overnight at 30°C. Whereas the control R_1 cured lawn showed lysis where the R_1 resident phage was deposited the R_1 relysogenised strain (designated R_{1r}) did not. This derivative was restreaked twice and examined further, for lysogenicity. A strain was considered to have been lysogenized when : (i) it was immune to the lysogenizing phage

(ii) the supernatant of a broth culture lysed the parent strain (R_1 cured),

(iii) it was induced by small doses of UV light.

9. Phage preparation for the electron microscope

Ultraviolet light induced lysates and phage stocks prepared as described earlier were first centrifuged at 6,000 rpm for 10 minutes to remove bacterial debris and then centrifuged again at 8,000 rpm for 2 hours (Spinco rotor 30). The final pellet was resuspended in 0.1ml 0.05 M ammonium acetate (pH = 7.2) overnight at 4°C. A drop of the phage suspension was then deposited on a carbon-coated grid for 8-10 minutes. The excess was then removed with filter paper and the phage stained negatively with a mixture of equal valumes of 2% potassium phospho-tungstate (KPT) pH = 4.0 and 2% ammonium molybdate. Excess liquid was withdrawn with a filter paper. Specimens were examined in a Phillips EM 200 electron microscope. Dimensions were measured on prints at a final magnification of X128,000. The tail length was measured on intact phages only and included the mid-point of the base plate.

10. Separation and purification of phage from UV induced lysates of R_1

C_sCl density gradient centrifugation was used for separation of the phages observed in the electron microscope in the UV induced lysate of strain R_1. The UV induced lysate was prepared as described before and concentrated by centrifugation at 24,000 rpm for 2 hours. Coliphage lambda was added as a density 'reference'. To the pellet was added 1.0ml CsCl solution of density 1.48g/cm^2 and allowed to resuspend overnight at 4°C. The CsCl solution was prepared by mixing 4.5g CsCl and 5.5.g buffer. (The buffer was 0.05M Tris buffer pH = 7.2 + 0.2M NaCl). The resuspended sample was then placed in a 5ml nitrocellulose centrifuge tube containing 3.7ml of

the same CsCl solution. 0.3ml of liquid paraffin oil was then added at the top and centrifuged at 24,000 rpm for 36 hours at 4°C in the SW39 rotor of the Beckman Model L ultracentrifuge. 25 fractions of 0.2ml each were collected in an ISCO density gradient fractionator (Model 640). The density of each fraction was then measured refractometrically in a Bausch and Lamb Abbe - 3L refractometer with horizontal prisms.

Each fraction was then titered for plaque-forming units on M17 agar plates by the soft agar overlay method described previously, on strains R_1 cured and 368, indicators for the phages liberated by R_1 and Escherichia coli for Lambda.

CHAPTER FOUR: RESULTS

1. Sensitivity of S. cremoris strains to different inducing agents.

1.1 Effect of the dose of UV on the survival of different strains of S. cremoris

The UV killing curves of strains R_1, AM_1, P_2 and 104 are compared in Figure 2. The UV survival curves of strains P_2 and AM_1 are single hit curves whereas 104 has a 3-hit curve and R_1 a 10-hit curve. This is not surprising since microscopic examination of logarithamically growing cultures of strains P2 and AM_1 show that they mainly exist as monococci or diplococci and so their killing curves describe the inactivation of single or double cell organisms in contrast with 104 which forms mainly short chains of 3-4 cells and R_1 which forms longer chains.

1.2 Effect of Mitomycin C on the growth of S. cremoris strains R_1, P_2, AM_1, AM_2 and 104.

Logarithmically growing cultures of the five strains in M17 medium were centrifuged, resuspended in phosphate buffer and spotted on plates of M17 medium containing different concentrations of mitomycin C, and incubated at 30°C for 24 hours. The results are presented in Table IV. It is clear that under these conditions, the growth of strains AM_1, 104, P_2 and AM_2 was inhibited by concentrations of MC of 0.10-0.12 µg/ml and that of R_1 by 0.12-0.14 µg/ml.

1.3 Effect of Temperature on the survival of S. cremoris R_1.

It was found that when 0.1ml of a log culture of strain R_1 was diluted into 10ml M17 broth at 44°C and then assayed at intervals of 10 minutes for 60 minutes on M17 agar plates

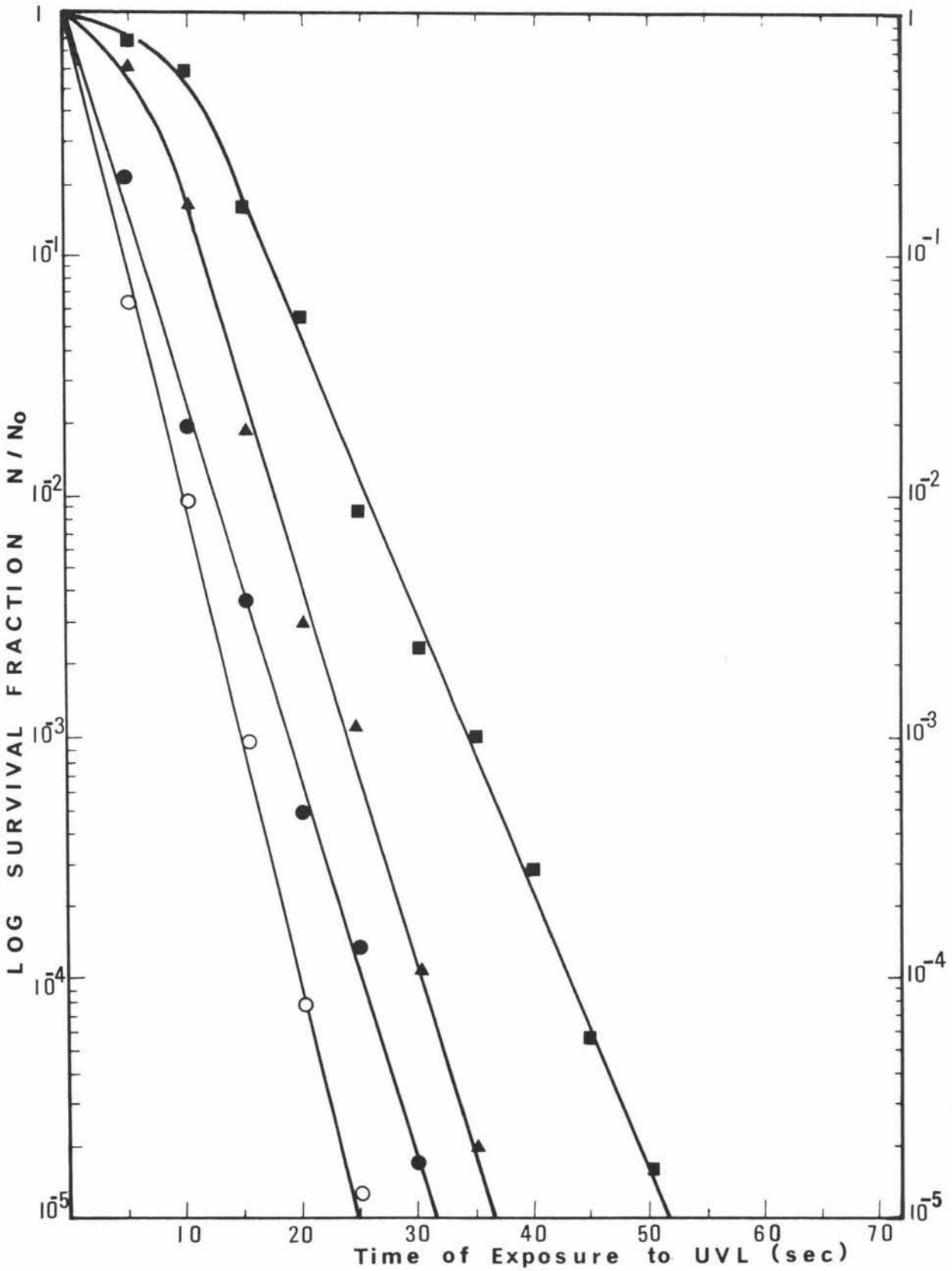

Figure 2: UV killing of different strains of *S cremoris.* Broth cultures in the mid log phase (2 x 10^8 cells/ml) were resuspended in ince-cold phosphate buffer and irradiated with various doses of UV as described in Methods. Photoreactivation was avoided by carrying out the post-irradiation procedures under a dim yellow light and incubating in the dark.
No : initial cell Number. N : number of surviving cells, after irradiation.

■, R_1; ▲ 104; ●, P_2; ○ AM_1

TABLE IV. Growth of different strains of S. cremoris on M17 plates supplemented with different concentrations of mitomycin C.

Strain	Concentration of MC (µg/ml)							
	0.00	0.05	0.06	0.08	0.10	0.12	0.14	0.20
R1	+++	+++	+++	+++	+++	++	+	-
104	+++	+++	+++	++	+	-	-	-
P2	+++	+++	++	++	++	-	-	-
AM_2	+++	+++	++	+	-	-	-	-
AM_1	+++	+++	++	+	-	-	-	-

[+++ : heavy growth; ++ : moderate growth;
+ : slight growth; - : no growth.]

and incubated O.N. at 30oC there was no significant change in the number of survivors during the 60 minute heat treatment, i.e. the cells were neither dividing nor dying. However when 0.1 ml of the same culture was plated directly on M17 plates and then incubated O.N. at 44oC the results shown in Table V were observed.

Microscopic examination of one small colony from the plate incubated at 30°C and one small colony from the plate incubated at 44°C showed no morphological difference, with chains of 5 to 10 cells each. However microscopic examination of one of the mucoid colonies picked from the 44°C plate showed predominantly chains of only one, two or three cells. A culture made from one mucoid colony was further tested for lysogeny by examining in response to UV light. It did lyse in $2\frac{1}{2}$ hours after 10 sec. exposure to UV and the resulting lysate gave plaques on 368. In addition, the new strain, designated R_{1t}, was still immune to the lysate of the parent R_1.

2. Measurement of induction of lysis of S. cremoris strains.

In order to study induction, we must select some phenotypic change in a lysogen that is associated with induction and is convenient to measure. I used the production of viable phage and the clearing of a lysogenic culture owing to cell lysis as indicators of induction, although these criteria are not necessarily appropriate when the prophage is defective.

2.1 Ultraviolet induction of strains P_2, AM_1, 104 and R_1

Logarithmically growing cultures of the strains P_2, AM_1, 104 and R_1 were irradiated for 10 seconds as fully described in 'METHODS', and examined for lysis. Figure 3 gives the results obtained with the four strains. S. cremoris strain P_2 showed lysis after 3 hours of incubation at 30°C; strain AM_1 showed a bit of lysis after 4 hours of incubation. S. cremoris 104 and and R_1 were both readily inducible by U.V light after 10 seconds exposure. Lysis was not observed in any of the strains when the exposure to UV lasted only 5 seconds.

TABLE V Survival of strains R_1 after an O.N. incubation at 44°C and morphological and other characteristics of the survivors.

Temperature of O.N. incubation	Dilution	Count	Titer	Morphology of colonies	Morphology of cells	Lysogenic
30°C	$1x0.1x10^{-4}$	350	$3.5x10^7$	Small smooth colonies	Chains	+
	$1x0.1x10^{-6}$	4	$4.0x10^7$	" "		
44°C	1x0.4un	320	$8.0x10^2$	Small smooth colonies	Diplococci	+
	1x0.1xun	80	$8.0x10^2$	Some larger mucoid colonies		

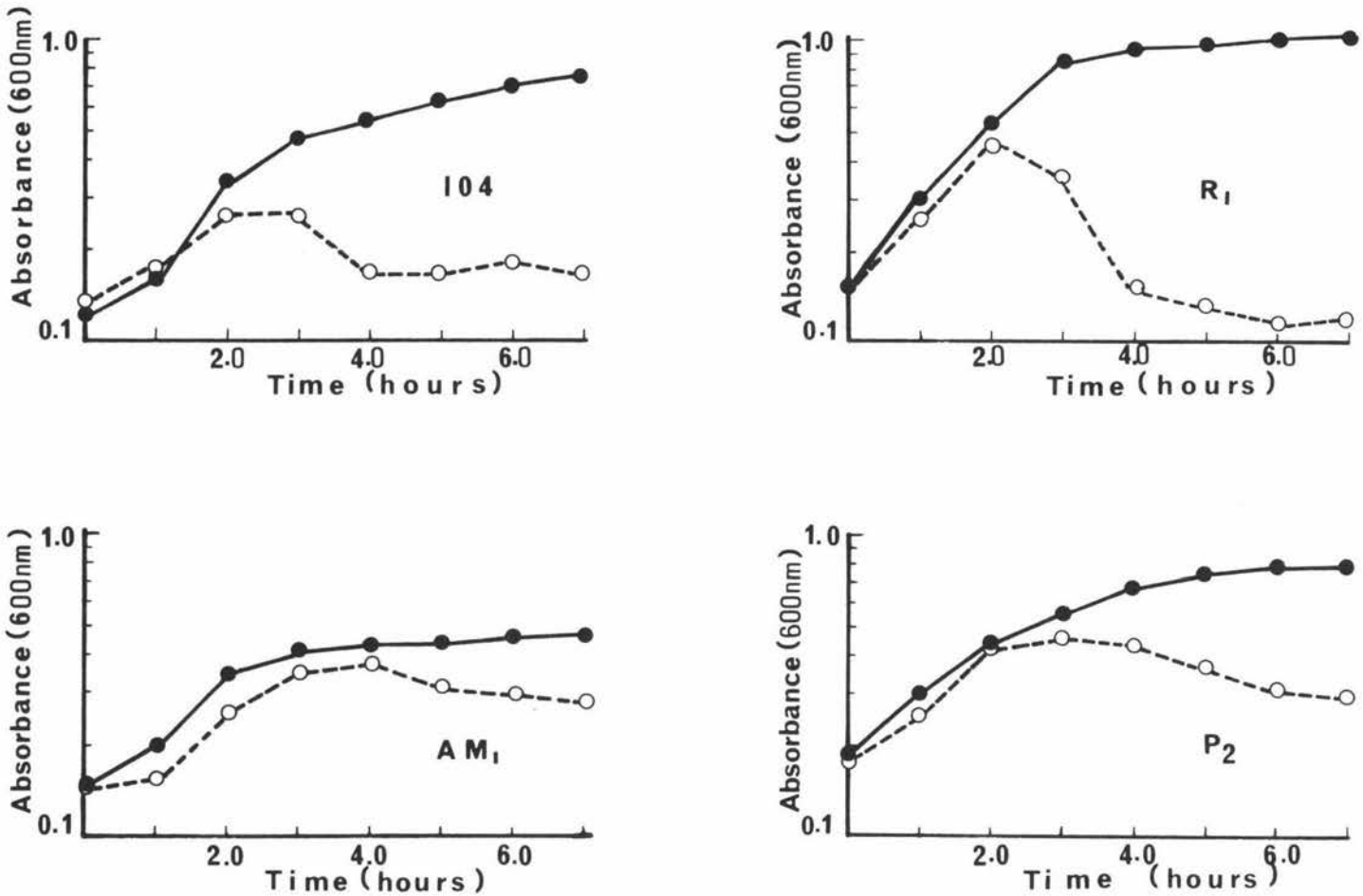

Figure 3: UV induction of lysis of *S. cremoris* strains P2, AM1, 104 and R1. Logarithmically growing cultures of each strain were washed, resuspended in chilled phosphate buffer and irradiated with UV light for 10 seconds. They were then diluted into M17 broth supplemented with Ca^{2+} and incubated at 30°C until lysis occured or until cells reached the stationary phase of growth. A non-irradiated sample was kept in parallel as a control. Readings at OD_{600} were made at 30 min intervals on a Bausch and Lomb Spectronic 20.

2.2 Mitomycin C induction of strain R_1

Logarithmically growing cultures of the strain R_1 in M17 medium were subjected to mitomycin C at a final concentration 0.1µg/ml. The cultures were then divided into two aliquots; one of them was incubated at 30oC, the other was washed after 20 minutes (to remove the MC), resuspended in fresh medium, and incubation continued at 30°C. Extinction measurements were made as described under METHODS and the results are presented in Figure 4. It is clear that no significant difference between the washed and non-washed cultures was observed and that in both cases MC induction of strain R_1 resulted in a pronounced release of mature phage particles as found by assaying the R1 lysate obtained on the indicator strain 368. It was also found that the age of the culture might play a significant role since no lysis was observed when an overnight culture was diluted to an optical density of 0.15 at 600nm ($2x10^8$ chain/ml) and treated with MC as with the log phase cultures (Fig 4).

2.3 Heat induction of S. cremoris R_1

The heat induction was done as described in Methods and the optical density of the heated cells was followed for 6 hours after the heat treatment. No cell lysis was observed during that time as can be seen from Figure 5.

It is clear from the above results that the UV and MC sensitivity is not necessarily correlated with temperature sensitivity. From the results presented here we can conclude that in S. cremoris R_1 the prophage induction is heat stable and UV and MC sensitive.

3. Isolation of a 'cured' strain of S. cremoris R_1

Among the five given strains AM_1, P_2, 104, 158 and R_1 of S. cremoris strains, R_1 was finally selected for curing because it is a known lysogen (Lowrie 1974), it shows the best response to the inducing agents (Figures 3 and 4) and its resident prophage has an indicator strain S. cremoris 368. In addition, R_1 is a rather slow acid producing strain and

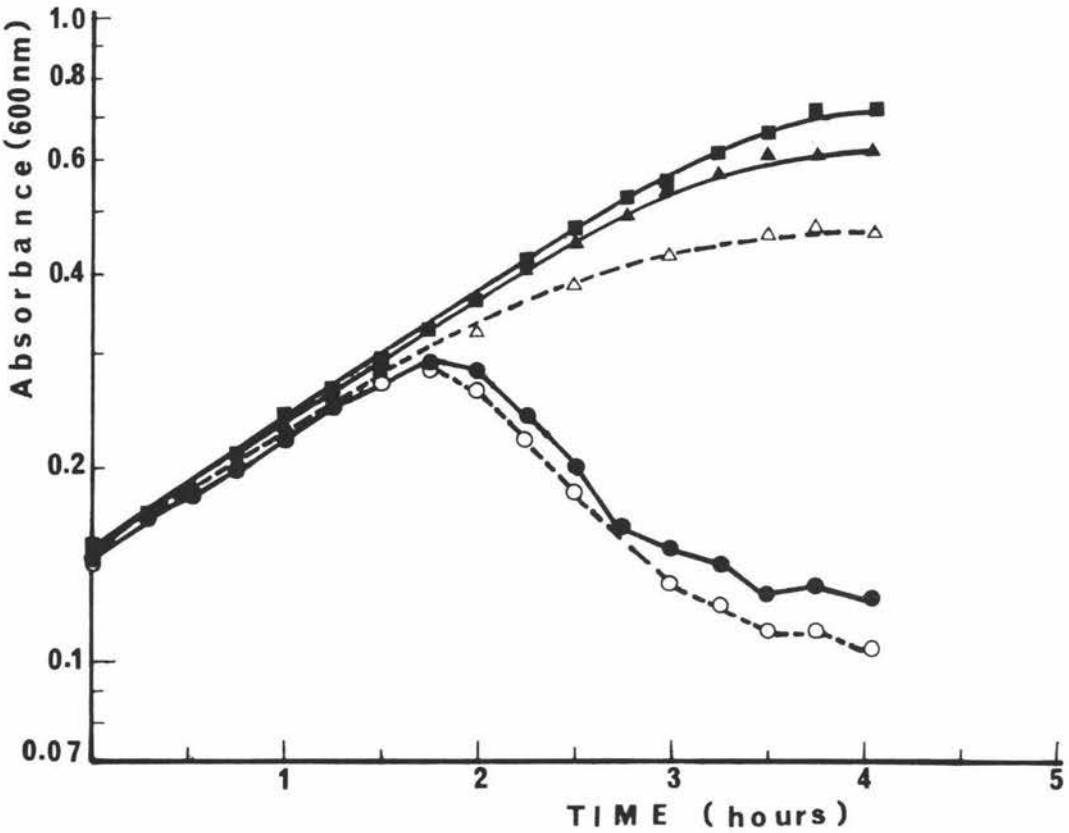

Figure 4: Induction of lysis of strain R_1 by MC. Logarithmically growing cultures of R_1 were subjected to MC at a final concentration of 0.1 μg/ml. The culture was then divided into two aliquots; one of them was incubated at 30°C, the other was also incubated at 30°C, but then washed after 20 minutes to remove the MC, resuspended in fresh broth and incubation continued at 30°C. A control without MC was grown in parallel as a control. An O.N. culture was diluted in to fresh M_{17} broth to an O.D. of 0.15 and also subjected to 0.1 μg/ml MC and incubated at 30C for 4h. (■—■ control culture, ●—● non washed culture, ○---○ washed culture, ▲—▲ O.N. culture (control), △--△ O.N. culture subjected to MC).

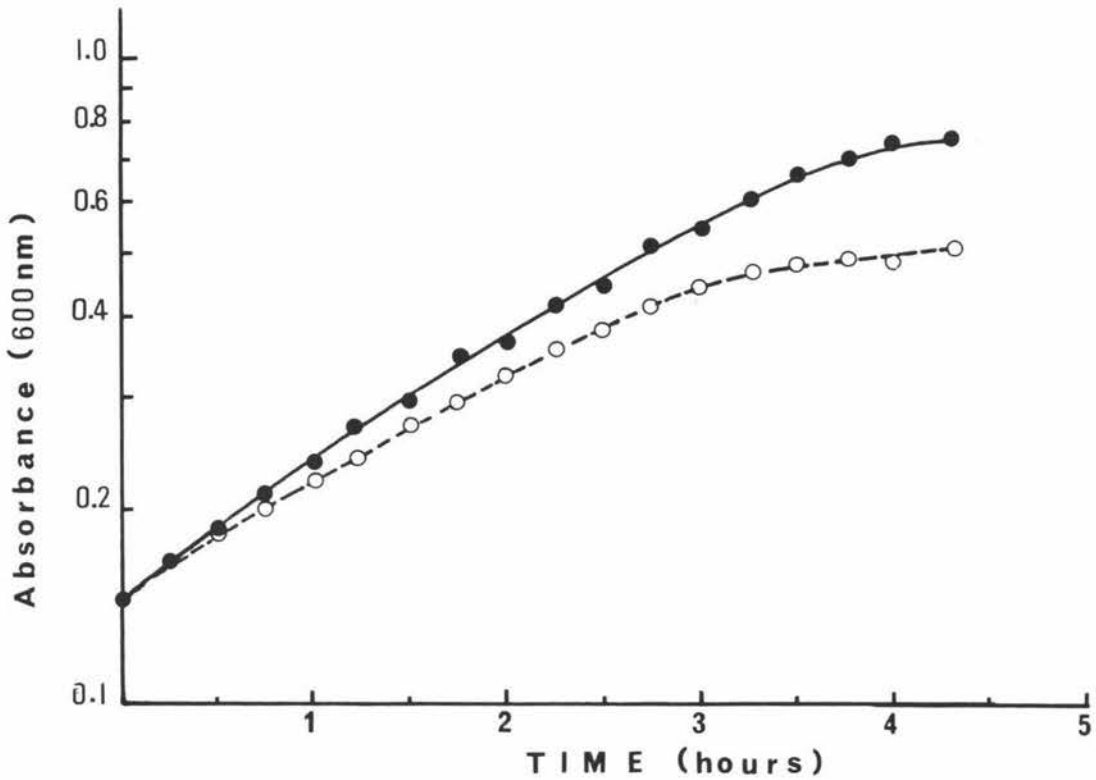

Figure 5: Induction of lysis of *S. cremoris* R_1 by heat. Logarithmically growing cells (3½ hrs, 30°C) were diluted 10-fold into fresh M17 broth and incubated for 12 min at 43°C. Incubation continued at 30°C for 5 hours and the OD was followed at intervals of ½ hour at OD_{600} ● , Control; ○ , heated culture;

it was interesting to see whether the removal of the prophage had any effect on acid production or on any other feature of cell physiology as was claimed by Sandine et al (1962).

A cured derivative was finally produced as described in METHODS and it will be designated as R_1 cured. This strain showed the following differential characteristics:

(i) It was not inducible by either UV light irradiation or MC treatment.

Figure 6 shows the growth and induction curves of strain R_1 and its cured derivative.

No lysis was observed even after 5 hours following 10 sec of irradiation of the cured strain, in contrast with the parent R_1 which started lysing 2 hours after UV irradiation and had completely lysed after 4 hours incubation at 30oC.

(ii) Loss of immunity by the cured derivative

If the induced lysate of R_1 is plated with the cured derivative the results of Table VI are obtained. In the same table the results of plating the induced lysate of R_1 with its indicator 368 are also presented.

TABLE VI Plaque forming ability of the induced lysate of strains R_1 and R_1 cured.

UV induced lysate of strain	Bacterial strain		
	R_1	R_1 cured	368
R_1	-	3.3×10^4	2.6×10^6
R_1 cured	-	-	-
Non induced R_1 (free phage)	-	1.2×10^2	3.4×10^2

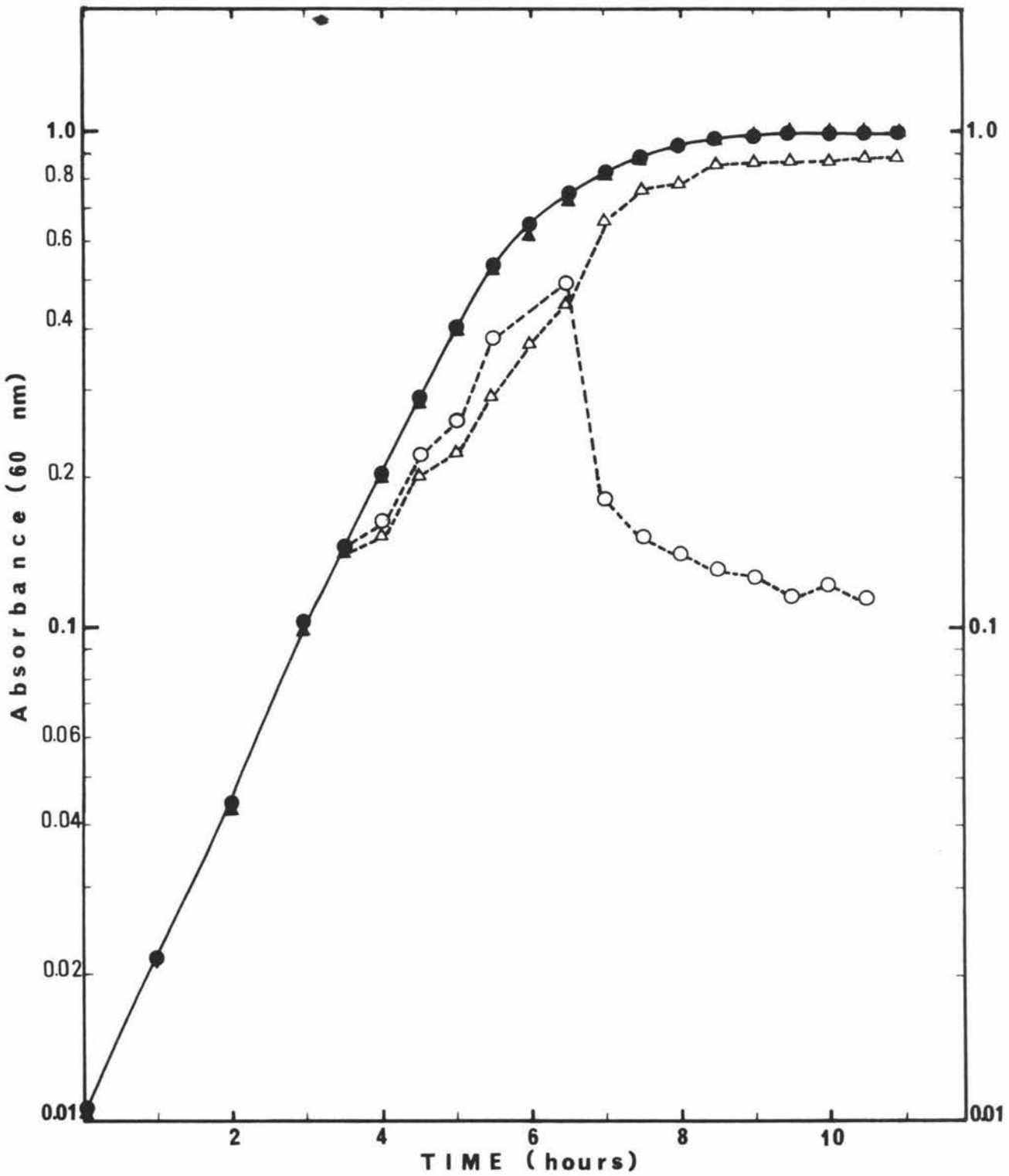

Figure 6: Growth and UV induction curves of strains R_1 and R_1 cured. Twenty-milliliter of M17 broth were inoculated with the appropriate strain (1% inoculum from 16 -h, 22C cultures) and incubated for 3.5 hrs at 30°C. They were then centrifuged, resuspended in 10ml chilled phosphate buffer and divided into two portions each: one portion of each culture was irradiated for 10 seconds and the other, being unirradiated, served as the control. All cultures were then inoculated into 7ml M17 broth supplemented with Ca^{2+} and incubated for 5 hours at 30°C. Photoreactivation was avoided by carrying out the post-irradiation procedures under a dim yellow light and incubating in the dark. Growth and lysis were measured as changes in OD.
●, *S. cremoris* R_1 unirradiated; ○, R_1 irradiated; ▲, R_1 cured unirradiated; △, R_1 cured irradiated.

It is important to note here that only about 10^5 plaque forming units (PFU) /ml were induced. Several explanations can be given for this low efficiency of plating of the induced lysate of R_1:

(i) Rapid adsorption of the induced lysate to cell debris. In the case of group H streptococcal temperate phage system, Parsons and Colon (1973) showed that rapid adsorption of the induced phage to cell debris may cause losses as high as 99.9% of PFU/ml. However, in the case of R_1 I did not find such loses since an electron microscopic examination of the cell debris revealed no phage particles.

(ii) The induced phage has high efficiency of lysogenization of both strains 368 and R_1 cured. However all attemps to lysogenise 368 were unsuccessful and the efficiency of lysogenization of R_1 cured was not high enough to explain the low EOP of the R_1 lysate.

(iii) A high proportion of the induced phage particles from R_1 are defective.

(iv) Production of bacteriocin together with the phage after exposure to UV or MC.

Clearly it is important to establish the identity of the products of a UV induction, a task in which the electron microscope is of considerable value.

4. Electron microscopic examination of the UV induced lysate of R_1.

Electron microscopic examination of the UV induced lysate of R_1, prepared as described already in METHODS did not show the presence either of a defective prophage or of bacteriocin, but instead revealed the presence of two morphologically different bacteriophages which can be seen in Plates 3 & 4. Both have heads which are isometric and appear to be octahedral

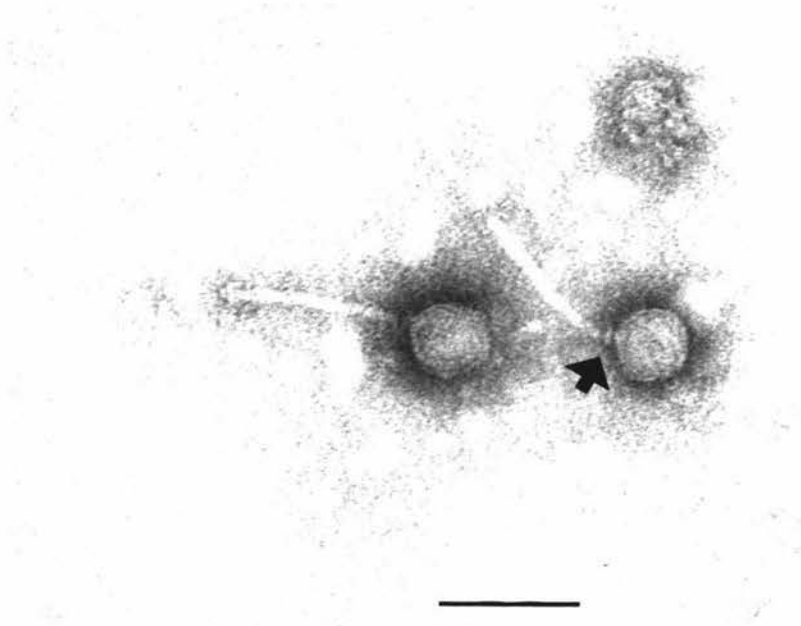

Plate 3 Phages r_1t_1 and r_1t_2 liberated from R_1 after UV irradiation. Arrow indicates the presence of a collar. Bar indicates 100nm.

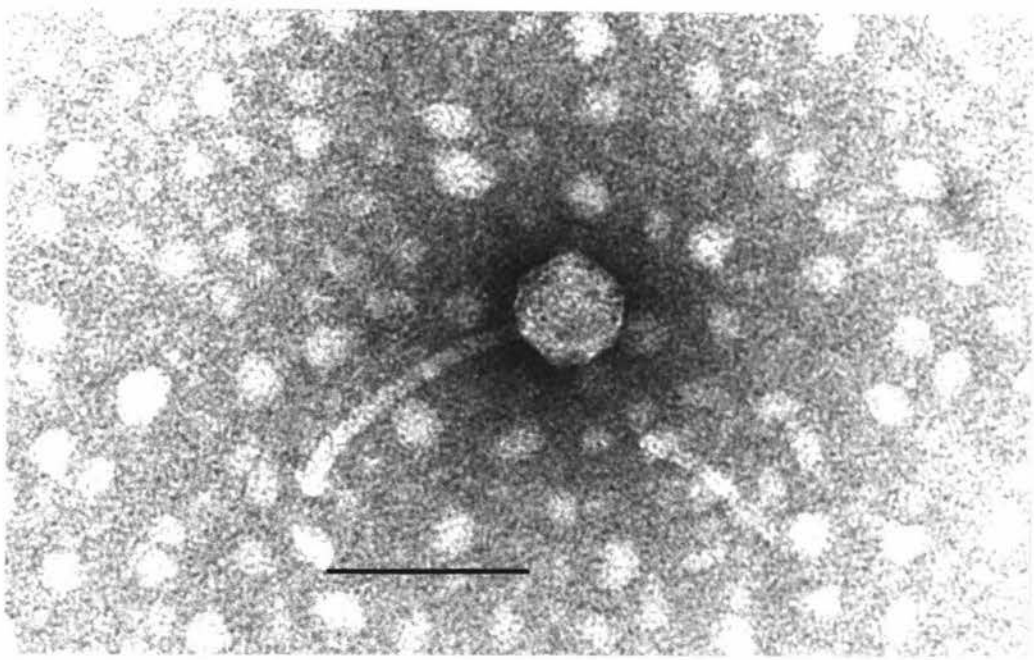

Plate 4. Phage r_1t_2 liberated from R_1 after UV irradiation. The bar represents 100nm.

in shape, measuring 55nm across, and are differentiated by flexible noncontractile tails of different lengths. One of them has a tail length of 122nm and a well defined collar. The tail length of the other phage is longer measuring 133nm and it has no collar.

Microscopic examination of the lysate of the cured derivative showed no trace of phage particles of either type, and no signs of other phage or bacteriocin related material was observed.

5. Density gradient analysis of the induced lysate of R_1

The CsCl density gradient profile of phages present in the induced lysate of R_1 is shown in Figure 7. It can be seen that when the fractions are plated on 368 the PFU peak has a buoyant density of approximately 1.474g/cm3. But when the same series of fractions are plated on strain R_1 cured the peak now has a buoyant density of 1.500g/cm^3 although a small shoulder is always found at a density of 1.474g/cm^3. In order to confirm these results phage stocks were prepared on 368 and R_1 cured, concentrated as described in METHODS, and subjected to CsCl density gradient centrifugation. Titration of each fraction from each phage stock on its propagated host gave the results seen in Figure 8, and are in excellent agreement with the earlier determinations. The phage induced from R_1 and propagated on 368 was the one with buoyant density of 1.474g/cm^3.

6. Liberation of phages from lysogenic R_1 and their apparent modification and restriction by strains R_1 cured and 368.

As was shown before an induced lysate of R_1 prepared as described under Methods contains two different bacteriophages distinguishable in the electron microscope and in CsCl density gradients. These two phages have as indicators the nonlysogenic strain R_1 cured and strain 368. Analysis of the infection of these two indicator strains with the phages liberated from R_1 revealed that both phages could undergo a host induced modification. (Figure 9).

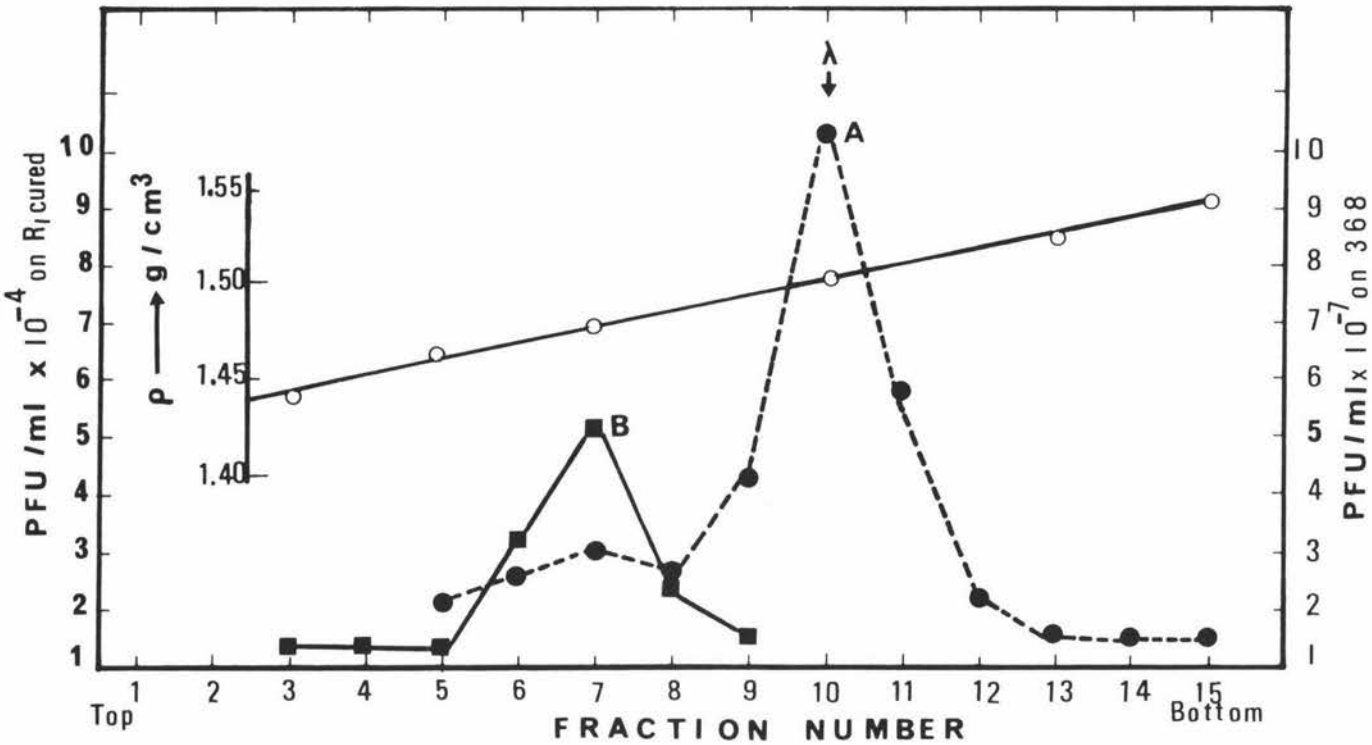

Figure 7: Density distribution in a CsCl gradient of phage particles from an induced lysate of R_1. A lysate of strain R_1 induced as in Methods, was concentrated by high speed centrifugation and banded in CsCl of average density = 1. 48g/cm^3. The gradient included bacteriophage lambda as a density marker. Twenty seven fractions were collected from the top of the tube and the density of each measured refractometrically. Peaks were located by spotting on lawns of the strains 368 and R_1 cured and also the indicated on *E. coli* A B 3013. Peak fractions were then diluted and plated with the same host strain.

■ lysate R_1 on 368 ; ● lysate R_1 on R_1 cured.

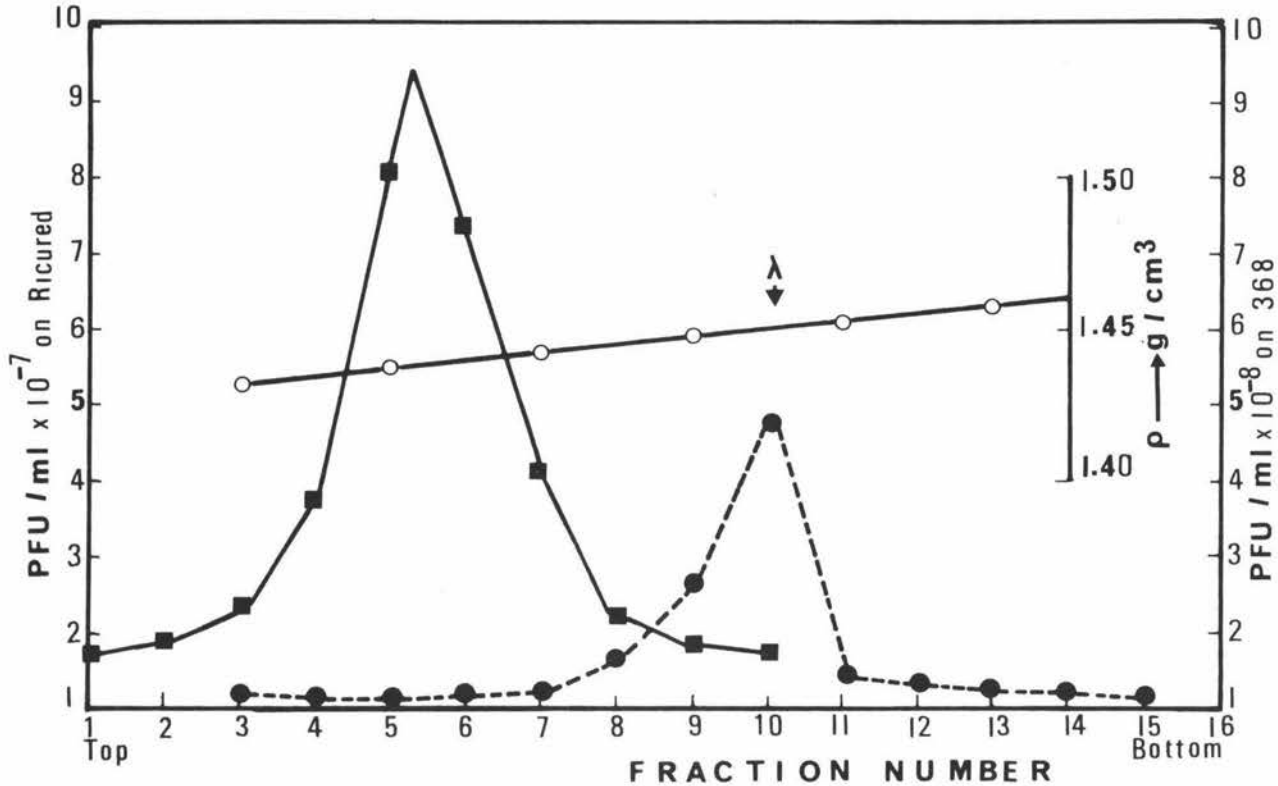

Figure 8: Density distribution in a CsCl gradient of phages liberated from R and propagated on R_1 cured and 368 Phage from peak A and B (Fig 2) were grown as plate stocks on R_1 cured and 368 respectively, harvested, and the phage density distribution analysed as described in Fig. 7.

- ● phage propagated on R_1 cured
- ■ phage propagated on 368

When a logarithmically growing culture of strain R_1 is induced by UVL irradiation, complete lysis is observed after almost 4 hours. If every cell in the culture produces phage then the induced lysate will contain a minimum of $2x10^8$ PFU/ml. However when this lysate is assayed on strains 368 and R_1 cured the titer of the lysate is found to be $1.8x10^6$ and $3.3x10^4$ respectively. If one of the plaques that formed on host R_1 cured is picked, repurified on R_1 cured and a phage stock made, this phage called $r1t_1$ plates on R_1 cured with a defined EOP of 1 but only with an EOP of $5.2x10^{-4}$ on 368. If now one of the rare plaques which form on 368, is picked, repurified on 368, and used for making a stock on 368, it is found to consist of particles which plate on 368, with a defined EOP of 1 but with an EOP of only $6.0x10^{-7}$ on R_1 cured. Figure 9 shows this apparent host induced modification and restriction of both phages.

7. Serological examination of the liberated phages.

The serological criterion is one of the most important of the taxonomic criteria of bacteriophages because it is experimentally simple and it has been shown to be the most useful single test of phylogenetic relationship. The neutralization of the infectivity of one phage strain by the antiserum to a second phage strain indicates a close biological relationship between the two strains.

Unfortunately it was not possible in our laboratory to prepare phage antiserum against the phages liberated by R_1. So we decided to do the neutralization tests using antisera against virulent phages similar in morphology to the two temperate phages liberated by R_1, and which was kindly supplied by Dr A. Jarvis. The antisera was prepared by Dr A. Jarvis as described by Adams (1959). The phages used for the preparation of antisera were the isometric $853/AM_1$ which has a collar and shows morphological similarity with the collared temperate phage induced by R_1 (designated r_1t1), and the isometric phage 936/158 which lacks a collar and shows morphological

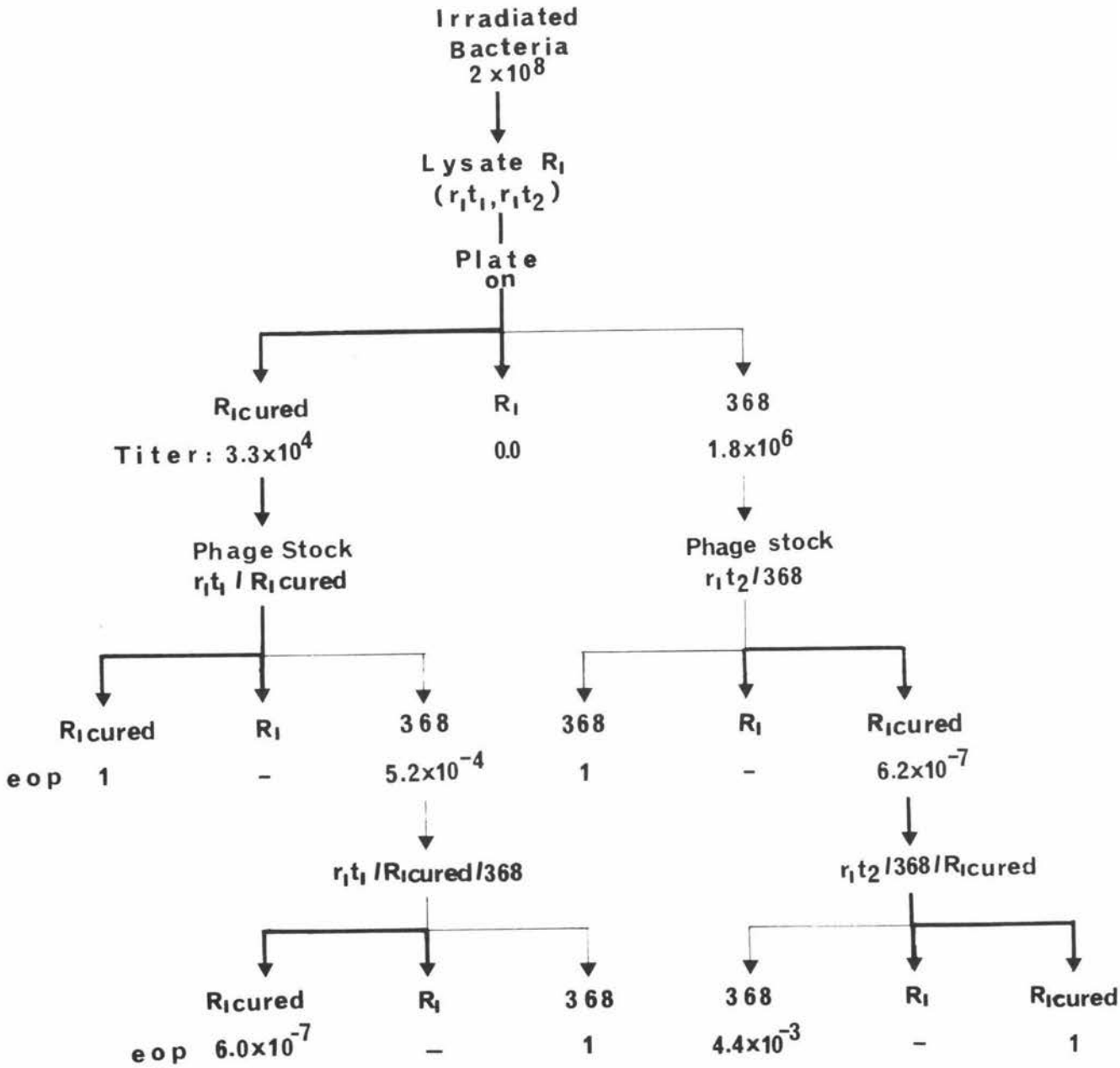

Figure 9: Host induced modification and restriction of phages liberated by R_1 on strains R_1 cured and 368.
A logarithmically growing culture of R_1 was centrifuged, resuspended in chilled buffer and irradiated for 10 seconds as described before. The induced lysate was then plated on the indicator strains R_1 cured and 368, and individual plaques were reisolated and phage stocks prepared as described in the text. Each phage stock was then assayed on its homologous and heterologous host. The numbers give the EOP of the phage variants r_1t_1 r_1t_2.

similarity with the collarless temperate phage r_1t_2, liberated by R_1. Phages r_1t_1 and r_1t_2 were treated with anti-853/AM_1 and anti-936/158 sera. No serological relationship was revealed by cross neutralization tests with anti-853/AM_1 and anti-936/158 sera between r_1t_2 and either of the two virulent phages. No serological relationship was found between r_1t_1 and 935/158. However phage r_1t_1 showed 40-50% neutralization with anti 853/AM_1 serum which is an indication of serological relationship between the two phages. The results obtained from this experiment are shown in Table VII.

8. Physiological and Microbiological examination and characterization of strains R_1 and R_1 cured.

8.1. Physiological characterization

8.1.1. Morphology

No difference was observed in morphology between the R_1 and R_1 cured when a culture of each is grown in M17 broth at 30oC for $3\frac{1}{2}$-4 hours, and then examined in the phase contrast microscope. Under these conditions both strains give medium to long length chains (15-35 cells per chain). No difference in colony morphology was observed when the two strains were diluted and plated on M17 agar/plates. Both strains have colonies that are usually small uniform, smooth and round with a whitish or grayish appearance.

8.1.2. Sensitivity of R_1 and R_1 cured to different inducing agents.

(i) Sensitivity to Mitomycin C.

Sensitivity of cells of the two strains to MC was tested by spotting on nutrient soft agar containing different concentrations of the drug and incubating overnight at 30oC. MC was sterilised by filtration and added to the autoclaved medium at 45°C before pouring plates. Table VIII presents the results obtained by spotting the two strains on MC containing plates.

TABLE VII Cross neutralization test of r_1t_1 and r_1t_2

Phage	Control	After neutralization* with	
		Anti-853/AM_1***	Anti-936/158***
r_1t_1	$6.3x10^{6}$**	$3.10x10^{6}$	$6.2x10^{6}$
r_1t_2	$2.9x10^{6}$	$2.7x10^{6}$	$2.9x10^{6}$

* Incubation at 37C for 15 min.

** PFU /ml

*** Dilution 1:500

TABLE VIII Mitomycin C resistance of R_1 and R_1 cured
(+++ : heavy growth; ++ good growth; + slight growth
- : no growth)

Concentration of μ^gMC (μg/ml)	R_1	R_1 cured
0.10	+++	+++
0.12	++	+++
0.14	+	+++
0.20	+	+++
0.50	+	++
1.00	-	++
2.00	-	++

Cells of R_1 were unable to grow in the presence of 0.5 g/ml MC in contrast to strain R_1 cured which did grow even in the presence of 2.0μg/ml MC. This was expected since MC is a good inducing agent and by induction of the prophage the host is killed. In the case of the R_1 cured the cells presumably do not carry prophage which can be induced by the MC and so R_1 cured is resistant to higher concentrations of MC.

In the course of this experiment a mutant of R_1 was isolated from the plate containing 0.5μ g/ml MC. This mutant will be designated R_1MC and will be described later together with the mutant R_{1t} isolated as described before.

(ii) Ultraviolet light and temperature sensitivity of R_1 and R_1 cured.

As shown in Fig 10, the UV survival of both strains manifested multiple-hit kinetics of killing of colony forming units, which is to be expected of a chain forming organism. No significant difference was observed in UV sensitivity (slope of the survival curves), although the cured strain appeared to require

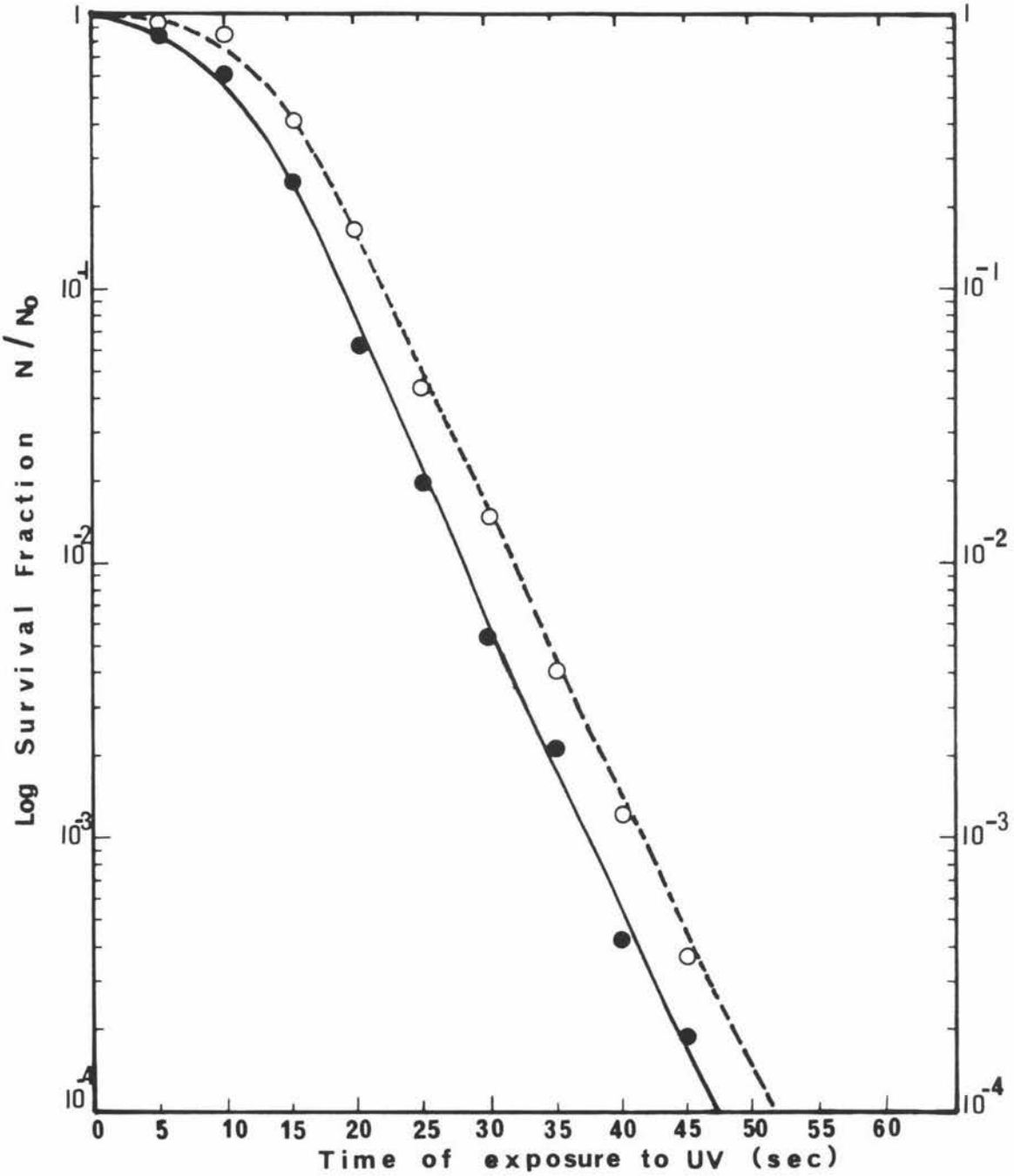

Figure 10: Survival of *S. cremoris* R1 and its cured derivative after exposure to UV irradiation. Logarithmically growing cultures were centrifuged (8,000 rpm, 10 min), resuspended in phosphate buffer and irradiated at different time intervals. Irradiated samples were diluted and plated on M17 agar plates as soon as possible after irradiation.

● *S. cremoris* R1, ○ *S. cremoris* R1 cured

twice as many hits to inactivate a colony forming unit. As this difference is not reflected in the average chain length it must be due, in some unspecified fashion to the difference in lysogenic state of the two strains.

No difference was observed in temperature sensitivity of the two strains and this was expected since it was shown before that the prophage resident in R_1 is not thermoinducible.

8.1.3. Acid production studies

(i) Acid production in milk of strains R_1 and R_1 cured.

Flasks containing 200ml milk medium (11% reconstituted non-fat milk solids (NFMS) were autoclaved for 12 min at 120°C) inoculated with 1% milk culture of each of the above stains. The flasks were incubated at 30°C in a thermostatically controlled water bath. At the time of incubation and at selected time intervals, 20ml aliquots of milk were asceptically withdrawn and the pH of the culture was measured. The results are shown in Figure 11. No significant difference was observed between the two strains.

(ii) Acid production in broth

The effect of varying the sugar source (lactose, glucose, galactose and sucrose) on the acid production of strains R_1, and R_1 cured was measured in lactic broth prepared as described in Materials and Methods. The broth cultures were incubated at 22°C for 15 hours and the final pH was measured. At this time 0.1ml of an appropriate dilution was plated on M17 lactose agar plates for colony counts. Table IX shows the results of acid production in broth containing different sugars.

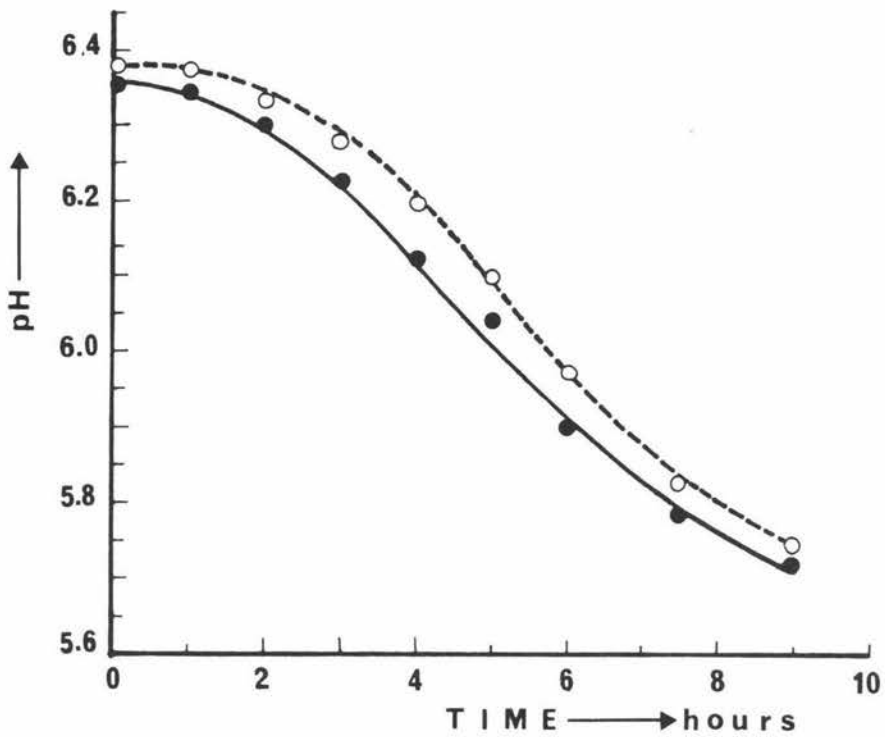

Figure 11: Lactic acid production in milk by *S. cremoris* R_1 and R_1 cured. Flasks containing 200ml milk medium were inoculated with 1% milk O.N. culture of each of the above strains. The flasks were incubated at 30°C and at selected time intervals 20ml aliquots of milk were asceptically withdrawn and the pH of the culture was measured.

● R_1 ○ R_1 cured

TABLE IX Final pH of broth cultures after 15 hours at 22°C

Strain	Substrate					
	Lactose		Maltose		Sucrose	
	Initial pH	Final pH	Initial pH	Final pH	Initial pH	Final pH
R_1	7.00	5.80	6.80	6.40	7.20	7.15
R_1 cured	"	5.85	"	6.47	"	7.19

(iii) Examination of lactose metabolism of R_1 cured.

Lactic agar containing 1% lactose as the primary carbon source supplemented with 0.004% BCP, served as the indicator medium. Plates were spread with 0.1ml of the appropriate dilution of both the R1 and its cured derivative, and incubated at 30°C for about 48 hours. On this medium, lactose fermenting colonies are yellow in contrast to the white non-lactose fermenting variants. Both strains were found to be *lac*$^+$ although McKay *et al* (1972) suggests that the lactose marker might be a prophage gene and that the loss of the prophage would then result in a *lac*$^-$ varient.

(iv) Isolation of lactose-negative mutants of *S.cremoris* R_1 cured

The idea of this experiment was to produce *lac*$^-$ variants of the cured strain and then to attempt to transduce the *lac* character from the lysogenic strain to its cured derivative using bacteriophage from an induced lysate of R_1.

Attempts were made to produce *lac*$^-$ variants by exposing the cells to different concentrations of acriflavin (AF) using the method devised by McKay *et al* (1972). The results obtained are presented in Table X. These results indicate that no *lac*$^-$ variants could be isolated from R_1 cured and no *lac*$^-$ variants were observed to occur spontaneously.

TABLE X Effect of acriflavine(AF) on S. cremoris R_1 cured at different temperatures

Acriflavine (µg/ml)	21°C			30°C		
	CFU/ml	Colonies examined	No of *lac*⁻ variants	CFU/ml	Colonies examined	No of *lac*⁻ variants
0.0	$2.5x10^8$	250	0	$2.1x10^8$	210	0
2.0	$1.8x10^8$	180	0	$9.2x10^7$	92	0
4.0	$2.0x10^7$	200	0	$2.0x10^7$	200	0
6.0	$1.5x10^5$	150	0	$3.4x10^5$	340	0
8.0	$4.0x10^3$	40	0	$1.2x10^3$	12	0

Since one way AF causes mutations by eliminating extrachromosomal deoxyribonucleic acid (Hirota, 1960), results given in Table X indicate that the lactose marker in S. cremoris R_1 is not located extrachromosonally. However, no conclusion can be made since the data obtained up to the present time are not definitive and more studies should be made.

8.1.4. Production of ammonia from anginine

0.1ml sample of the appropriate dilution of the cultures of R_1 and R_1 cured were spread evenly on the surface of plates of BCP agar medium and inoculated for24-48 hours at 30°C as described in Materials and Methods. Both strains formed yellow colonies which indicates that neither strain is capable of deaminating arginine. This is characteristic of S. cremoris species.

8.1.5 Examination of the cured derivative on GMA medium.

GMA is a glycerophosphate buffered milk-based agar medium which has been developed for the differentiation of fast from slow coagulating organisms in starter cultures and has been described in Materials and Methods. Both strains R_1 and R_1 cured were found to grow well on GMA after three days incubation at 30oC and no difference was observed between the colony size of the two strains. Subsequent examination of the ability of the two strains to coagulate autoclaved milk, showed that both strains were rather slow with strain R_1 coagulating the autoclaved milk in 20 hours at 22°C and R_1 cured in 22 hours when used as a 1% freshly coagulated inoculum.

8.2. Microbiological Characterization.

This chapter deals with the effect of the resident prophages on the susceptibility of S. cremoris R_1 to various virulent and temperate phage types. From previous studies in other bacterial-phage systems it was shown that the prophage can confer on a bacterial cell an inability to support adsorption or

multiplication or both of some phages. For example Holloway and Cooper (1952) reported that Pseudomonas aeruginosa lysogenised with phage D3 showed changes in somatic antigens and loss of the ability to adsorb phage D3i. Barnet and Vincent (1970) reported that Rhizobium trifolii, lysogenic for phages 7 and 7cr, showed changes in somatic antigens and loss of the ability to adsorb phages 7, 7cr and 8. Lederberg (1957) found no difference in the adsorption of T phages on certain strains of Escherichia coli and Shigella dysenteriae lysogenic for phage P2, but he did find differences in the multiplication of these phages on the lysogenic and sensitive cells.

8.2.1. Effects on the adsorption and multiplication of various virulent phages of S. cremoris on the lysogenic strain R_1 and its cured derivative.

To examine the above effects six serologically and morphologically different virulent phages were used.

(i) Adsorption of various virulent phages.

The adsorption experiment was done as described under Methods and the results obtained are shown in Table XI. It is clear that there is no significant difference in the adsorption of the different virulent phages between the lysogenic and non lysogenic strain, and all phages were found to adsorb to both strains to the same extent. This suggests that there is no prophage directed alteration in the surface phage receptor sites.

(ii) Multiplication of various virulent phages

No change in the phage pattern was observed following curing of strain R_1. The efficiency of plating of phages 879/368 and 652/166 remained almost the same in both hosts and the inability of R_1 to propagate the phages $690/SK_{11}$, $949/AM_1$, 799/368 and 949/402 remained unchanged after the removal of the prophages (Table XII). These results are in agreement with those reported by Lowrie (1974) who artificially lysogenised Str. cremoris Sk_{11} with a temperate phage liberated also from R1 and found no change in the sensitivity of the SK_{11}

TABLE XI Adsorption of virulent bacteriophages on strains R_1 and R_1 cured

Phage Strain	Fraction of unadsorbed phage*	
	S. cremoris R_1	S. cremoris R_1 cured
879/368	0.18	0.08
690/SK_{11}	0.14	0.14
652/166**	0.00	0.06
949/AM_1	0.02	0.00
799/368	0.05	0.02
949/402	0.47	0.38

* Ratio of the number of PFU due to free phage remaining unadsorbed at 10 min to the number of PFU added.

** 652 is referred to as phage r_1v by Lowrie (1974).

TABLE XII Lytic spectrum of virulent phages

Infecting phage		Host strain	
		R_1	R_1 cured
289/368	I	1.1×10^{-7}	4.1×10^{-7}
690/SK_{11}	P	0.0	0.0
652/166	I	2.8×10^{-6}	1.8×10^{-5}
949/AM_1	I	0.0	0.0
799/368	I + C	0.0	0.0
949/402	I	0.0	0.0

NOTE: the values on the ratios of the plaque titers on host strains to the titer on the phage propagating strain.
P : prolate ; I : isometric; C : collar.

lysogens to the two serologically unrelated virulent phages $601/AM_1$ and $690/SK_{11}$. However from the results that Lowrie gave it is not clear that lysogenization did occur and the immunity of SK_{11} to the R_1 resident phage might equally well be ascribed to the carrier state.

In contrast to the above results Terzaghi and Sandine (personal communication) claimed that artificial lysogenization of SK_{11} with a phage that it did not carry previously confers immunity to the attack of the three serologically and morphologically unrelated phages $601/AM_1$ (isometric), $690/SK_{11}$ (prolate) and $691/SK_{11}$ (isometric with collar).

(iii) Liberation of phage 652 by the lysogenic strain R_1 and its cured derivative.

The purpose of this experiment was to examine whether the presence of the prophages in strain R_1 confers any restriction to the multiplication of the virulent phage 652, similar to the restriction which E. coli B and Shigella dysenteriae lysogenised with bacteriophage P_1 confers to the virulent phage T_1 (Lederberg, 1957).

Table XIII shows that phage 652 propagated on strain 166 plated with equal efficiencies on strain R_1 and R_1 cured. Plaques of 652/166 grown on R_1 and R_1 cured were isolated and propagated on each of the two strains. The resultant lysates $652/166/R_1$ and $652/166/R_1$ cured plated on R_1 and R_1 cured with about equal efficiencies which shows that there was no difference between the lysogenic and cured strain in respect to modification or restriction of phage 652. However the efficiency of plating of phage 652 was always lower when the phage was propagated on the lysogenic R_1, although the difference was not significant.

TABLE XIII Lytic spectrum of bacteriophage 652 grown on strains 166, R_1 and R_1 cured

Phage	INDICATOR STRAIN		
	166	R_1	R1 cured
652/166	1	2.8×10^{-6}	1.8×10^{-5}
652/166/R_1	n.d	5.0×10^{-2}	1
652/166/R_1 cured	n.d	2×10^{-2}	1

NOTE: The 652 phage suspensions were phage stocks grown successively on the hosts as indicated by the symbols (the last host at the right). The values given are the ratios of the phage titers on host strains to be titer on the phage propagating strain
n.d. not determined.

8.2.2 Effects on the adsorption and multiplication of temperate phages on the lysogenic strain R_1 and its cured derivative.

There is evidence that the phages harboured by lysogenic strains usually are active only upon other bacterial strains belonging to the same broad group, as determined by phage typing (Wahl and Fouace, 1954; Rosenblum and Dowell, 1960). There is also evidence that certain changes in phage typing patterns, which follow artificial lysogenization are the result of serologically specific prophage immunity. The object of this investigation was to determine whether the presence of the two temperate phages in S. cremoris R_1 is associated with any changes in the adsorption of the phages for which it is normally lysogenic, and also if it is responsible for the inability of this strain to act as a host for phages induced by other strains. Failure to adsorb its homologous phages would suggest structural differences in the cell wall following lysogenization (Boyd,1954; Halloway and Cooper, 1962). If adsorption is unaltered the inability of the lysogenic strain to multiply temperate phages for which its cured derivative is sensitive would suggest that these phages are all closely related, at least to the extent of being homoimmune.

(i) Adsorption of the temperate phage, for which R_1 is normally lysogenic by R_1 and R_1 cured.

The adsorption experiment was done as described in Methods and the results are shown in Table XIV. No difference in the adsorption of r_1t_1 and r_1t_2 was observed between the strains R_1 and R_1 cured.

(ii) Multiplication on R_1 and R_1 cured of phages induced from other S. cremoris strains.

Initially 0.02 ml from overnight M17 broth cultures of 24 strains of lactic streptocci (22 S. cremoris and 2 S. lactis)

TABLE XIV Adsorption of phages r_1t_1 and r_1t_2 by strain R_1 and R_1 cured

Phage	Fraction of unadsorbed phage	
	S. cremoris R_1	S. cremoris R_1 cured
r_1t_1	0.05	0.10
r_1t_2	0.22	0.20

NOTE: Phage stocks of r_1t_1 and r_1t_2 were prepared from a UV induced lysate of R_1 plated on strains R_1 cured and 368 respectively. Single plaques of each were reisolated twice and phage stocks were prepared with the plate method as described in text.

which were previously found to be lysogenic (Terzaghi and Sandine, personal communications) were spotted on lowns of the naturally lysogenic strain R_1 and its cured derivative on M17 agar plates supplemented with Ca^{2+}.

I used overnight cultures for spotting instead of supernatants of these cultures because free phages which are always present in lysogenic cultures, seem to adsorb onto bacterial cell walls of some strains. Accordingly, after low speed centrifugation (6,000 rpm for 10 min) a large proportion of the phage may be removed and can be found in the pellet (Parsons et al, 1972).

UV induced lysates of cultures from each of the 24 strains spotted onto the lawns of strain R_1 and R_1 cured gave qualitatively the same results which are presented in Table XV. Strain R_1 is immune to the infection of the phages induced from the lysogenic strains 108, 158, R_1, H_1, 398, 240, 114, 130 and 286 for which the R_1 cured is sensitive. The most straight forward interpretation of these results is that the lysogenicity of R_1 confers extended immunity to a series of temperate phages liberated from various other lysogenic strains. It was of interest to compare the morphology and dimensions of the temperate phages which lysed R_1 cured with those phages for which R_1 is lysogenic. This comparison is shown in Table XVI. From Table XV and XVI we can say that the phages liberated by the strains examined can be put in two groups according to their morphology: one with shorter tail and collarless and another with longer tail and collar and that strain R_1, doubly lysogenic for phages from each group is immune to the attack of a wide range of morphologically similar phages.

TABLE XV Changes in phage patterns of strains R_1, R_1 cured and R_1r by phages induced from other lysogenic strains.

Phage resident in	Lawn of R_1	Lawn of R_1 cured	Lawn of R_1r
ML_8	-	-	-
368	-	-	-
134	-	-	-
108	-	+++	-
AM_1	-	-	-
P_2	-	+	-
AM_2	-	+++	-
158	-	+++	-
166	-	-	-
R_1	-	+++	+
H_2	-	-	-
266	-	-	-
316	-	-	-
E_8	-	-	-
H_1	-	++	++
104*	+++	+++	+++
398	-	+	-
SK_{11}	-	-	-
402	-	-	-
240	-	+++	-
386	-	++	-
114	-	+++	-
130	-	+++	-
286	-	+++	+++

+++ pronounded lysis; ++ moderate lysis; + slight lysis (2-3 turbid plaques); - no lysis.

* possibly an antibiotic effect rather than a phage effect

R_1: double lysogen; R_1 cured: cured derivative of R_1; R_1r: relysogenised with the phage r_1t_1 (see text for explanation).

TABLE XVI Structural features and dimensions of temperate phages of lactic streptocci

Phage induced from strain	Phage dimensions (in nm) ± 10%		Presence of	
	Head	Tail	Collar	Filament
R_1 H_1	57	120	-	+
R_1, 108, 114, 168	57	135	+	+

Note: The phage dimensions for strains H1, 108, 114 and 158 were obtained from B.E. Terzaghi (personal communication).

8.2.3. Relysogenization of R_1 cured.

It has been shown that R_1 is a double lysogen and that there are perhaps two types of temperate phages to which R_1 cured is sensitive. Therefore it was of interest to ask if relysogenization of R_1 cured with one of its temperate phage would allow differentiation of these two types of phage.

Lysogenic derivatives were obtained from the turbid center of plaques of R_1 lysate plated on R_1 cured, and purified by three successive streakings. The relysogenized strain will be designated R_{1r}.

In order to determine whether R_1r was lysogenic, induction of cultures grown from purified colonies was attempted with UV. The UV induction was done as described in Methods and the results are presented in Figure 12. The double lysogenic strain R_1 showed obvious lysis after 2.30 hours and complete lysis 4.30 hours after UV irradiation. Strain R_1r showed lysis also 2.30 hours after irradiation. Strain R_1 cured, as expected, did not show any response to the UV irradiation.

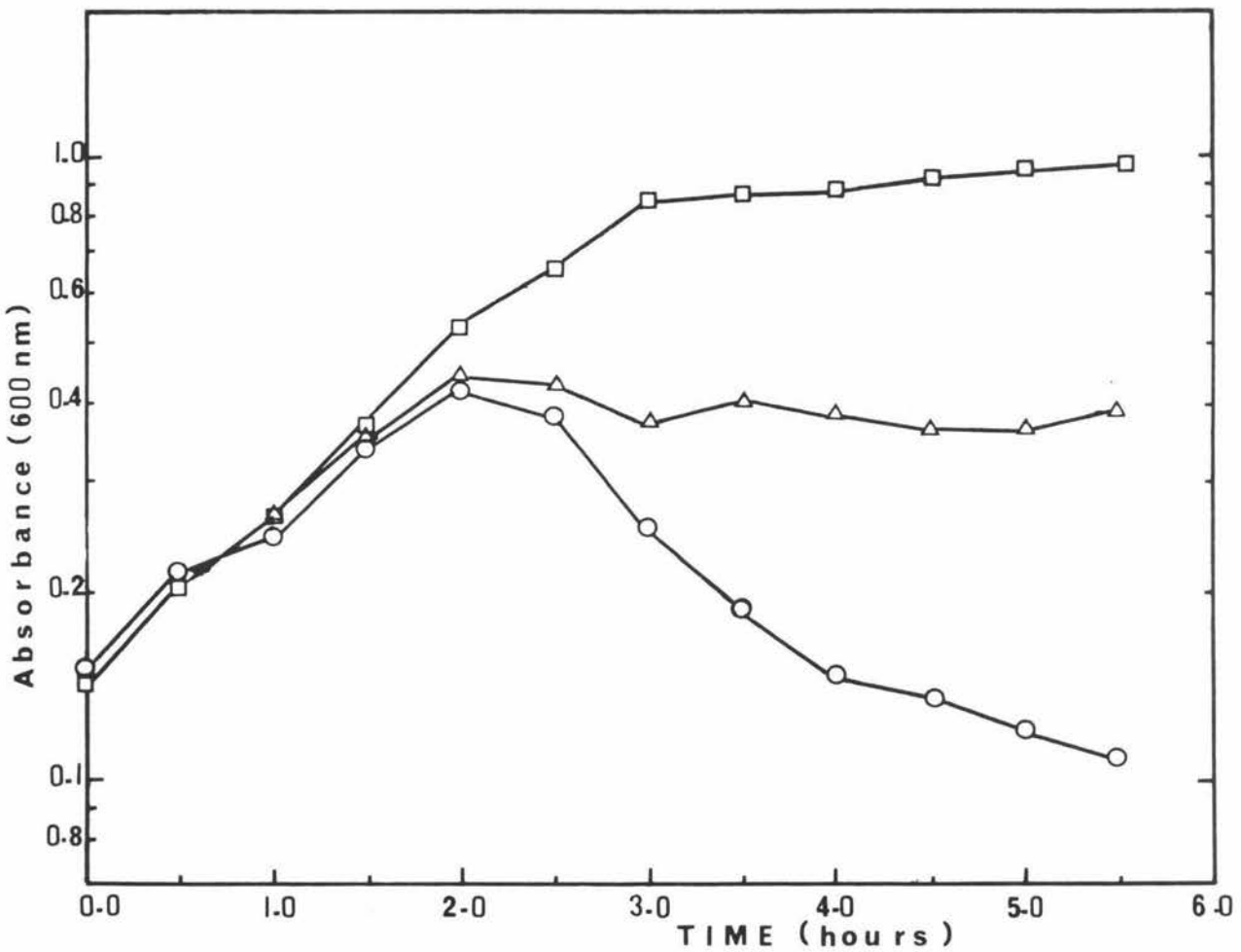

Figure 12: UV induction of lysis of *S. cremoris* R_{1r}. Logarithmically growing cultures of R_{1r}, R1 and R_1 cured were washed, resuspended in chilled phosphate buffer and irradiated with UV light for 10 seconds. They were then diluted in M_{17} broth supplemented with Ca^{++} and incubated at 30°C. The absorbance was measured at 600nm every half hour.

○ R^1

△ R_{1r}

□ R_1cured

To examine the immunity of R_1r to the phages liberated by R_1 the induced lysate of R_1 was spotted on R_1r. Also, to ensure that R_1r liberated phage by UV induction the lysate of R_1r was spotted on R_1 cured. Strain 368, indicator for phages liberated by R_1, was also included. The results obtained are shown in Table XVII.

TABLE XVII Infection of R_1, R_1r and R_1 cured with phages liberated from each strain respectively

Bacterial lawn	lysate R_1	lysate R_1r	lysate R_1 cured
R1	-	-	-
R_1r	+	-	-
R_1 cured	+++	+++	-
368	+++	-	-

+++ : confluent lysis; + : slight lysis;
- : no lysis

The results from Table XVII and Figure 12 suggests that R_1r was lysogenised by only one of the two phages liberated from R_1 and that the two phages were heteroimmune. To confirm this the lytic spectrum of the temperate phages liberated by the 24 lactic streptococci strains was examined on R_1r. The results are included in Table XV. R_1r was still sensitive to phages liberated by the strains H_1 and 286 but immune to those liberated by 108, P2, AM_2, 158, 398, 114, 386, 240, and 130. In addition, slight lysis was observed from spotting R_1 on R_1r; this is another indication that R_1r was lysogenized by only one phage and is still sensitive to the other.

9. Unusual derivatives of S. cremoris R_1.

During the course of the experiments involved in the isolation of a cured strain two unusual derivatives were isolated and designated R_1mc and R_1t. R_1mc was isolated from a plate containing 0.5 µg/ml Mitomycin C and R_1t from a plate incubated at 44oC overnight. (The isolation of R_1t and R_1mc mutants was described in chapters 1.3 and 8.1.2 respectively). These two derivatives had in common the morphology of their cells. As mentioned earlier S. cremoris R_1 forms long chains of 10-35 cells per chain, whereas the two mutants do not form chains but exist only as monococci and diplocci. Plate 5 and 6 shows the morphology of R1 and R_1mc respectively. Unfortunately it was beyond the scope of this project to examine whether any changes on the surface antigen of strain R_1 prevented the chaining.

9.1 Mutant R_1t

This mutant which was isolated from a mucoid colony of R_1 under the conditions described before, it was still lysogenic for the phages of R_1 as was showed from the electron microscopic examination of the UV induced lysate of R_1t. Its growth and induction curves were approximately the same as the parent strain, and the resulting lysate gave plaques when plated on the indicator strain 368. In addition the new strain was immune to the lysate of its parent R_1, and it did not adsorb the virulent phages 879/368, 949/AM1 and 799/368 which R_1 adsorbs. The most peculiar characteristics of this strain were those obtained from the biochemical tests and which makes the classification of this strain very questionable. These are summarised in Table XVIII. The new strain fermented sucrose and maltose whereas the parent strain and generally the members of the species of S. cremoris do not. In addition in contrast to strain R_1 it hydrolysed arginine and coagulated milk in 20 hours at 44°C. Growth at 44°C was as good as at 30°C.

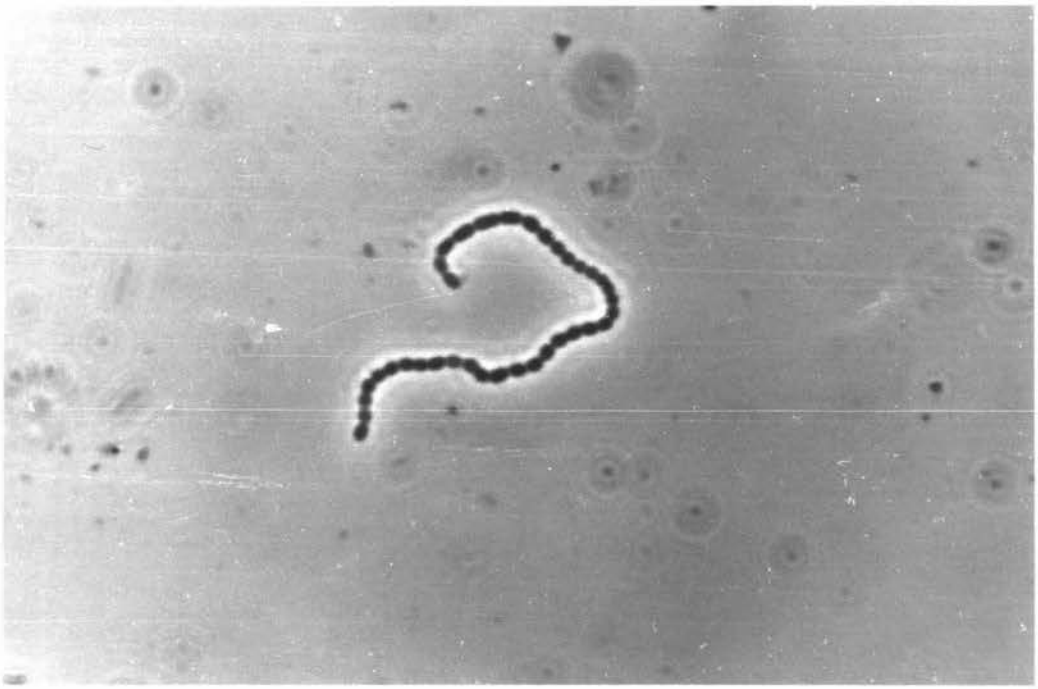

Plate 5 S. cremoris R_1 Chain formation characteristic of the strain. Photo taken in a phase contrast microscope from a 4-hour culture in M17 broth X 800

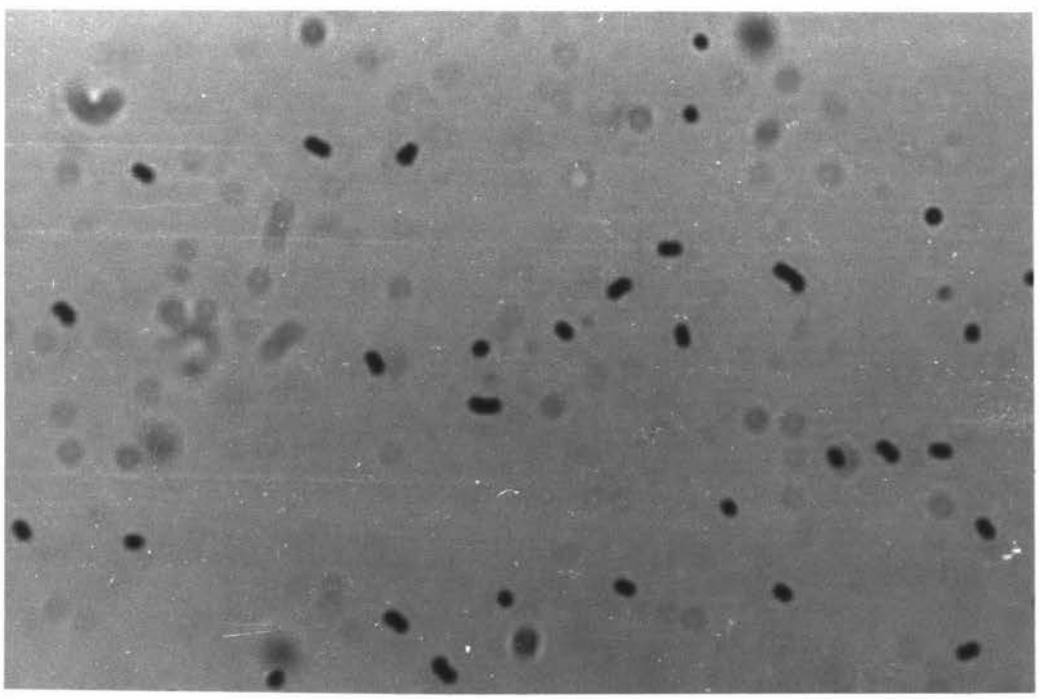

Plate 6 S. cremoris R_{1mc} - Note diploccal & monococcal morphology; smear taken from a 4-hour culture in M17 broth and stained with Methylene blue X 800

TABLE XVIII Differentiating characteristics of strains R_1 and R_1t

Characteristic	S. cremoris R_1	S. cremoris R_1t
Acid from		
Lactose	+	+
Glucose	+	+
Maltose	-	+
Galactose	+	+
Sucrose	-	+
Growth at 44oC	-	+
NH_3 from arginine	-	+
Clot milk at 30oC	18 hours	24 hours
" " " 44oC	-	20 hours
UV inducibility	+	+
Morphology	Long chains	Diplococci
Gram stain	Gram +	Gram +

9.2. Mutant R_1mc

Strain R_1mc had all the biochemical characteristics of its parent R_1, which are typical for the species S. cremoris. In respect to the ability to clot milk it can be classified as 'fast', since it clotted the milk in 15 hours at 22oC, although the parent is rather slow, clotting the milk in 20 hours at 22oC. No difference in phage sensitivity between R_1 and R_1mc was observed.

The most peculiar characteristicof this mutant was the presence of a characteristic motion of the cells, when examined in a phase contrast microscope, which did not seem to be a Brownian movement. Although the motility test using the semisolid medium technique (stab cultures of soft M17 agar) and incubating at room temperature was positive, attempts to stain flagella using the method of O'Toole (1942) failed.

Subsequent electron microscopic studies also failed to show positively the presence of flagella although flagella like formations were observed. Motile strains are quite common among varieties of S. faecium (group G streptococci) but in no other group has motility been encountered, as far as I am aware.

CHAPTER FIVE : DISCUSSION

This investigation was undertaken to examine the effects of lysogeny in group N streptococci. The phenomenon of lysogeny in this group of microorganisms has recently been investigated in several strains in different laboratories. However although more than twenty seven years have passed since Reiter (1949) first reported the occurence of lysogeny in lactic streptococci it is surprising to note that only recently a few reports deal with the incidence of this phenomenon in lactic streptococci and none on its effects on the host cell.

Sandine et al (1962) reported lysogeny in S. cremoris strain W and he stated that the resident temperate phage may play a role in metabolic variations among lactic streptococci such as for example, in the rate of acid production. Although he stated that subsequent studies will concern an examination of the role that lysogenization may play in variation among lactic streptococci no report of such studies have been published since.

McKay et al (1972), during studies of lactose metabolism in lactic streptococci, presented data suggesting that lactic streptococci could be carrying a genetic element which is responsible for the cells ability to ferment lactose. He further stated that lysogeny may be necessary to obtain lactose metabolism and the loss of the prophage could then result in a lac$^-$ variant. This was shown in Shigella dysenteriae, normally a lactose-negative organism, which can be converted to a lactose positive strain by incorporation of prophage (Barksdale, 1959).

More recently Park and McKay (1975) studied twelve commercial starter cultures and found nine to be susceptible to induction of lysis by UV irradiation or MC. However no indicator strains were found and in only two of the induced lysates was the presence of phage confirmed by electron microscopy. The same authors stated that ".....it may be

possible to isolate cured strains and if these possess no metabolic defects, to incorporate them into starter cultures. Such strains would not only guarantee phage-free cultures but also possibly reduce the phage problem."

In view of these speculations concerning the possible consequences of lysogeny among the group N streptococci there are several points to be made and discussed concerning the properties of the cured strain derived in the course of these studies.

(i) R_1 is a double lysogen carrying two phages that are morphologically and serologically dissimilar.

(ii) As R_1 is the first strain of a lactic streptococcus to be demonstrably cured of resident prophage(s), we have for the first time the possibility of assessing the effects of prophage loss.

(iii) No morphological and biochemical differences were observed between the R_1 and R_1 cured strains.

(iv) No suppression of heterologous virulent phage growth by prophage was observed.

(v) R_1 induced phages are homoimmune to temperate phages resident in other strains.

(vi) Treatment with several standard curing agents (heat and Mitomycin C) produced variants which were atypical for the species.

These points merit further discussion because of the absence of other reports on curing and lysogenic conversion of lactic streptococci.

(i) Strain R_1 is a double lysogen. Induction of a bacterial culture of R_1 yields a population of phage particles which are physically of two types : one with a short tail and a collar, and a density of 1.50 g/cm^3 ($r_1 t_1$), and the other with a longer tail, no collar and a density of 1.47 g/cm^3 ($r_1 t_2$). They are further distinguished on the basis of their antigenic characteristics and their host specificity.

The antigenic characteristics of the two phages were examined by a serum neutralization test using antisera raised against the virulent phages 936/158 (collarless, isometric) and 853/AM_1 (collared, isometric). No serological relationship was revealed by cross-neutralization between 936/158 and r_1t_1 or r_1t_2, nor was there any cross-neutralization between 853/AM_1 and r_1t_2. However phage r_1t_1 showed 50% neutralization with antiserum raised against 853/AM_1 which suggests that the two phages might be related serologically.

Further examination of the plating efficiencies of the phages liberated by R_1 and grown on the two indicator strains R_1 cured and 368 showed an apparent strain specific modification-restriction system (Fig 9). Host specificity may be considered as a system of immunity directed against unmodified genetic material. It is reasonable to assume that bacteria gain advantages by protecting themselves from foreign nucleic acid. Most viral infections, for example, cause the death of the infected cell. Restriction may thus seem a quite efficient way to defend a cell's integrity against such invasions (Arber, 1971). However the presence of a modification-restriction system can also make it difficult to detect a potential phage host interaction.

Modification-restriction was first reported for lactic streptococci in S. cremoris by Collins (1956) and although several reports have dealt with this phenomenon since then, no more work has been done to verify that modification-restriction of DNA is in fact occurring, nor have there been any attempts to characterize its biochemical basis.

One feature of the data given in Fig. 9 which is puzzling is the low efficiency of plating of the induced lysate of R_1 on both strains R_1 cured and 368. If a logarithmic culture of R_1 is subjected to 10 seconds UV irradiation complete lysis of the culture is observed after about 4 hours of incubation at 30°C. If we assume that 4×10^8 CFU/ml were induced then the lysate should contain at least 4×10^8 PFU/ml. Electron microscopic examination showed no trace of incomplete phage particles or

bacteriocin, in the induced lysate and the frequency of lysogenization was not high enough to explain the low EOP of the lysate on both indicator strains. Attemps to understand this low EOP in terms of differences in modification restriction specificities of R_1, R_1 cured and 368 and/or different specific sites in the R_1 phages (r_1t_1 and r_1t_2) failed to yield an hypothesis consistent with the results presented in Fig. 9.

(ii) As R_1 is the first strain of a lactic streptococcus to be demonstrably cured of resident prophage(s), we have for the first time the possibility of assessing the effects of prophage loss.

Lysogeny is usually a very stable property of a bacterial strain, as stable as any of its hereditary characters. Thus in most populations of lysogenic cells only a very small percentage of individuals appear to have lost their prophage that is, have been cured of their lysogeny. Nevertheless, nonlysogenic derivatives of erstwhile lysogenic bacteria can be isolated. This was shown in many other species (E. coli, B. megaterium, etc.,) but with Streptococcus species, the case was more difficult since members of this genus are generally chaining organisms. But in order to learn anything about the physiological effects of lysogeny, one must have two strains of bacteria of which one is the lysogenic derivative of the other.

The procedure used for curing of R_1 was as described under Methods, and was based on irradiation with high doses of UV of a logarithmically growing culture of R_1, followed by examination of the survivors for curing; this is substantially the same procedure as has been used for curing of other genera. However a few precautions needed to be taken before irradiation. Firstly, examination of the supernatant of a log culture of R_1, by plating on strain 368 showed the presence of free phage particles at a level of 1×10^2 PFU/ml. So, it proved necessary to eliminate

as many of the free phage as possible in the culture, by centrifugation and washing, as these phages will infect any newly arising sensitive (cured) cells. To overcome the problem of chaining, the cells were first blended so that the chains were reduced to predominantly diplococci. Martley (1972) found that fragmentation of the chains is brought about during the first 15 seconds of blending and that blending for longer than 30 seconds results in only a slight further decrease in average chain lenth. However using R_1, I found no significant difference in the chain length after 30 seconds of blending; in order to reduce the chains, R_1 needed blending for 120 seconds, during which time the viability, as judged by colony counts was not affected.

The last precaution taken was to use Ca^{2+} free medium for plating the highly irradiated cells. In a Ca^{2+} free medium the nonlysogenic sensitive cells would probably be protected from the free bacteriophage present, since Ca^{2+} is found to be a necessary cofactor for the penetration of the virus in to the host cell. The importance of calcium ions for bacteriophage infection in lactic streptococci is well established (Shew, 1949). Lowrie and Pearce (1971) found that different bacteriophages differ in their requirement for calcium ions, with S. cremoris phages being more exacting in their requirements than S. lactis phages. I found that for the temperate phages liberated by R_1 and grown on strains 368 and R_1 cured the Ca^{2+} ions were extremely necessary.

Screening procedures designed for the isolation of cured strains from stably lysogenic ones most often involve induction of broth cultures of individual bacterial strains followed by centrifugation and subsequent spotting of the induced lysates on possible indicator strains. By using the method we described in Materials and Methods as many as 24 cultures could be induced at the same time and this saves time and effort.

iii. No morphological and biochemical differences were observed between the R1 and R_1 cured strains.

From the work described in Results it is clear that the cured derivative of R_1 is identical to its parent strain and that it gave all the reactions typical for the species. No changes in colony morphology were observed, nor any changes in acid production, carbohydrate metabolism, or hydrolysis of arginine. These are good results, since most of the strains used in New Zealand were found to be lysogenic (Terzaghi and Sandine, ms in preparation) and so any metabolic defect as a result of the presence or absence of the prophage would be undesirable and uneconomic. The only difference observed between R_1 and its cured derivative was the resistance of the cured strain to the antibiotic Mitomycin C when both strains were spread on plates containing different concentrations of the antibiotic. This result was expected since MC can induce the development of active phage by inactivation of the prophage repressor of the lysogenic strain R_1 with subsequent lysis of the culture. The resistance of the cured strain to certain concentrations of MC might be used for the detection of cured cells in those cases where an indicator strain for the resident phage is not known. This could be done, for example, by replica plating the survivors of a high dose of UV onto a series of plates containing increasing concentrations of MC. The cured colonies should grow at higher concentrations of MC than the lysogenic ones whose resident prophage will be induced, and so either lyse completely or will grow as colonies with nibbled edges.

In contrast with the above results there are a number of instances in which the establishment of lysogeny is accompanied by changes in characteristics such as colony morphology, toxin production and carbohydrate metabolism changes. For instance Ionesco,(1973),observed that, due to the presence of prophage, the normally smooth colonies of Bacillus megaterium became rough and wart-like. Antigenic changes were described by Uetake, Nakagawa and Akiba (1955), Uetake, Luria and Burrows (1958), Staub, Tinelli, Luderitz and Westphol (1959), in

Salmonelae after they became lysogenized and Mankiewicz et al (1969) reported that lysogenization of Mycobacterium phlei not only showed the expected phage immunity but also a marked difference in colonial appearance, pigmentation, growth rate, antigenic composition and pathogenicity. Changes in antibiotic and phage sensitivities and inhibition of coagulese activity were observed by Jollick,(1974),as a result of lysogenic conversion in a strain of Staphycococcus aureus.

iv. No suppression of heterologous virulent phage growth by the resident prophage was observed.

In order to examine the question of whether lysogeny influences the interaction of hosts and heterologous phage, the R_1 cured and parental R_1 strains were compared for their ability to support the growth of six representative morphological types of virulent phages. As mentioned before no difference was observed either in the adsorption or the lytic pattern of these phages, which clearly shows that in this case, the presence of the prophage does not apparently serve host purposes, by conferring virus resistance to the host. From Table XII it can be seen that the resistance of R_1 to the phages $690/SK_{11}$, $949/AM_1$, 799/368 and 949/402 does not change upon removal of the phage. These results are in agreement with a report of Lowrie,(1974) whose data however does not clearly show that lysogeny had been established in the one strain examined. The data he gave suggested rather the carrier state. Completely different results were found by Terzaghi and Sandine which claim that lysogenization of S. cremoris SK_{11} with a phage that it did not carry previously confers resistance to the attack of three serologically and morphologically unrelated virulent phages.

There are several reports in the literature showing that lysogenic conversion often involves exclusion of superinfecting phage. Thus the susceptibilities to heterologous phages in Salmonella typhosa (Craigie, 1946; Anderson and Felix, 1953), in Shigella dysenteriae (Bertani, 1953a,b) in E. coli

(Benzer, 1955), and in Staphylococcus aureus (Asheshov and Rippon, 1959; Blair and Carr, 1961) have been shown to be determined at least in part by a prophage. Lederberg, (1957), showed that phages T_2, T_4, T_5 and T_6 fail to multiply in most cells of certain strains of E. coli or Sh. dysenteriae lysogenic for phage P2. Likewise, phages T1, T_2, T_7 and P_2 cannot multiply in cells lysogenic for P_1. P_1 lysogenic cells on which T_1 has adsorbed but has not multiplied, survive, and grow normally. These properties are lost when the prophage is lost.

v. Homoimmunity of R_1 induced phages with temperate phages liberated by other strains.

The results presented in Tables XIV and XV show that the presence of the prophages in R_1 do not change its ability to adsorb phages r_1t_1 and r_1t_2 and that they confer a wide range of immunity to the host strain. As soon as these prophages are removed the immunity is lost and the strain becomes susceptible to the phages liberated by several other strains. That is, the R_1 resident prophages appear to be homoimmune with the phages resident in strains 108, P_2, AM_2, 158, H1, 398, 240, 114, 130 and 286. Electron microscopic examination of these temperate phages reveals that they have similar if not identical morphology with the two phages resident in R_1. Relysogenization of the P_1 with one of the two phages confers immunity to phages liberated from H_1, 286 and the parent strain R_1 which as I showed is a double lysogen. From the results presented we concluded that these phages can be classified into two immunity groups: those with a shorter tail and a well defined collar and those with a longer tail and no collar. Phages within each group are presumed to produce a similar type of repressor (and hence are homoimmune) but the two groups are suspected of being heteroimmune, perhaps by virtue of each producing a different type of repressor.

The results presented here are in agreement with those presented by Kozak et al (1972) who suggested that phages 40, 41 and 45 of S. lactis, produce a similar type of repressor, because cross-immunity to these phages was observed. He also reported a different type of repressor produced by phage 37 of S. lactis strain 37. A similar situation was observed in Staphylococcus aureus where lysogenization by a serological type F phage confers apparent immunity to the serological type A phage 54 and 75. This represents an example of cross immunity between phages of unrelated antigenic structure.

Strain 104 lysed all three strains equally (Table XV), and examination of the lytic area showed the presence of an antibiotic substance rather than a plaque-forming phage. Further examination of that substance was not pursued, since its behaviour against the lysogenic and non lysogenic R1 was the same.

vi. Treatment with several standard curing agents (heat and MC), produced variants which were atypical for the species.

In the course of this work, two unusual mutants have been isolated which were designated R_1t and R_1MC.

S. cremoris R_1t, the high temperature derivative, differed physiologically from its parent strain R_1 in respect to the fermentation of maltose and sucrose and hydrolysis of arginine. These properties are characteristic of most of the strains of S. lactis, from which it differs in that it clotted the milk at 44°C. Growth between 40 and 45°C is also a property of another species S. thermophilus which is often used as a starter culture in the manufacture of swiss cheese and yoghurt. Both S. cremoris R_1t and S. thermophilus survived 65oC for 30 minutes, but they differ in that R_1t fermented maltose whereas S. thermophilus did not. As no serological and genetic studies have been done no conclusions can be made in regard to the identity of R_1t.

There are several reports in the literature referring to atypical strains of streptococci but none in group N. An example of this is a strain isolated by James and McFarland (1971) which although it was a bacitracin sensitive M type

18 group A streptococcus, it was extremely mucoid and not hemolytic. Others have reported isolation of similar nonhemolytic group A streptococci from clinical cases. Rammelkamp and Dingle (1948) reviewed instances where nonhemolytic group A streptococci have presented problems. Others have shown that under certain cultural conditions β - hemolytic variants can be obtained from α -hemolytic group A strains (Colebrook et al; 1942). Sherman et al (1943), recognized that among the nonhemolytic streptococci of the human throat there was a homogeneous cluster of strains all of which formed levan from sucrose, fermented inulin, raffinose, salicin and esculin, grew at 45°C, failed to ferment mannitol, sorbitol, and glycerol, and did not hydrolyse arginine. Later collections of strains were not so homogeneous and Carisson (1968), for instance, assigned to a new species those levan producing strains that did not grow at 45°C or ferment inulin. More recently Colman and Williams (1972) examined ten strains assigned to the same species, and found all of them producing levan, but not dextran, from sucrose, hydrolyced esculin, but not arginine, most fermented one or both inulin and raffinose but only one strain grow at 45°C. Sherman's strains possessed all these properties.

In contrast with R_1t, S. cremoris R_1MC manifested all the typical biochemical reactions of the species. No difference in colony morphology, carboydrate metabolism, temperature tolerance or phage sensitivity was observed so that if R_1MC was not examined microscopically in a phase contrast microscope no difference from its parent R_1 could be discerned. Microscopic examination of R_1MC showed cells existing only as monococci and diplococci and having a characteristic motion which did not seem to be a Brownian movement.

The lack of chain formation, which was an inherited character, was not affected by the culture conditions and will be discussed first. As mentioned earlier R_1MC was isolated from a plate containing 0.5 μg/ml of the antibiotic mitomycin C, which is a well known mutagen, acting specifically on

DNA, but not affecting either RNA or protein synthesis (Levin, 1961). So we can suppose that exposure of the cells to the mutagen produced a mutation to a gene which now directs the formation of a chain-splitting enzyme. Another possibility is that the 'normal' state is that of the short chains that is, the gene which directs the formation of the chain splitting enzyme is the wild type which, by mutation, was inactivated or repressed, and by the action of MC it was derepressed and started directing the production of the chain-splitting enzyme again. Evidence for the existance of a chain splitting enzyme in group A streptococci was first given by Ekstedt and Stollerman (1960a). Another explanation is that MC causes alteration in the antigenic structure of the cell wall. It is interesting to note that in the case of group A β - hemolytic streptococci factors tending to promote preservation of the intercoccal junctions and thus to exaggerate chaining includes not only conditions which impair growth, but also the presence of antibodies that react with cell wall antigens. The presence of anti- M antibody, for example, which reacts with the M protein surface antigen of the streptococcal wall, causes the organisms to grow in long chains in broth culture. Similar results have been obtained with antibodies to another surface protein, the R antigen (McCarty, 1973).

The mechanisms that control bacterial morphology and division are, in general, not clearly understood. Most mutations affecting these processes might be expected to be lethal. However, in other bacterial genera, recent reports show that conditional and non-conditional morphological mutants can be obtained and used to learn something about their physiology and genetics. Genetically determined morphological changes in bacteria have been observed mainly with conditioned mutants, (Rodolakis et al, 1973). This is not surprising, since most non-conditional mutations affecting production or activity of wall-synthesizing enzymes, or other processes involved in division or leading to unbalanced formation of

wall are expected to be lethal.

In the case of motility no final conclusions can be made. I have found this strain to be motile by microscopic observation of young (4 hour) cultures in M17 broth and also in stab cultures of soft (0.3%) M17 agar when incubated at room temperature. Incubation at 30oC showed only growth but no indication whatever of motility. Direct examination of wet mounts of young cultures grown in liquid or solid medium showed no sign of movement. Electron microscopic examination of the cells did not reveal positively the presence of flagella although flagella like formations and filaments were observed.

The determination of motility of an organism is sometimes difficult because this property may be conditioned by such cultural factors as pH, temperature, composition of medium and time of incubation. The detection of flagellation is fraught with even more difficulty (Carrier and McCleskey, 1961).

Busing, Doll and Freytag (1953) described a diphtheria like organism which they named Corynebacterium vesiculars sp.n. A culture of this organism was sent to the American Type Culture Collection (A.T.C.C.) where it was described as a non-motile and gram-positive species. However when Carrier and McCleskey (1961) obtained a culture from the A.T.C.C. they found the species to be motile. In their report, Carrier and McCleskey explained the fact that other workers and the A.T.C.C. described that species as non-motile because the flagella of C. vesiculars are unusually susceptible to detachment from the cell and are very difficult to stain.

As mentioned earlier R_1MC differed from R_1 in two features: (1) the lack of chain formation and (2) the characteristic motion of the cells. At least two possible hypotheses were entertained to account for this double change: (a) the two altered phenomena might be manifestations of a single gene which was suppressed, and when derepressed by the action of MC had the pleiotropic effect of forming the chain-splitting enzyme and

direction of flagella formation.
(b) two independent mutational events have taken place simultaneously. However, as it was beyond the scope of this project, no genetic studies have been made.

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