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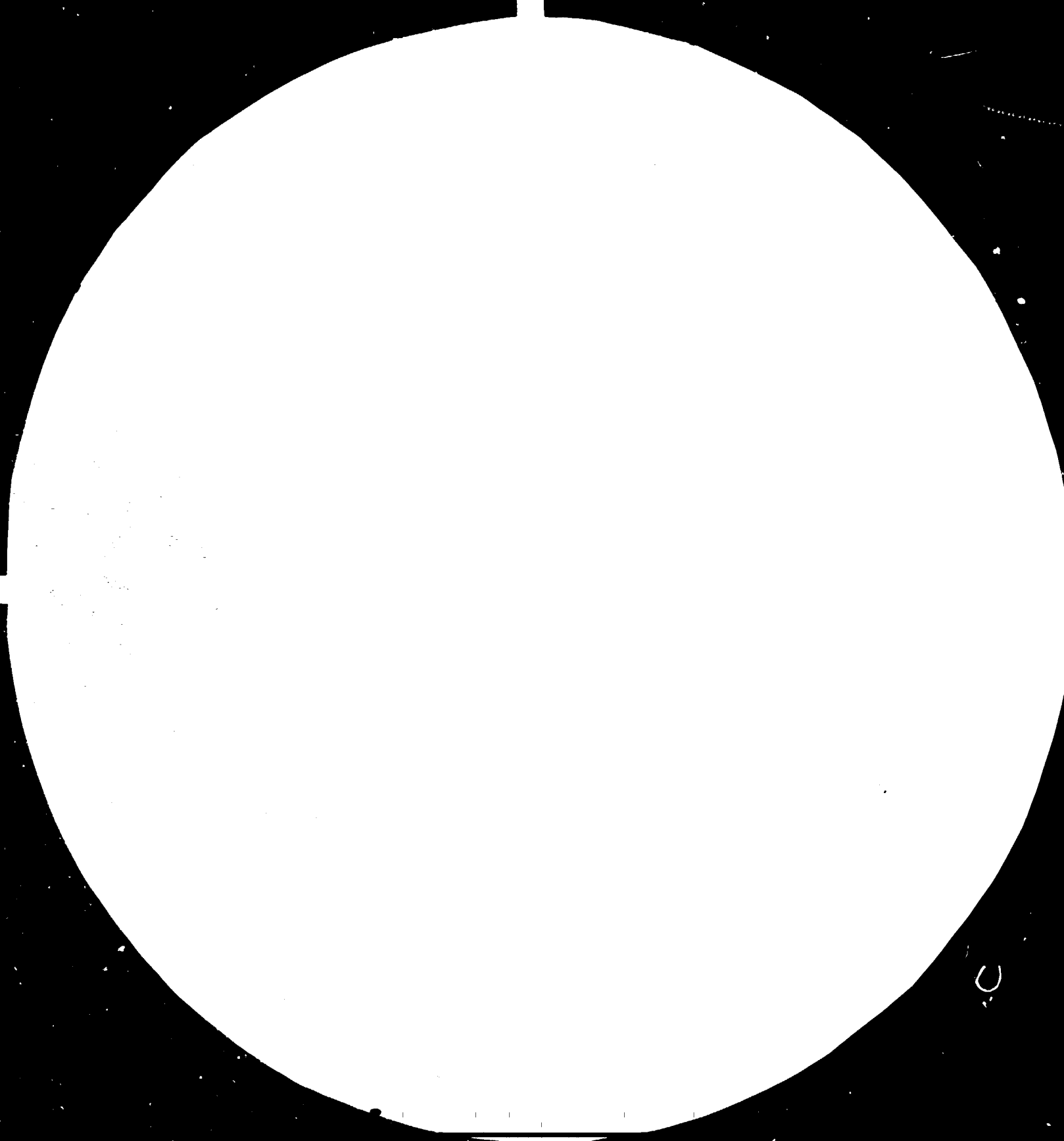

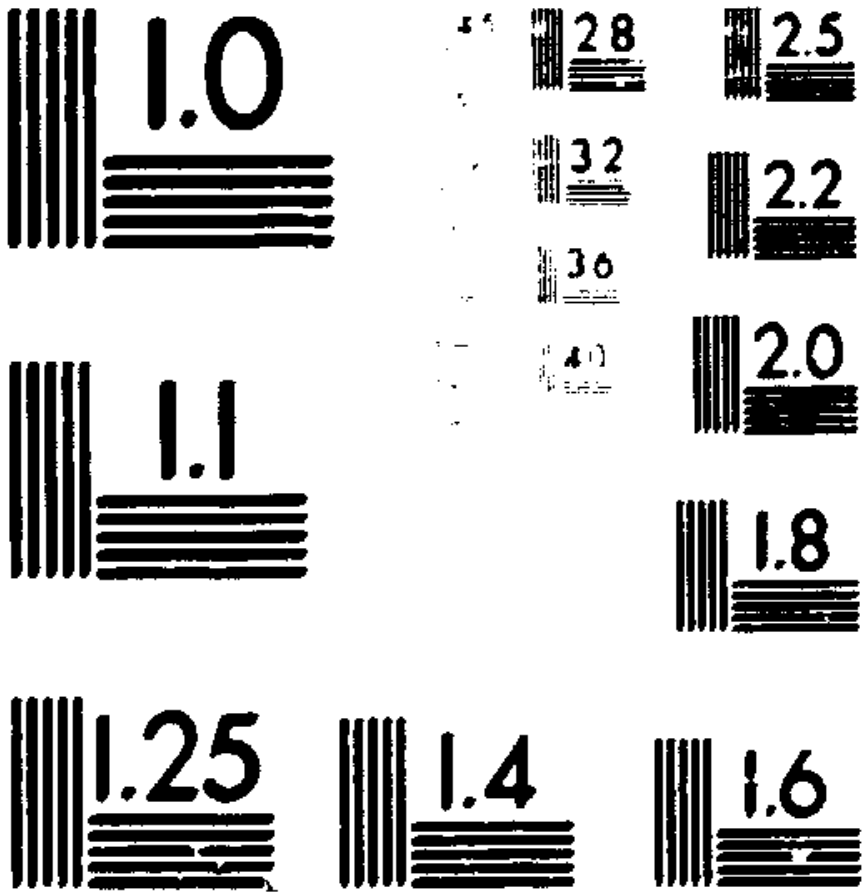

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CONTROL OF PHAGE INFECTIONS IN DAIRY FERMENTATIONS*

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CONTENTS

I. BACKGROUND

A novel approach to control phage infections in dairy fermentations is presented. It involves construction of lactic streptococcal strains with increased phage resistance. This could be done by cloning genes coding for phage resistance in lactic streptococci. An example of this approach is reported. Streptococcus cremoris IL839 possessed one restriction/modification (r/m) system. In this strain, phage growth was restricted by a 10^5 factor. Strain IL839 contained 8 plasmids designated pIL20 to pIL27 with respective sizes of c.a. 3,5, 6, 9, 13, 14, 17 and 46 kb. Using protoplast-induced method, we obtained derivatives cured in turn for each of the plasmids except for pIL21 retained in all derivatives. Phage sensitivity of these derivatives indicated that none of the cured plasmids encoded for the r/m system. To determine the role of pIL21, we transferred it into a plasmid-free, r-/m- strain of Streptococcus lactis using cotransformation with an indicator plasmid (pHV1301) conferring erythromycin resistance. Transformants containing pIL21 did not show r/m activity. So we were able to conclude that r/m genes were located on the chromosome. Work is currently in progress to clone chromosomal gene sequences which are implicated in the r/m system.

II. INTRODUCTION

Since thousands of years, the man has used lactic bacteria naturally present in milk for making cheese and others milk products, without understanding the role of these microorganisms. The knowledge of what was happening when such naturally inoculated substrates clotted came much later, in 1878, when Lister isolated Streptococcus lactis, the most common and abundant lactic acid bacteria in raw milk (22).

The taxonomic studies on lactic acid bacteria started at the beginning of this century. Actually, most of these microorganisms are grouped in four genera : Streptococcus, Leuconostoc, Pediococcus and Lactobacillus (23). For industrial milk acidification, Streptococcus genus is the most important because of the ability of two main species, S. lactis and S. cremoris to realize efficiently a bioconversion of milk sugar.

Milk processing originated from the necessity of preserving qualities of raw milk. In order to obtain long-lasting forms of consuming dairy products, milk acidification was first used empirically. Later, consequently to the hygienic improvement represented by pasteurization, it became necessary to replace the wild lactic flora destroyed during this heat treatment. This was achieved through addition of so-called starters. Generally, starters are made of strains of one or more species acidifying milk at the wished rate to obtain a dairy product with the required organoleptic characteristics (flavour-texture).

Industrialization of cheesemaking led to noticeable progress in starters production and use because of the needs to reach day after day repeatable products from large volumes of milk varying in their quality and in their composition. Socio-economical conditions of work and use of expensive equipments in large cheese plants have raised up special attention to acidifying ability of lactic starters. Indeed, lactic acid production in the right time not only is largely essential for the quality of the final cheeses but also permits an optimal utilization of man-power and cheese vats.

Several factors can affect starters development which is directly related to lactic acid production as growth temperature, inoculation rate, strain genetic characteristics, composition of the inoculated milk and, the

most important, bacteriophage contamination. Phage infection of lactic starters is still responsible for serious losses of dairy productions and prevents rationalization of milk transformation processes. Numerous attemps have been made to prevent and control phage development in dairy fermentation (8, 13, 14, 25). This led to the elaboration of manufacturing practices as direct processing vats inoculation, use of phage inhibitory media for starters multiplication and utilization of several starters cultures systems.

According to Cox (3) and to Lawrence et al. (13) there are three commercialized types of starters systems based on their composition :

1. Mixed strains starters : mixtures of strains of S. cremoris, S. lactis, S. lactis subsp. diacetylactis and Leuconostoc in variable proportions are used mostly in the European dairy industry and in South America.

2. Multiple strains starters : qualitatively and quantitatively defined mixtures of usually 3, 4 or more single strains of mainly S. cremoris and S. lactis are used mostly in USA.

3. Single strains starters : single strains of S. cremoris or of S. lactis are used alone in Australia and paired in New Zealand.

The mixed strain starters had proved to be successful but they used unknown mixtures of wild cultures. Therefore, improvement of starter properties is very difficult. Limsowtin et al. (15) have pointed out that the second starter system (multiple strain starters) offers a lot of advantages. But, it needs rotations, and the number of starter strains unrelated for phage sensitivity is too low for efficient rotation in large factories.

A new concept trying to use advantages of mixed and multiple strains starters was developed in New Zealand and Australia. Strains suitable for cheesemaking and highly resistant to bacteriophages may be isolated from mixed starters as a result of natural selection. These strains may be used alone or in defined mixtures of 2 to 6 successfully during periods of a week to several years (16) before getting sensitive to a phage. Then, they are replaced by a phage resistant mutant.

Generally, there is no way of predicting how long it will be before a phage does appear that will attack the strain. The ways in which a new phage might arise include temperate phage from lysogenic starters (13), phage from wild strains in raw milk (7), modification of existing phages (19), host range mutants (9) and possibly recombinants of virulent and temperate phages (14). Then, the problem is very complex and none of the existing starter systems is completely satisfying.

III. RESULTS

The increasing possibilities of genetic manipulations in lactic acid bacteria offers a new approach to control the phage development in dairy industries. It became possible to consider the construction of improved strains by cloning genes coding for phage resistance mechanisms.

Several recent papers are dealing with genetic determinism of phage-resistance mechanisms in group N streptococci. The most studied phage resistance mechanism in lactic group N streptococci is the host-controlled restriction of entering phage DNA. This common bacterial defence mechanism that functions by recognition and degradation of a foreign DNA was first described in that group of streptococci by Collins (2). Later, a type II restriction enzyme was isolated from Streptococcus cremoris (5). Limsowtin

et al. (16) found that the maintenance of a restriction/modification (r/m) system was unstable in one strain of S. cremoris and speculated on the possible extrachromosomal location of r/m genes. Sanders and Klaenhammer (20) linked a r/m system to a 10 Mdal plasmid. Chopin et al. (1) established that two plasmids of 28 and 31 kb each encoded one r/m system in Streptococcus lactis IL594. Other phage resistance mechanisms involving a prevention of phage adsorption (14, 21) or a temperature-sensitive restriction (17) were described. In all the cases, they were plasmid-linked.

On the other hand, there is a rapid development in gene cloning techniques available in groupe N streptococci. Protoplast transformation was described by Kondo and McKay (11, 12). Recently, Kok et al. constructed a shuttle plasmid vector for lactic streptococci which also replicate in Bacillus subtilis and E. coli (10).

As an example of this new approach to control phage development in dairy industries, we report results we obtained with Streptococcus cremoris strain IL839. This strain exhibited a r/m system (Table 1) and contained 8 plasmids designated pIL20 to pIL27, with respective sizes estimated as 3, 5, 6, 9, 13, 14, 17 and 46 kb (Fig. 1).

In order to determine whether the r/m activity was related to one or several of these plasmids, we isolated plasmid-cured variants using a protoplast-induced plasmid-curing method (6, 18). Sixteen different types of derivatives were obtained, all lacking some of the plasmids present in the parental strain except for pIL21 (Fig. 2).

All plasmid-cured derivatives were still phage-resistant, indicating that none of the 7 cured plasmids was coding for r/m system. To study pIL21 incidence on the r/m system, we used a technique recently developed in lactic streptococci (24). Plasmid pIL21 was introduced in a

plasmid-free Streptococcus lactis strain by using cotransformation with an indicator plasmid (pHV 1301) conferring erythromycin resistance. Indicator plasmid was later spontaneously segregated from doubly transformed cells (Fig. 3).

Transformants bearing pIL21 alone did not acquire r/m system. These results indicated that the r/m system present in S. cremoris IL839 was determined by chromosomal genes. We have improved transformation efficiency to 10^4 - 10^5 transformants per ug of recombinant DNA. So, we are now cloning r/m genes in a S. lactis plasmid-free, r-/m- strain using a bacteriophage to select recombinant clones expressing r/m activity. Through this way, it will be possible to construct strains which will contribute to minimize phage problem in dairy industry.

Table 1

Evidence for a Restriction/Modification Mechanism Active on Phage δ in Streptococcus cremoris IL839

Host Strains	Plating Efficiency of		
	Phage δ (IL835)	Phage δ (IL835, IL839)	Phage δ (IL835, IL839, IL835)
IL835	1	1	1
IL839	2×10^{-5}	1	6×10^{-5}

Figure 1

Plasmid content of Streptococcus cremoris strain IL839

The CCC forms were ascertained using bidimensional electrophoresis according to Hintermann et al. (1981)

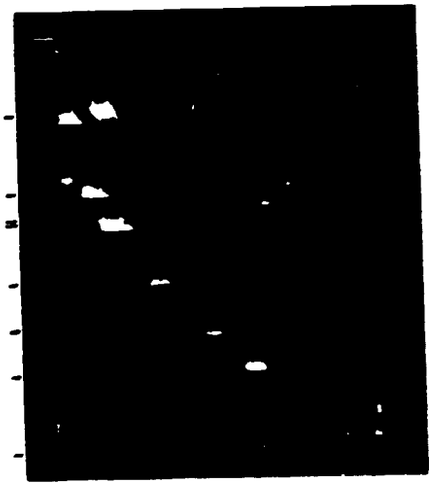


Figure 2

Streptococcus cremoris IL839 variants obtained by a protoplast-induced plasmid-curing method

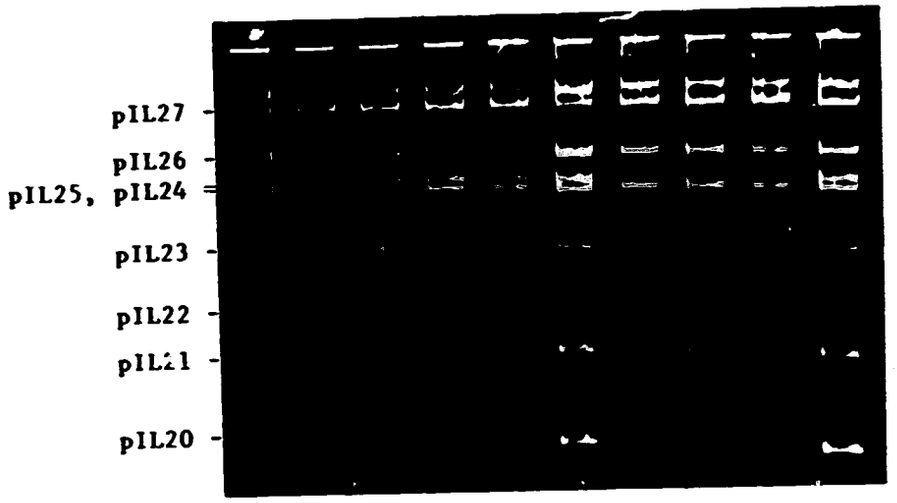
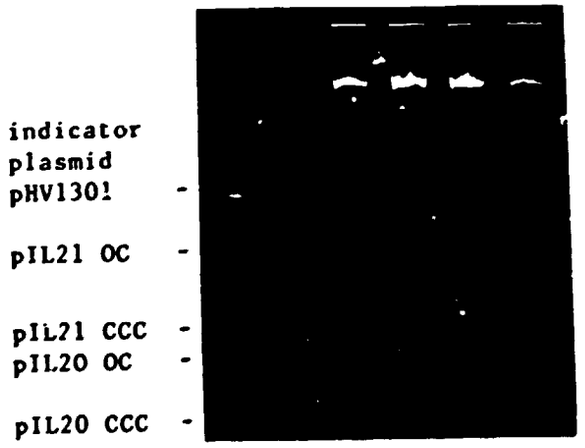


Figure 3

Transfer of a cryptic plasmid from Streptococcus cremoris IL839 into Streptococcus lactis IL1403 using a cotransformation procedure



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