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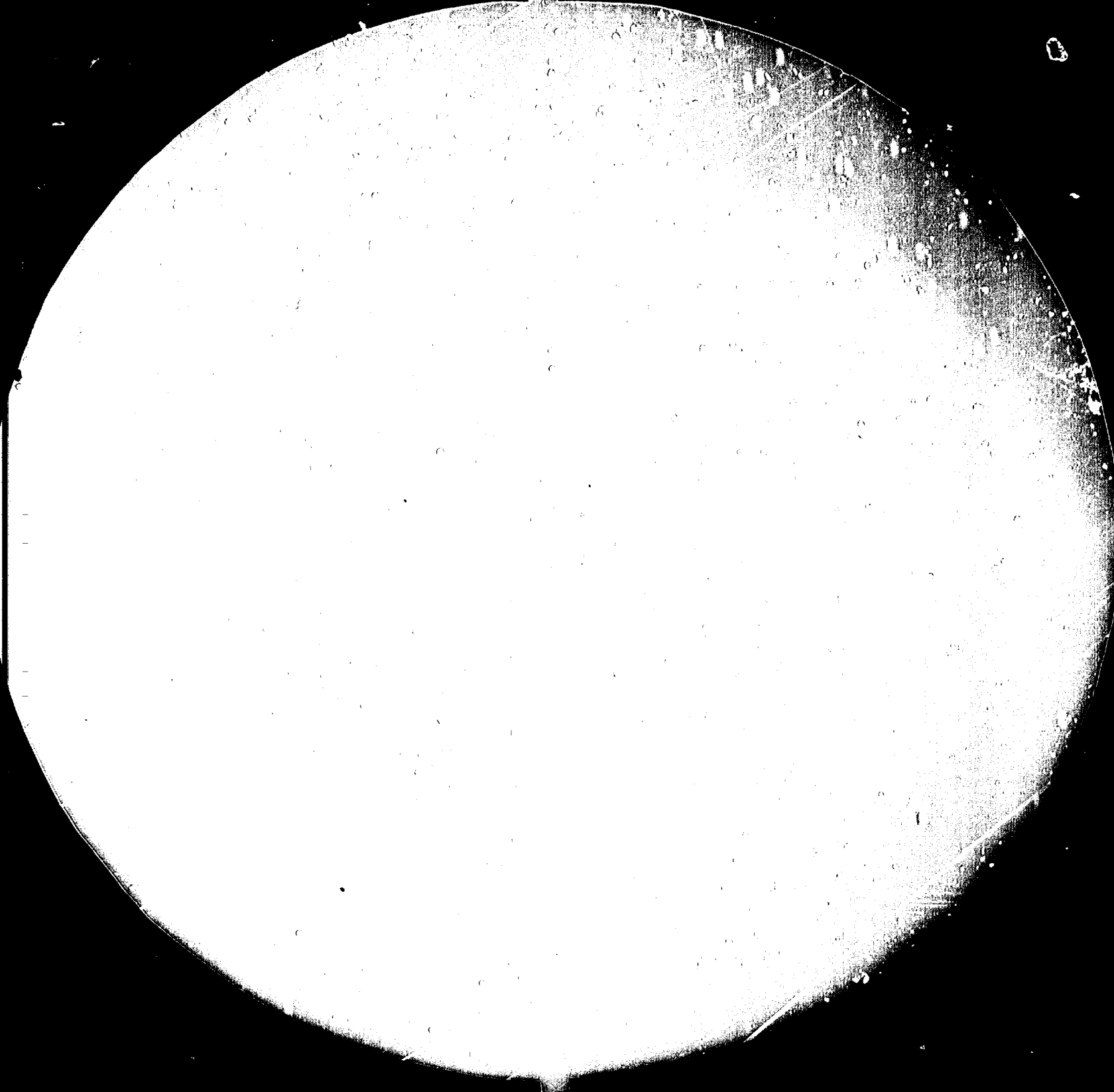

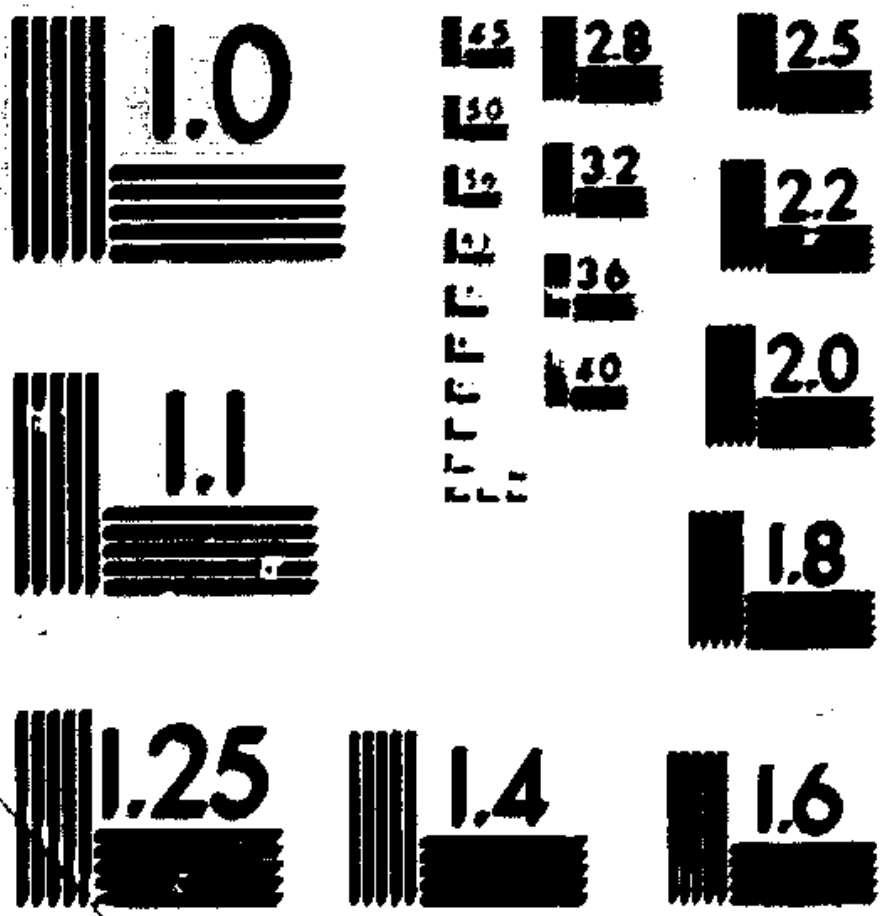

14434

Distr.
LIMITED

ID/WG.431/4
28 February 1985

ENGLISH

United Nations Industrial Development Organization

Symposium on the Importance of Lactic Acid Fermentation in the Food Industry

Mexico City, Mexico, 27 - 29 November 1984

PRESENCE OF PHAGES IN STARTER CULTURES*

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V.85-23349

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CONTENTS

I. BACKGROUND

1. The primary cause of slow acid production by lactic bacteria during industrial fermentations is lysis by bacteriophages. Many virulent and temperate phages have been isolated and characterized by electron microscopy but the genome structure was determined for only few phages. A classification of phages has been proposed according to their host-range, their morphological type or their antigenicity but DNA homology seems to be a more precise criterion to determine relatedness among lactic bacteria. It is not yet known to which extent temperate phages contribute to bacteriophage problem. Transduction with temperate bacteriophage has been demonstrated for the transfer of some genes of the lactic bacteria. Restriction-modification seems to be one of the main defenses of the lactic bacteria against their phages. Investigation into the mechanisms of phage resistance would provide information for construction of phage-resistant strains.

II. INTRODUCTION

2. Lactic acid bacteria such as lactobacilli and streptococci are major components of starter cultures essential to the production of a variety of fermented products. Protection of culture fermentation from bacteriophage contamination is important in the fermentation industry. As a matter of fact, the primary cause of slow acid fermentation is

lysis by bacteriophage (Lawrence, 1978). The occurrence of strains that demonstrate resistance to a wide variety of phages and maintain resistance during long-term commercial use is rare. Bacteriophages are commonly encountered in practice since almost all of the industrial fermentations have to be performed under non-sterile conditions including non-sterile substrates and equipment. Lysogenic starter strains may also act as a reservoir of phages. Because of their economic importance, the phages of lactic bacteria have been intensively studied but little is known of their molecular biology and their genetic systems.

III. RESULTS AND DISCUSSION

Morphology and differentiation of the lactic bacterium phages.

3. Electron micrographs of many phages of the mesophilic lactic streptococci have been published by many groups. In contrast, little information is available about phages that attack the thermophilic lactic bacteria. A comparison of the electron micrograph data is almost impossible because of the different procedures used.

4. The phages can be differentiated by head size and shape into small isometric, large isometric and prolate. They have a short or a long tail with or without a collar (Terzaghi, 1976; Accolas and Spillman, 1979).

5. A classification of some phages has been made according to their lytic action on mesophilic lactic streptococci. The lytic activity of

132 virulent phages isolated during slow acid production in french cheese factories has been determined on 291 strains by Chopin et al. (1976). Six groups of phages were formed.

6. Another criterion commonly used for the phage classification is based on serological studies. A good correlation was found between serological and host-range data by Jarvis (1977). Serological studies as well as lytic spectra of the bacteriophages or their morphology-type seem to be insufficient criteria to allow a rational phage classification since these studies reflect only part of the phage genome. Bradley (1971) considered that serological unrelatedness does not necessarily indicate a significant difference between two phages. On one hand, a single point mutation or insertion of exogenous DNA may change one morphological type into another one. On the other hand, the lysogenic state of a bacterium can greatly modify its sensitivity to various phages. In contrast, DNA-DNA hybridization techniques seem to be well adapted for determining relatedness between different phages. These studies have been undertaken by Jarvis (1984a).

7. Twenty five lactic streptococcal bacteriophages were differentiated by DNA homology into four species. Complete correlation was found between DNA homology groups and morphological characterization of the phages except for two phage-types. The complete lack of homology among the four proposed phage species indicates that they do not have a recent common phage ancestor and that one phage type is not derived by mutation from another.

Lysogeny.

8. Lysogenic strains of lactic streptococci have been described as early as 1949 (Reiter, 1949). The generally accepted criteria for lysogeny are : 1) phage inducibility; 2) immunity of a bacterial strain to its own temperate phage and closely related phages; 3) resistance of the lysogenic state to antiserum raised against the lysogenic bacteriophage (Barksdale and Arden, 1974).

9. Only the first criterion has been commonly used to prove the lysogenic state of lactic bacteria. Induction of lysis in cultures of lactic bacteria are currently carried out by mitomycin C treatment or by UV irradiation; the phage particles released are detected by electron microscopy. Following this method, lysogeny was shown to be widespread amongst lactic streptococci and lactobacilli (McKay and Baldwin, 1973; Lowrie, 1974; Huggins and Sandine, 1977; Yokokura et al., 1974; Terzaghi and Sandine, 1981; Reyrolle et al., 1982).

10. Relatively little is known about spontaneous induction or about the effect of environmental conditions on it. Lysis induced by mitomycin C or UV cannot be taken as evidence of lysogeny since strains that are lysed do not always release phage-like structures detectable by electron microscopy of the concentrated lysates. In addition, very often it is not possible to find susceptible indicator bacteria for phage particles seen in the electron microscopy but undoubtedly non-inducible prophage, defective prophage and multiple lysogens also exist.

11. A strict demonstration of lysogeny remains rare for the lactic bacteria and has been published only recently. Gasson and Davies (1980) achieved prophage curing in S. lactis (NCDO505 and 712) and S. cremoris (NCDO1428). They were able to relysogenize the cured derivatives by the homologous bacteriophage. True lysogeny was also demonstrated in S. cremoris MU0001(R1) by Georghiou et al. (1981) and the strain was relysogenized. Lactobacillus casei strain S1 was found to be a lysogen because the supernatant of a culture contained a small amount of free phage, however the temperate phage of strain S1 could not be induced by either UV irradiation or mitomycin C. A thermoinducible mutant of this phage was isolated. Prophage-cured strains were selected after heat induction of the mutated strain proving that strain S1 was lysogen (Shimizu-Kadota and Sakurai, 1982).

12. As pointed out by Lawrence (1978) an important part of the DNA in the lactic bacteria may consist of prophage. The distinction between "phage" DNA and "cellular" DNA may not be particularly meaningful and phage may be considered as an integral part of a culture. The molecular mechanisms of the host-phage relationship in lactic bacteria are still not well understood.

Transfection and transduction by bacteriophage.

13. Phage-based genetic transfer systems are important for the development of techniques used in genetic engineering. Geis (1982) has developed a high efficiency transfection procedure for lactic streptococci using isolate P008 DNA. Since this phage DNA possesses only one target site for two restriction endonucleases, it might be a suitable cloning vector.

14. Transduction with temperate bacteriophage has been demonstrated for the transfer of streptomycin resistance, tryptophan, lactose, mannose and protease genes (Snook and McKay, 1981; Snook et al., 1981). Interspecies genetic transfer of lactose-fermenting ability by a temperate phage was also successful. These results suggests that phages play a role in the mixing of the lactic bacteria genes.

Origin of the virulent phages.

15. Little attention has been paid to tracing the origin of the infecting phages. Do the virulent phages originate as temperate phages from lysogenic starter bacteria or as lytic phages either carried in the starter sultures or as contaminants from external sources?

16. Phages specific for mesophilic lactic streproccoci can be found from raw milk, pasteurized milk and whey. Equipment (cheese vats), floor, walls and air of rooms, personnels and their cloths may be source of phages. The system of distribution of cheese starter culture to most New Zealand factories was suited for research on phage origins by Terzaghi (1976). Phages were isolated from whey samples collected from 21 factories and tested against all the strains of lactic streptococci in the factories. The 109 phages isolated were examined for host-range and morphology. The appearance and distribution of phages in the factories was consistent with a common origin for most of the phages (Terzaghi, 1976)

17. These findings are in agreement with data presented in a recent study (Shimizu-Kadota et al., 1983). A virulent phage which was isolated

from abnormal fermentation products in single cultures of Lactobacillus casei was shown to be closely related to a temperate phage integrated into the chromosome of the starter strain. DNAs from the temperate and lytic phages were identical in restriction endonuclease pattern and the lytic phage DNA was able to hybridize with the chromosome of the lysogenic bacterial strain. The authors conclude that the virulent phage is derived from the prophage; therefore, curing of the prophage in the lysogenic strain is the most effective method of protection from contamination by the virulent phage isolated. Two possible mechanisms of the derivation of the lytic phage from the temperate phage were porposed : multiple point mutations or rearangement of the prophage DNA may occure leading to appearance of the lytic phage.

18. Other results emphasizing the importance of development of temperate phages as a cause of accidents in cheese making is given by studies of Reyrolle et al. (1982). It has been shown that the majority of the collection of 113 strains of mesophilic lactic streptococci classed on the basis of their sencitivity to virulent phages (Chopin et al., 1976) are lysogenic and there was a close correlation between the lytic spectra of temperate and virulent phages.

19. The opinion that most lytic phages arise from the temperate phages of the starter bacteria is contested by some searchers. Jarvis argue that it is hard to inderstand why induced phages have proved to be so difficult to propagate on starter strains if phages released from lysogenic starters are an importance source of the phages detected in cheese plants.

20. Three temperate phages were induced from Streptococcus cremoris and compared by DNA-DNA hybridization with 25 lytic phages isolated on cheese starters (Jarvis, 1984b). Little or no homolology was found between DNA from the temperate and lytic phages. In contrast, temperate phages showed a partial relationship with one another. The author conclude that the release of temperate phages from starter cells currently in use is unlikely to be the predominant source of lytic phages in cheese plants. Teuber and Lembke (1982) also propose that temperate phages are of minor importance.

21. Whether the phages derived from the lysogens depends on the frequency with which spontaneous induction occurs and on whether indicator strains are present to allow such phage to replicate. In conclusion, it is not yet known to which extend temperate phages contribute to bacteriophage problems in the industrial fermentations.

Characterization of the genome of the bacteriophages.

22. Only little information concerning the molecular biology of lactic bacteria is available. The physical map of the DNA of the Streptococcus lactis bacteriophage P008 was constructed by Loof et al. (1983) in order to be suitable as a reference molecule DNA.

23. The molecular biology of the temperate lactobacillus phage ϕFSW has also been undertaken by Shimuzu-Kadota and Tsuchida (1984). The physical structure of the virion is circular; in the lysogen, the DNA of the bacteriophage was found to be linearized at a specific site and

integrated into a specific locus of the host genome. The prophage integration appears to occur into the host genome without large deletion. The structure of the prophage predicts that ϕFSW has a Campbell-type integration and excision system with a site-specific recombination (Campbell, 1971).

24. In our laboratory we have used the techniques of the genetic engineering to study the genome structure of the virulent phage LLH of Lactobacillus lactis. We made a restriction map of the phage DNA and we subcloned the restriction fragments of the phage DNA into plasmid pBR322 or derivatives. The hybrid vectors were introduced into Escherichia coli and the expression of the cloned phage genes was examined by the immunoblotting technique. This procedure allows to assign the structural protein to a part of the genome. The dominant protein having a molecular mass of 36 000 is encoded by a restriction fragment of 2 300 base pairs (Trautwetter et al., unpublished results). The exact location of the genes coding for the other structural proteins is in progress.

Restriction and modification.

25. The phenomenon of restriction-modification is considered to play a role in phage insensitivity. In studies of phage-host interactions, alterations in restriction patterns of phage DNA modified by growth in different hosts have been observed (Daly and Fitzgerald, 1982). These authors have demonstrated the presence of a type II restriction endonuclease in Streptococcus cremoris F. Two other strains of S. cremoris were also shown to possess modifying and restricting activity. One of these strains showed restricting ability against 8 of 24 phages examined with an

efficiency of plaquing as low as 10^{-9} in some cases. Restriction may be overcome by modification of yet unknown nature. Two other restriction/ modification systems were found in Streptococcuc lactis and was related to the presence of two plasmids (Chopin et al., 1984).

26. Boussemaer et al. (1980) suggest that restriction/modification is one of the main defenses of the lactic streptococci against their phages. The important role of restriction/modification explains why host range cannot be considered to be a useful criterion for phage taxonomy.

Mechanisms for phage insensitivity.

27. The fact that a strain is not attacked by a phage is due to lack of adsoprtion, lysogenic immunity, restriction-modification systems or other unknown mechanisms. In practice, phage resistance is a genetically unstable property. This is not surprising since the phage sensitivity depends on both evolving genetic entities, the bacterium and the phage.

28. Successful phage infection begins with phage adsorption to the host cell surface. Many studies on levels of phage adsorption to the lactic streptococci have been described but little is known about the specific nature of their phage receptor sites. Phages-resistant mutants isolated from cultures of cells infected with virulent phages have generally shown a decreased ability to adsorb the phage (King et al., 1983); a chromosomal mutation probably leads to changes in phage receptor sites.

29. Recently, the involvement of a plasmid DNA in coding for products that affect the adsorption efficiency of phages has been reported in Streptococcus lactis (Sanders et Klaenhammer, 1983). Another plasmid-linked mechanism by which lactic streptococci could become resistant to phage is described by McKay and Baldwin (1984). A 40 megadalton conjugative plasmid in S. lactis subsp diacetylactis was shown to carry genetic determinants for resistance to c2 phage at 21 and 32°C but not at 37°C. The temperature sensitivity was not at the step of c2 phage adsorption to the cells and appears to be different to a typical modofocation-restriction system. These results provide evidence for novel plasmid-coded phage defense mechanisms. Some plasmids were also shown to determine restriction/modification systems (see above). Therefore, maintenance of starter cultures in a manner that would minimize the genetic variability resulting from plasmid loss seems essential for preventing phage appearance.

30. Lysogenic conversion has been shown to result in surface structure alterations that leads to the loss of phage receptors. This phenomenon was described for the lysogenization of Salmonella anatum by phage ε15 (Losick and Robbins, 1967). This type of lysogenic conversion is thought to provide a mechanism for superinfection immunity and may be applied to lactic bacteria since lysogeny is widespread among these microorganisms.

31. In practice, commercial phage-inhibitory media are used, but are reported to vary greatly in effectiveness. In these media, calcium

ions availability has been reduced by the addition of ammonium and/or sodium phosphate which bind calcium ions. Unfortunately, phages have been isolated which lyse sensitive cultures in the absence of available Ca^{++}.

32. In conclusion, investigations into the mechanisms of phage resistance could provide information essential to the selection, characterization and genetic construction of phage-resistant strains for commercial use. Therefore, an exact genetic analysis of the bacteria and phages involved will be indispensable. For such purposes, the techniques of genetic engineering will be very useful. These studies will perhaps provide alternative for the actual empiric solution used in the factories to overcome the phage problem, i.e. the rotation of starter strains.

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