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.

Demetra Christophorou Georghiou

1976

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.

ACKNOWLEDGEMENTS

The author is indebted to the Department of Microbiology and Genetics for providing the facilities.

In particular I would like to thank:

My supervisor Dr E.A. Terzaghi for his general help, encouragement and helpful criticisms during the preparation of this manuscript; Professor D.F. Bacon and the other academic and technical staff of the Department of Microbiology and Genetics; Dr B.E. Terzaghi of the Dairy Research Institute for the gift of cultures and bacteriophage stocks and many useful discussions; Mr R.M.T. Cursons for general and photographic assistance; Mr R.W. Cleaver for technical assistance.
Mrs F.S. Wicherts for the excellent typing.
The Central Photographic Unit, Massey University
The Electron Microscopic Unit, Applied Biochemistry Division, D.S.I.R.

Acknowledgement is also given to the New Zealand Government for the award of the British Commonwealth Scholarship, and the Dairy Research Institute for providing the materials.

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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

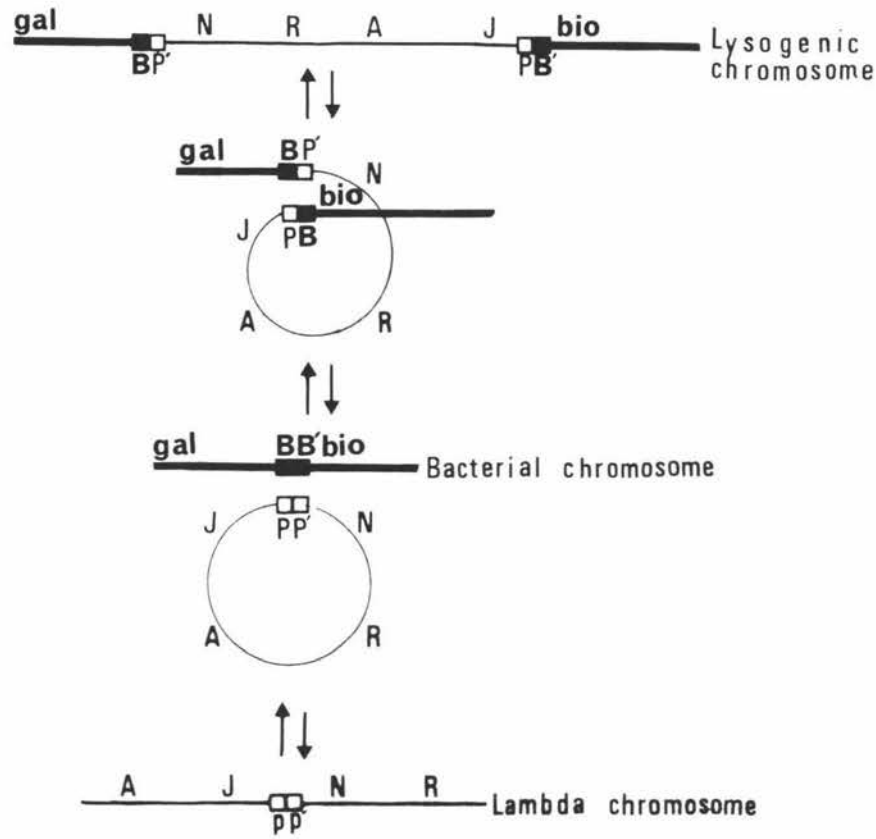

Figure 1: Prophage insertion and excision. The light lines represent the phage and prophage chromosomes and the heavy lines the bacterial chromosome. The rectangles represent the attachment sites, A, J, N and R are phage genes. P.P' and B.B' are respectively the phage and bacterial sites of insertional 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.