REVIEW

Bacteriophage Resistance in *Lactococcus*

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Abstract

Lactic acid bacteria are industrial microorganisms used in many food fermentations. *Lactococcus* species are susceptible to bacteriophage infections that may result in slowed or failed fermentations. A substantial amount of research has focused on characterizing natural mechanisms by which bacterial cells defend themselves against phage. Numerous natural phage defense mechanisms have been identified and studied, and recent efforts have improved phage resistance by using molecular techniques. The study of how phages overcome these resistance mechanisms is also an important objective. New strategies to minimize the presence, virulence, and evolution of phage are being developed and are likely to be applied industrially.

Index Entries: *Lactococcus*; bacteriophage infections; phage.

1. Introduction

Bacteriophage infection of lactic acid bacteria used as starter cultures for dairy fermentations is a significant problem worldwide. Phage infection is the major cause of slow acid production in dairy fermentations *(1)*. Slow or nonfermenting vats ultimately result in economic losses to the industry. Dairy fermentations are particularly susceptible to phage attack for several reasons, including the following: The medium for culture growth (pasteurized milk) is not sterile and may contain phage; the fluid nature of the medium allows quick dispersion of phage; repeated use of pure cultures provides constant hosts for phage proliferation.

The dairy environment is a unique arena in which to study phage–host interactions. Of the species comprising the lactic acid bacteria, bacteriophage attack is most often reported for *Lactococcus lactis,* and thus, this review will focus on phage resistance mechanisms in this species. Because of their economic significance, substantial research has been conducted to characterize lactococcal phages and the mechanisms that protect lactococcal cells from phage infection. In the past decade, the techniques of molecular biology have been applied to lactococci and their phages, resulting in more knowledge about the genetic basis for phage resistance. More recently, details revealing how phages overcome these mechanisms have become available. In order to be successful in the long term, future starter culture designs must consider the molecular interactions behind host and phage evolution and take steps to minimize the genetic routes leading to the appearance of new virulent phages.

2. Bacteriophage Resistance Mechanisms in *Lactococcus*

The phenotypic, genetic, and biochemical characterization of phage resistance mechanisms has led to extensive progress in the understanding of the lactococci and in the ability to improve them for fermentations. The main approach to studying phage resistance has been to characterize naturally occurring resistance mechanisms that have evolved in the dairy environment after repeated exposures of bacteria to bacteriophages. Another approach that will be discussed is the development of new phage resistance mechanisms using biotechnology. All mechanisms discussed are shown in Fig. 1.

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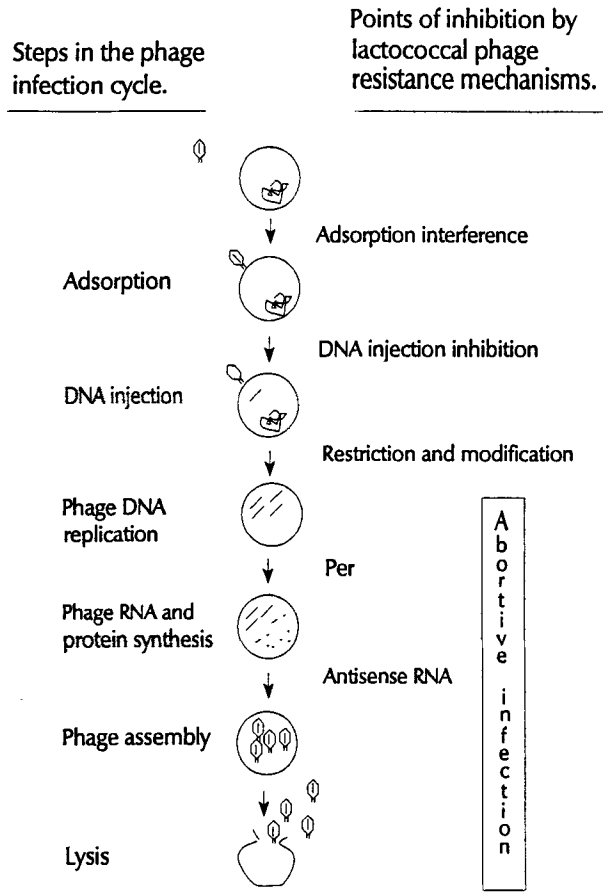

Fig. 1. Generalized phage infection cycle showing the points of inhibition by lactococcal phage resistance mechanisms.

2.1. Naturally Occurring Phage Resistance Mechanisms

Currently, four groups of naturally occurring phage resistance mechanisms are recognized based on their general modes of action: adsorption interference, DNA injection inhibition, restriction and modification systems, and abortive phage infection mechanisms. Continued characterization of these mechanisms undoubtedly will result in addition of new groups or subdivision of existing groups. The following are descriptions of the four groups, citing several well-characterized examples of each. This is not intended to be a comprehensive listing of all of the phage resistance mechanisms studied in lactococci. For recent reviews more thoroughly covering this area, *see* the reviews by Hill *(2)* or Klaenhammer and Fitzgerald *(3)*.

2.1.1. Interference with Phage Adsorption

The first line of defense a cell has against a phage is to prevent phage attachment to the exterior of the cell, thus halting the phage infection cycle at its first step. Cells with reduced phage adsorption may result from lack of or a defect in the cellular receptor for the phage or from the presence of another compound that blocks the receptor. Two approaches have been pursued in studying adsorption interference: studying strains with a mutation in their genome that prevents phage adsorption and studying plasmid-encoded mechanisms that prevent adsorption of phages to cells containing the plasmid. Both approaches have provided some insight into the components of the lactococcal cell surface that are necessary for or can block phage adsorption.

Many of the bacteriophage-insensitive mutant strains used in cheese making do not permit phage adsorption to the cell surface *(4–7)*. The study of these mutants and of laboratory strains has provided some clues about the nature of phage receptors on lactococcal cells. For most of the phage receptors identified, carbohydrate moieties in the cell wall play a key role in phage adsorption. Loss of nearly all of the cell wall-associated galactose in *L. lactis* subsp. *cremoris* KH correlates with the loss of binding to cell walls by five phages tested *(8)*. Rhamnose was also essential for adsorption of two of these five phages. Rhamnose and glucose have been implicated as part of the receptor for the phage sk1 on *L. lactis* subsp. *lactis* C2 *(9)*. Metaperiodate treatment and acid hydrolysis of cell walls of *L. lactis* subsp. *lactis* biovar. *diacetylactis* F7/2 and *L. lactis* subsp. *cremoris* Wg2-1 suggest that a carbohydrate component is also part of the receptor for phages P008 and P127, respectively *(10)*.

In one of the first studies of phage adsorption in lactococci, Oram and Reiter *(11)* describe a phage receptor for phage m13 that is present in the plasma membrane of *L. lactis* subsp. *lactis* ML3. Phage ml3 did not adsorb to the cell wall fraction of any strain tested, although five other phages of *L. lactis* subsp. *cremoris* adsorbed to cell walls. The m13 plasma membrane receptor later was determined to be a lipoprotein *(12)*.

Valyasevi et al. *(13)* have also described a membrane protein of *L. lactis* subsp. *lactis* C2 that appears to be required for phage infection. Phage-resistant derivatives of strain C2 adsorb phage normally to the cell wall and have the same cell wall carbohydrate composition as wild-type cells, but do not allow phage proliferation. Although the cell membrane fraction from resistant mutants had no effect on infectivity of c2, membranes from wild-type cells inactivated phage c2 such that it was no longer infectious. The proteinaceous nature of the membrane component was demonstrated by its sensitivity to proteinase K. This protein was partially purified and is 32 kDa in size. Electrophoresis under nondenaturing conditions suggests that the protein is part of a large complex of 350 kDa.

In subsequent study, Geller et al. *(14)* cloned a gene *(pip)* from the wild-type C2 genome that reverts the phage-resistant mutant to sensitivity to phage c2. The protein predicted from the DNA sequence of this gene has a M_r of 99,426, four to six potential membrane spanning regions, and a putative signal sequence at its amino terminus. The relationship between the 32-kDa protein and the protein encoded by the *pip* gene (PIP) is unknown. Recently, it was demonstrated that seven phages adsorb reversibly to a rhamnose-containing component of the C2 cell wall *(15)*. Apparently following adsorption to the cell wall, these phages adsorbed irreversibly to the membrane protein encoded by the *pip* gene. The phage-resistant derivative of C2 discussed has a defective PIP, therefore membranes from this strain do not adsorb these phages. Ejection of phage DNA rapidly followed adsorption to the membrane *(15)*.

Gopal and Crow *(16)* have characterized a material that is loosely associated with the cell surface of *L. lactis* subsp. *cremoris* E8 and its phage-resistant derivative, 398. The loosely associated material (LAM) from the parent strain binds the two phages in the study, whereas LAM from strain 398 did not. This indicated that the LAM is directly interacting with the phage and that a difference in the composition or structure of the LAM caused the phage resistance observed in strain 398. Chemical analysis revealed that LAM from 398 is two to three times more abundant and contains five times more rhamnose and two times more galactose than that of E8. Also, LAM from strain 398 lacked a 21-kDa protein that is present in the parent strain. The direct function of any of these components in phage adsorption was not determined.

Another approach to studying adsorption inhibition has been to characterize plasmid-encoded mechanisms. Sanders and Klaenhammer *(17)* reported the first incidence of plasmid-encoded adsorption inhibition in lactococci. Their study demonstrated that phage-sensitive derivatives of the prototype phage-resistant strain *L. lactis* ME2 lacked a 31-MDa plasmid, pME0030, and adsorbed ϕ18 at significantly higher levels.

The adsorption inhibition mechanism encoded by pSK11 was discovered by correlating the absence of this plasmid from the cell to sensitivity to phage sk11G *(18)*. Further characterization of this mechanism has revealed differences between the cell surface characteristics of the pSK11-carrying, phage-resistant strain, *L. lactis* subsp. *cremoris* SK110, and its phage-sensitive derivative, SK112. The cell surface of SK112 was more negatively charged and more hydrophobic than that of SK110. The carbohydrate composition of the cell surface was examined using lectins with affinities for specific sugars. The resistant strain, SK110, agglutinated in the presence of a lectin specific for terminal β-D-galactosyl residues and did not agglutinate with a lectin specific for glucosyl residues. Agglutination results with strain SK112 were opposite than that with SK110 *(19)*. Sugar content analysis of material extracted from whole cells indicated that the SK110 cell surface material contains a significantly higher amount of galactose. Mild alkali treatment of strain SK110 resulted in phage sensitivity, suggesting that the phage receptor remains intact but is shielded by a galactosyl-containing component that is removed by this treatment *(20)*. Subsequent chemical analysis of the lipoteichoic acid (LTA) from both strains indicated the absence of galactose in LTA from SK112, whereas LTA from SK110 contained galactose *(21)*. It was proposed that galactosyl-containing LTA is involved in preventing phage adsorption.

Plasmid pCI528, isolated from *L. lactis* subsp. *lactis* UC503, also causes reduced bacteriophage resistance by an alteration in the cell surface *(22)*. Introduction of pCI528 into *L. lactis* subsp. *lactis* MG1363 resulted in complete resistance to prolate- and small isometric-headed phages. Since this mechanism interferes with adsorption of a variety of phages, it is not specific to one phage receptor. Cells containing pCI528 produced a "hard" colony morphology on agar media and a loose, "fluffy" pellet on centrifugation. Electron microscopy indicated the presence of a thick, irregularly distributed extracellular substance on the surface of cells containing pCI528. Cells without the plasmid had a smooth, regular surface. Treatment of pCI528-containing cells with detergents, acid, and proteases had no effect on phage resistance. However, alkali treatment of the cells allowed phage adsorption and restored the wild-type pellet phenotype on centrifugation. Cell surface polysaccharides from parental and pCI528-containing cells before and after alkaline treatment were analyzed by gas-liquid chromatography. The levels of galactose and rhamnose were much greater in the untreated pCI528-containing sample than in the NaOH treated plasmid-containing strain or the plasmid-free parental strain.

Harrington and Hill *(23)* reported a spontaneous phage-insensitive mutant of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* DPC220 that occurred after infection with phage ϕD1. This mutant, strain DPC721, was shown to contain pAH90, a 26.75-kb plasmid not present in DPC220. This plasmid was formed as a result of cointegration of pAH82 (20.75 kb) and pAH33 (6 kb) that are present in DPC220, but absent from DPC721. The appearance of pAH90 correlated with phage resistance by adsorption inhibition and by restriction and modification. It was proposed that cointegration resulted in activation of a silent resistance mechanism.

Despite many studies, inhibition of phage adsorption is still a poorly understood phenomenon at the molecular level. Fortunately, recent work has begun to reveal some details about the cell surface components that play a role in or interfere with phage adsorption. Adsorption inhibition completely excludes phage from the cell, and therefore, it is a powerful resistance mechanism. A better understanding of adsorption inhibition will contribute to future efforts to develop phage-resistant strains for long-term use in dairy fermentations.

2.1.2. Inhibition of Phage DNA Injection

Two reports have described inhibition of phage proliferation by blocking the injection of the phage genome into the cell. In these cases, the phage adsorbs to the cell surface normally, but the phage DNA does not reach the cytoplasm, and the completion of the infection cycle is prevented.

The first description of inhibition of DNA injection in lactic acid bacteria was in the genus *Lactobacillus*. Watanabe et al. *(24)* described a derivative of *Lactobacillus casei* YIT9002 that was resistant to the phage PL-1. Using electron microscopy, it was shown that the phages infecting the resistant strain adsorbed normally, but retained their DNA inside the phage capsids. On the other hand, phages infecting wild-type cells displayed increasing numbers of empty heads over increasing length of incubation with the cells.

A more detailed study was reported by Hill *(2)* in which phage c2 infection of *L. lactis* MG1614 containing pNP40 was studied. pNP40 encodes two Abi mechanisms, conjugal transfer ability and the injection blocking mechanism described. Phage c2 adsorbs normally to cells containing pNP40, but c2 DNA cannot be detected in the cell after adsorption. The resistance mechanism was bypassed and successful infection was achieved when phage DNA was electroporated into the cell. This indicated that phage DNA injection was blocked. Blocking phage DNA injection has not been studied extensively as a phage resistance mechanism, but because it acts early in the infection cycle, this mechanism may become important in construction of phage-resistant strains.

2.1.3. Restriction and Modification Systems

Restriction and modification (R/M) systems are widespread throughout lactic acid bacteria. The restriction enzyme component of the system recognizes and cleaves DNA that is not modified at a specific site. Thus, incoming unmodified phage

DNA is cleaved, and phage proliferation is prevented. Host DNA is protected from cleavage owing to enzyme-specific modification by the methylase component of the system. An R/M system is frequently cited as the cause for an observed phage resistance phenotype in lactic acid bacteria based on host-dependent replication of the phage *(23,25–38)*. In most reported cases, the genetic determinants for these R/M systems are borne on plasmids. Their location on plasmids has simplified the identification and characterization of these mechanisms. Although many R/M systems have been identified, in only a few cases have the genetics and biochemistry of the R/M system been studied. The following discussion will focus on these.

The first type II restriction endonuclease purified from lactic acid bacteria was *Scr*FI from *L. lactis* subsp. *cremoris (39)*. The optimal conditions for the enzyme and its cleavage site (CC/NGG) were determined. It is interesting to note that the temperature optimum for *Scr*FI, 40°C, is the same as the cooking temperature of many cheeses. Two restriction endonucleases from *L. lactis* subsp. *cremoris* W9 and W56 have also been purified. *Lla*AI and *Lla*BI recognize and cleave the sites /GATC and C/TRYAG, respectively *(40)*. Another restriction enzyme, *Lla*I, which appears to have a similar recognition site as *Scr*FI (CC[A/T]GG), was partially purified from *L. lactis* subsp. *lactis* NCDO 497 *(41)*. Three type II restriction endonucleases have been purified from *Streptococcus thermophilus* strains. *Sth*134I *(41a)*, *Sth*117I *(41b)*, and *Ssl*I *(41c)* were analyzed to determine optimum conditions and cleavage sites. Their cleavage sites (C/CGG, CC/WGG, and CC/WGG, respectively) are almost identical.

Two genes encoding methylases involved in the *Scr*FI system have been cloned based on their ability to protect the cloning vector from digestion by *Scr*FI in vitro *(42)*. No endonuclease activity was detected from either of the cloned fragments. One of the methylase genes was sequenced. Computer analysis of the expected M-*Scr*FI protein sequence indicated characteristics of m^5C methylases and homology with other known methylases.

One complete R/M system has been cloned and sequenced. *Lla*I originated from the plasmid pTR2030 from *L. lactis* subsp. *lactis* ME2 *(43)* and should not be confused with the enzyme from NCDO 497 to be redesignated as *Lla*497I. *Lla*I is effective against several small isometric-headed phages. The first component to be sequenced was the methylase gene, *llaIM (44)*. The predicted protein sequence of *Lla*IM has two functional domains and shows organizational similarities to type II-S methylases. Sequencing of the regions surrounding the methylase gene revealed a small upstream open reading frame (ORF) and four consecutive downstream ORFs *(45,46)*. Frameshift mutations demonstrated that three of the four downstream ORFs are required for restriction activity, making this an unusual multisubunit restriction enzyme. The function of the last ORF is unknown. Northern analysis revealed that the upstream ORF, the *llaIM* gene, and the four downstream ORFs form an operon.

The small upstream ORF, designated *llaIC*, encodes a protein with homology to a class of regulatory genes for type II R/M systems. At 40°C, C-*Lla*I has been shown to be a negative regulator of transcription of the operon. Also, C-*Lla*I enhances *Lla*I restriction activity in vivo at 30°C, but at 40°C, restriction activity is significantly retarded. The results provide a molecular mechanism to explain the temporary loss of restriction activity by starter cultures held at the cooking temperature of cheddar cheese manufacture.

R/M systems are very effective in blocking the phage infection cycle at an early stage without sacrificing the viability of the cell. The major drawback to these systems is the ease with which phage can overcome them. A phage may escape cleavage by the endonuclease if it becomes modified by the methylase before cleavage occurs. Modified progeny phage DNA is not recognized as foreign by the cell, so the phage may replicate unrestricted within that cell and within neighboring cells harboring the same R/M system. This problem prevents R/M systems from being effective alone during prolonged use of a culture, although they are extremely effective when com-

bined (naturally or artificially) with other R/M systems or Abi mechanisms *(see the following)*.

2.1.4. Abortive Infection

Abortive infection occurs when the phage infection cycle is inhibited at some point after adsorption, DNA entry, and early phage gene expression occur *(47)*. Abortive phage infection (Abi) mechanisms are typically plasmid-encoded and result in reduced burst size, efficiency of plaquing (EOP) and efficiency of center of infection formation. As a consequence of aborted phage infection, the cell dies, but the release of progeny phage is minimized or eliminated *(48)*. It is unclear if cell death is caused by the resistance mechanism itself or by the initial stages of the phage infection cycle prior to abortion.

Many plasmids encoding Abi mechanisms have been identified and studied *(3,49)*, but none tested show significant DNA homology with any other Abi determinants. This suggests that there are many types of Abi mechanisms that may act to inhibit different parts of the phage infection cycle. This discussion will focus on specific genes that encode abortive phage infection.

2.1.4.1. AbiA

The first lactococcoal abortive phage infection mechanism to be studied in detail was AbiA from the plasmid pTR2030. AbiA was originally designated Hsp because it causes heat-sensitive phage resistance. The mechanism is now designated AbiA to promote consistency in the nomenclature of abortive infection mechanisms. The AbiA phenotype was first observed by Klaenhammer and Sanozsky *(43)* in *L. lactis* ME2. Conjugation experiments using ME2 as a donor indicated that AbiA was associated with the 46-kb self-transmissible plasmid, pTR2030 *(43,50,51)*. Through AbiA and *Lla*I, pTR2030 confers complete resistance to small isometric-headed phages, whereas, large isometric- or prolate-headed phages are unaffected or only slightly affected by this plasmid *(49,52,53)*. The AbiA mechanism, subcloned without *Lla*I, reduces the number of infected hosts that generate viable phage and reduces the burst size of any successful infections. Though phage proliferation is inhibited in infected cells, cell death occurs at about the same percentage as in infected AbiA$^-$ cells *(49)*.

The genetic determinants for AbiA were localized by mapping a spontaneous deletion in pTR2030 that resulted in sensitivity to phage *(54)*. DNA sequence determination of a 3-kb region indicated an open reading frame of 1887 basepairs encoding a protein of predicted molecular mass of 73.8 kDa *(55)*. As with the phenotypic designation, this gene was originally called *hsp* and has been renamed *abiA*. Coffey et al. *(56)* reported the same DNA sequence for the gene encoding the Abi mechanism isolated from pCI829. Primer extension has defined a transcriptional start upstream of *abiA (46)*. A PCR generated fragment containing only the *abiA* ORF and 240 bp upstream also conferred phage resistance *(57)*, confirming that this is the sole locus responsible for the AbiA phenotype.

The phage resistance conferred by *abiA* is dependent on gene dosage *(57,58)*. A single copy of *abiA* integrated into the lactococcal chromosome does not confer significant phage resistance to the cell, whereas amplification of the insert results in decreased EOP and burst size of the small isometric-headed phage 712 *(58)*. Placing the *abiA* gene on three plasmids with different copy numbers caused decreasing efficiencies of plaquing of several phages with increasing copy number of the plasmid *(57)*.

The mechanism by which AbiA prevents phage proliferation is unknown. Hill et al. *(59)* have provided some insight by using DNA hybridization to observe phage DNA during infection of cells with and without AbiA. In AbiA$^+$ cells, the presence of phage DNA was detected intracellularly soon after infection, but the DNA did not increase in amount over time as did the phage DNA in AbiA$^-$ cells. This suggests that AbiA has a direct or indirect effect on phage DNA replication. Moineau et al. *(60)* also reported that capsid protein production of the phage ul36 was almost totally inhibited in AbiA$^+$ cells.

2.1.4.2. Abi-416

The genetic determinant encoding Abi in *L. lactis* subsp. *lactis* IL416 has also been cloned and sequenced *(61)*. Fragments of about 10 kb

resulting from partial *Sau*3AI digestion of the total DNA from IL416 were cloned into a plasmid vector and transformed into *L. lactis* subsp. *lactis* IL1403. Transformants were screened for resistance to the phage bIL66. A clone containing a 5-kb insert was chosen for further characterization. Deletion analysis left a plasmid (pIL611) containing a 2-kb fragment encoding phage resistance. The phenotype conferred by pIL611 was consistent with other abortive infection mechanisms. The number of phages executing successful infections was decreased to 0.04% of the input adsorbed phages. The burst size of the infections was reduced by 80% when compared to the Abi^- strain.

Sequencing of the 2-kb fragment in pIL611 revealed an Iso-IS*S1* element upstream of an open reading frame encoding 250 amino acids. This gene was designated *abi-416*. When a portion of the ORF was deleted, the Abi^+ phenotype was abolished. Deletion of a putative promoter which resides in the Iso-IS*S1* element also results in loss of the Abi^+ phenotype, suggesting that the promoter for *abi-416* is provided by this IS element.

2.1.4.3. AbiC

The gene encoding another Abi mechanism from the phage-insensitive strain *L. lactis* subsp. *lactis* ME2 has been identified, cloned, and sequenced *(62)*. This mechanism was originally designated Prf, for phage resistance five, and was the fifth mechanism characterized from ME2. As with AbiA, this mechanism was renamed AbiC for uniformity in naming Abi mechanisms. The plasmid pTN20 encodes AbiC and an R/M system. Subcloning of the entire pTN20 plasmid into the plasmid pSA34, which encodes erythromycin resistance and replicates in *E. coli,* inactivated the R/M system and revealed the abortive mechanism, AbiC. AbiC is effective against small isometric-headed phages. As with other Abi mechanisms, EOP and burst size are reduced. The plaque sizes of the phage p2 propagated on a $AbiC^+$ strain are variable in size. Isolation and propagation of large plaques indicated that they had become resistant to AbiC. The mechanism by which AbiC prevents phage proliferation is unknown, although it differs from AbiA in that intracellular phage DNA replication occurred *(62)* and 50% of ul36 capsid protein was produced in $AbiC^+$ cells *(60)*.

Subcloning and Tn*5* mutagenesis localized the region encoding AbiC to 1.5 kb, which was sequenced to reveal a 1031-bp ORF, designated *abiC*. A putative ribosomal binding site was found, but no transcriptional expression signals were identified probably because the fragment which was sequenced ended 12 nucleotides upstream from the putative ribosomal binding site. Cloning this gene into a lactococcal expression vector resulted in the $AbiC^+$ phenotype, indicating that the *abiC* gene alone is sufficient for phage resistance.

2.1.4.4. Other Plasmids Encoding Abi

An Abi mechanism effective against the prolate-headed phage c2 was identified on the plasmid pKR223 *(32)*. The genetic locus for this Abi mechanism was determined to be within a 1.3-kb fragment. Attempts to clone the Abi determinant were unsuccessful possibly because its expression may be linked to upstream genes encoding an R/M system.

Other Abi mechanisms have been reported on pBF61 *(27)*, pCI528 *(63)*, pAJ2074 *(64)*, and a 131-kb plasmid from the transconjugant strain HID600 *(65)*. Many other examples exist where an Abi mechanism may be implicated in phage resistance, although the resistance types have not been completely characterized *(3)*.

Continued study of Abi mechanisms may reveal the ways in which these mechanisms prevent phage infection. Identifying the stage of the infection cycle inhibited by each of the Abi mechanisms will allow logical combination of resistance mechanisms within a cell to provide the maximum protection against phage.

2.1.5. Comments on Natural Phage Resistance Mechanisms

In the dairy environment, it is common to find lactococcal strains containing more than one phage resistance mechanism per cell, and in many cases, more than one per plasmid. It is easy to speculate on why industrial strains, which are constantly exposed to phage, would contain a battery of resistance mechanisms. For example, with

resistance mechanisms that are very effective, but easily overcome by the phage, such as R/M systems, it may be necessary for the cell to have alternative means of preventing phage infection later in the infection cycle. Also, some phage resistance mechanisms act against one phage or a small group of phages; therefore, increasing the types of mechanisms per cell would provide more diverse resistance.

Cases of naturally occurring combinations of phage resistance mechanisms are abundant in lactococci *(17,26,27,62)*. The prototype phage-resistant strain *L. lactis* subsp. *lactis* ME2 is an excellent example. ME2 was an industrial strain which performed consistently over long periods in commercial cheese production. ME2 has 13 plasmids *(17)*, three of which have been shown to be involved in phage resistance. The plasmid pME0030 encodes adsorption inhibition *(17)*, pTR2030 encodes an R/M and the AbiA abortive infection mechanism *(30,54)* and pTN20 encodes an R/M and AbiC *(29,62)*. This combination of plasmids renders ME2 insensitive to phages. The mechanisms in this strain act at different points of the infection cycle, providing the cell with several opportunities to prevent successful phage infection. Adsorption inhibition is its first line of defense, followed by the R/M systems with different specificities. The two abortive mechanisms prevent proliferation of phages that escape the earlier mechanisms. One abortive mechanism (AbiA) acts early in the cycle by inhibiting phage DNA replication *(59)*, whereas AbiC appears to affect a later stage in phage development *(62)*. The combination of phage resistance mechanisms on the same or on different plasmids in the laboratory has also demonstrated that most mechanisms have additive effects on phage resistance when combined *(37,66–68)*.

Many phage resistance plasmids also encode conjugative functions, a feature that has been exploited in the identification and isolation of many of these plasmids. Plasmids pTR2030 *(43)* and pTN20 *(29)* were both identified after conjugal transfer to plasmid-free strains. Other conjugal plasmids that encode more than one type of phage resistance include pNP40 *(69)* and pAJ1106 *(70)*. Conjugation has been applied to obtain phage-resistant lactococcal strains for the dairy industry *(71,72)*. Strains that are genetically improved by conjugation, as opposed to transformation or electroporation, are acceptable in food-grade applications because the DNA is introduced by natural means *(73)*. Little is known about the transfer (Tra) functions on these plasmids, but they may play an important role in dispersion of plasmids encoding phage resistance and other industrially important traits *(74)*. Conjugal elements may also affect phage resistance levels. Romero and Klaenhammer *(75)* demonstrated increased phage resistance upon interaction of a plasmid containing pTR2030-derived sequences with an unknown conjugal element.

Transposable elements have also been associated with phage resistance genes, suggesting that these genes are able to move intracellularly. The R/M and AbiA determinants on pTR2030 are flanked by copies of IS*946* that may allow this unit of DNA to move as a composite transposon *(76,77)*. The expression of the *abi-416* gene was shown to be dependent on a promoter in an iso-IS*SI* element *(61)*. Last, insertion of a copy of IS*981* was responsible for the loss of Abi activity in some pGBK17-containing cells *(32,78)*.

2.2. Genetically Engineered Phage Resistance Mechanisms

Until recently, the information obtained about phage resistance depended on what was provided by and gathered from nature. Because of the increased ability to genetically manipulate lactococcal cells, scientists are now able to create novel phage resistance mechanisms using recombinant DNA technology. Currently, two mechanisms have been designed that are based on phage DNA sequences.

One approach has been to clone phage genes such that the antisense RNA strand is synthesized in the cell. Antisense constructions have been made to an ORF of unknown function *(79)* and to the major capsid protein of phage 4-1 *(80)*. Although expression of antisense RNA was not demonstrated in these studies, phage resistance was observed in strains containing the antisense

constructions (EOP of 10^{-2} and EOP of 0.5, respectively). The resistance observed resembles that seen with Abi mechanisms. However, recent homology studies showed that the sequences cloned in these antisense constructions are poorly conserved among lactococcal phages *(81)*. The effectiveness of this method will depend on the identification of genes vital to phage development, which are highly conserved among many phages.

A different approach was taken by Hill et al. *(82)* in a study that reports the cloning and sequencing of a phage origin of replication *(ori)*. The *ori* from phage ϕ50 was cloned into pSA3. The resulting plasmid, pTRK104, was introduced into *Lactococcus* where it replicated owing to the gram-positive plasmid *ori* on pSA3. Upon infection with ϕ50, however, plasmid replication is driven by the phage *ori,* resulting in an explosion in plasmid copy number. The plasmid-borne phage *ori* may titrate essential replication factors from the phage-borne phage *ori,* slowing phage DNA replication and retarding development of mature phage particles. This type of phage resistance also resembles that observed with the Abi mechanisms and has been called Per, for phage encoded resistance. The level of resistance offered by Per can be increased significantly by increasing the copy number of the plasmid containing the phage *ori,* thus providing more false targets for the phage replication factors. For example, the low copy number plasmid pTRK104 causes an EOP of 0.42 and reduced plaque size, whereas a high copy number plasmid containing the ϕ50 *ori* reduced the EOP to 2.5×10^{-4} *(83)*. This same phenomenon has been demonstrated using the phage ϕ31 *ori (83)*. Per50 is not effective against ϕ31, and Per31 is not effective against ϕ50, although both phages are members of the P335 species. On the other hand, Per50 does inhibit phage ul36 *(84),* suggesting that ϕ50 and ul36 share similarities in the factors required for the replication of their DNA. It remains to be determined how many phages share similarities in their replication factors. As with antisense technology, Per must inhibit more than one or two phages to be effective in protecting a cell from phage attack. New approaches and more information about the factors necessary for lactococcal phage infection will provide more avenues of interference with phage development.

3. Phage Counter Defenses

Lactococcal phages are genetically diverse and highly adaptable, probably owing to the extensive use of their hosts in milk fermentations worldwide. As cells evolve or are provided with new mechanisms to prevent phage infection, phages are also adapting to overcome these barriers *(85)*.

3.1. Phages Insensitive to R/M Systems

Two routes by which phages become insensitive to R/M systems have been studied. There is also the temporary resistance offered to a phage when its DNA is modified by the host-encoded methylase, but in this case, sensitivity is restored on propagation through a cell that lacks the R/M. Reports from the 1980s *(86,87)* suggest that restriction enzyme sites are lost in phage genomes over time, and thus the phage DNAs become less sensitive to R/M systems. The loss of these sites may be owing to substitution or deletion mutations. Recently, Moineau et al. *(88)* showed that seven phages isolated from cheese plants in North America since 1989 (P335 species) were more sensitive to lactococcal R/M systems in vivo than more common phages that have been studied for many years (936 and c2 species). The phages in the P335 species were also cleaved more frequently in vitro by restriction enzymes. Other lactococcal phages studied have also shown a paucity of restriction sites for many commercial endonucleases *(89–97)*.

In another study, in vivo genetic exchange between a phage genome and a plasmid was implicated in the evolution of a phage resistant to the R/M on pTR2030. Phage ϕ50 was isolated from an industrial fermentation that used a pTR2030 transconjugant as a starter culture. Phage ϕ50 is insensitive to both AbiA and the R/M encoded on this plasmid. A region of homology was detected between ϕ50 and pTR2030 suggesting genetic exchange between the two genomes *(98)*. After physical mapping and DNA sequencing it was determined that ϕ50 had acquired part of the *llaIM* gene from pTR2030 encoding the amino terminal

domain of the protein and the upstream regulatory sequences *(44)*. The amino terminal domain cloned on a plasmid was shown to be sufficient to modify another phage genome and protect it from cleavage upon infection of an R/M-containing cell. Thus, ϕ50, through genetic exchange with pTR2030, carries its own functional methylase, insuring that its genome is always modified and protected from the pTR2030-encoded R/M. Subsequently, phages al, bl, cs, and d1, which were isolated independently from ϕ50 in two separate locations, were also shown to have acquired the amino-terminal domain of the methylase encoded by pTR2030 *(99)*.

3.2. Phages Insensitive to Abortive Infection Mechanisms

Phages insensitive to Abi mechanisms have also been reported. The reasons for their insensitivity are generally not well understood. Point mutations leading to Abi insensitivity are the most common. Phages bearing point mutations resulting in insensitivity to the following Abi mechanisms have been isolated in the laboratory: AbiA *(57,59)*, AbiC *(62)*, Abi-105 *(100,101)*, Abi-416 *(102)* and the mechanism identified on a 131-kb plasmid from HID600 *(65)*. In one case, Abi-105, the phage mutation has been localized and sequenced *(101)*, but the function of the mutated gene is not known. Identification and characterization of more phage genes targeted by Abi mechanisms may lead to a better understanding of how these mechanisms inhibit phages. Phages insensitive to AbiA have also been isolated from industry. As mentioned, the AbiA-resistant phages ϕ50, ϕ48, al, bl, cs, and dl were isolated from cheese plants where starters containing a pTR2030 transconjugant were in use *(98,99)*. Since the ancestors of these phages are unknown, it is not understood how their insensitivity to AbiA came about.

A recent report also implicates a genetic exchange between phage and host genomes in the evolution of Abi- and Per-insensitive phages. When *L. lactis* NCK203 containing an AbiC-encoding plasmid is infected with the phage ul36, AbiC-sensitive phages emerge at an EOP of 10^{-3} *(84)*. At a lower frequency (EOP = 10^{-5}) large plaques containing an AbiC-resistant phage, ul37, were recovered. Phage ul37 is also insensitive to Per50, whereas the parental phage, ul36, is sensitive to this mechanism *(84)*. This suggested that ul37 may have acquired a new origin of replication. Characterization of these phages by electron microscopy indicated that ul36 and ul37 are morphologically distinct from each other and from an inducible prophage resident in the NCK203 chromosome. Most striking was that each of the three phages had a different base plate. Restriction enzyme analysis of the genomes of ul36 and ul37 showed some fragments common to both phages, but ul37 had acquired new fragments and lost some of the fragments present in ul36. Interestingly, the "new" DNA acquired in ul37 originates from the NCK203 chromosome, but not from the inducible prophage in this strain. Disruption of the chromosomal sequences contributing to ul37 formation prevents the appearance of this phage during subsequent infections with ul36 *(see the following)*.

Resistant phages that have altered restriction enzyme patterns have also been observed when ϕ31 is infected on NCK203 containing Per31 or AbiA on a high copy plasmid *(57,83)*. These phages are insensitive to both Per31 and AbiA. Again, this may indicate the acquisition of a new origin of replication. A total of four different "new" phages (ϕ31.1, ϕ31.2, ϕ31.7, and ϕ31.8) derived from ϕ31 have been isolated under the selective pressure of the high copy Per31 plasmid (*83*, O'Sullivan et al., unpublished data).

These data indicate that host sequences are contributing to the evolution of phages that are insensitive to Abi and Per mechanisms. This discovery may play an important role in future strain selection and development.

4. Strategies to Reduce the Emergence of Bacteriophages in Dairy Fermentations

4.1. Traditional Strategies

Strategies to reduce the emergence of phages in dairy fermentations are presented in Table 1 and discussed in the following sections. Tradition-

Table 1
Strategies to Reduce the Emergence of Phages in Dairy Fermentations

Isolation of bacteriophage insensitive mutants *(105)*
Multiple strain starter cultures *(103)*
Rotation of phage unrelated cultures *(103,111)*
Development of phage-resistant derivatives using genetic techniques *(3)*
Rotation of isogenic derivatives of a single strain containing different phage resistance mechanisms *(66,68)*
Inactivation of host sequences contributing to phage evolution *(84)*

ally, several approaches have been taken to protect starter culture strains from phage infection. The strain composition of starter cultures is an important factor in protecting the fermentation from phage. Today, starter compositions fall into three categories *(103,104)*. Single-strain starters are composed of single strains of *L. lactis* subsp. *cremoris* or subsp. *lactis* that are used alone or in pairs. Multiple-strain starters are composed of three to six defined strains of lactic acid bacteria. Mixed-strain starters consist of an unknown mixture of strains of *L. lactis* subsp. *cremoris* and subsp. *lactis,* and sometimes *L. lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc* species.

In the preparation of single- and multiple-strain starters, good acid- and flavor-producing strains are isolated and then tested against phages found in the dairy plant. Insensitive strains are incorporated into the starter culture and then tested daily during fermentation for the emergence of phages. A strain that becomes infected is withdrawn and replaced by another strain that is resistant to this phage or by a phage-resistant mutant of the original strain *(105)*. Although this strategy has been successful, isolation of phage-resistant mutants or other replacement strains with the same fermentation characteristics as the original strain can be difficult *(106,107)*.

Mixed-strain starters are much more complex. Their strain composition is unknown and can vary as these mixtures are propagated. Most mixed-strain starters also contain phages in the carrier state *(108,109)*. The presence of phage-sensitive and -insensitive strains of bacteria allow the survival of the phage, as well as survival of the culture *(110)*. These cultures contain highly phage-resistant strains that often lose their phage resistance when propagated in the absence of phage *(104)*. Also, propagation of the entire mixed starter in the laboratory without exposure to phages can alter the balance of the culture such that sensitive strains dominate and the culture as a whole is much more sensitive to phages.

Many starter culture programs also depend on the rotation of cultures to aid in minimizing phages in the plant. The rotation should include starters that are unrelated in terms of their susceptibility to phages. Therefore, a particular phage that accumulates on one day will be without a host on the next day. When a particular starter in the rotation succumbs to phage attack, it is replaced. Recently, Moineau *(111)* suggested a rotation series where strains are substituted on the basis of their sensitivity to different phage species. Certain lactococcal strains that propagate one or two phage species may not propagate a third or fourth species. Therefore, the rotation series would be based on strain combinations that are not only phage-unrelated, but also phage species-unrelated.

4.2. A Novel Rotation Program Based on Isogenic Derivatives of a Single Strain

Rotation programs and multiple-strain starters require many strains. Often it is difficult to isolate enough phage-unrelated strains with the fermentative characteristics necessary to produce a certain product. Recently, a rotation program has been designed to protect one specialized strain during continuous use over an extended time. This rotation scheme, called the phage defense rotation strategy, is based on isogenic derivatives of a single strain that harbor different phage resistance mechanisms *(68)*.

In the development of this strategy, phage-resistant derivatives of *L. lactis* subsp. *lactis* NCK203 were constructed by introducing, through conjugation or transformation, various plasmids encoding abortive infection and/or restriction and modification *(68)*. Selected phage-resistant strains were evaluated in different rotation sequences

through cycles of the Heap-Lawrence starter culture activity test (SAT) in milk contaminated with phage and whey from the previous cycle. Four rotation sequences were tested using the phage ϕ31. Sequences were terminated when strains failed to lower the milk pH below 6.0, and high levels of phage were detected.

Otherwise, nine cycles (three rotations of three strains) were completed. It was found that alternating the type and specificity of R/M and Abi mechanisms prevented phage proliferation, and, in some cases, greatly reduced the number of contaminating phages. The rotation that performed the best started with a strain containing pTR2030 ($AbiA^+$, R^+/M^+). This strain was followed by two derivatives containing different R/M and Abi systems. This sequence also performed well when contaminated with a composite of industrial phages.

Initiating the rotation sequence with NCK203 (pTR2030) reduced the number of ϕ31 present in the milk from an initial level of 10^6 pfu/mL to 10^3 pfu/mL after the first cycle. The phage titer is then at a level where R^+/M^+ strains are effective at holding down subsequent phage development. NCK203(pTR2030) is so effective because any phage which escapes cleavage by the R/M is prevented from completing infection by the AbiA abortive response that kills the cell. This traps the phage and removes it from the fermentation, thus lowering the numbers of phage.

In a subsequent study, a phage defense rotation strategy was developed in which the three rotated strains each contained a different abortive infection and a different restriction and modification mechanism *(66)*. Four Abi mechanisms were used in this study. Three of them, Per31, Per50, and AbiA, were placed on high copy plasmids in order to maximize the effect of these mechanisms on the phages. AbiC was on a medium copy plasmid. Mutant and recombinant phages resistant to these mechanisms were expected to emerge *(see earlier)*. The designed rotation system successfully controlled modified, recombinant, and mutant phages resistant to any one the defense systems by presenting a different set of R/M and Abi defenses the following day. The rotation series completed 9 days of consecutive tests when challenged continuously with high titer phage preparations. A combination of two laboratory phages and a composite of 10 industrial phages were tested.

The phage defense rotation strategy has several advantages. As mentioned previously, a specialized strain can be used repeatedly. Use of one strain may also decrease the diversity of the phage populations found in a cheese plant, and thus decrease the genetic pool available for phage evolution. And last, monitoring the emergence of new phages in a plant would be simplified since only one indicator strain is necessary.

4.3. Inactivation of Chromosomal Sequences Contributing to Phage Evolution by Site-Specific Integration

As described in Section 3.2., a recombinant phage, ul37, emerged after infection with phage ul36 of *L. lactis* subsp. *lactis* NCK203 containing the AbiC abortive infection mechanism. Phage ul37 is resistant to AbiC and Per50 and resulted from recombination between the ul36 genome and the NCK203 chromosome. Restriction enzyme fragments of the ul36 and ul37 genomes were individually cloned into a gram-positive suicide vector that was lacking a gram-positive origin of replication *(84)*. Integrants were recovered with one of eight clones containing a ul36 fragment. On the other hand, five of six plasmids containing a ul37 fragment yielded integrants. Site-specific integration was demonstrated by Southern blots. A plasmid encoding AbiC was introduced into the five ul37-based integrants, and the resulting strains were infected with ul36. The authors report that disruption of NCK203 chromosomal sequences by site-specific integration of a plasmid eliminated the appearance of ul37 from $AbiC^+$ NCK203 cells.

This experiment confirmed that NCK203 sequences can contribute to the evolution of phages. It remains to be determined if the genomes of other strains can contribute to phage evolution. The DNA involved in the NCK203 background is likely to be derived from a defective or noninducible prophage, but this topic awaits further study as well. Strain NCK203 was

derived from an industrial strain, thus the evolution of recombinant phages may also be significant in an industrial setting. If so, the sequence disruption strategy described herein will provide a novel method to prevent the emergence of phages that are insensitive to the resistance mechanism encoded by the host.

5. Future Considerations

Investigation of phage resistance mechanisms has led to several strategies by which phage resistance of dairy starter cultures may be improved. To apply these research accomplishments, phage resistance genes must be introduced into industrial strains. Since these bacteria will be part of the finished food product, they must not contain antibiotic resistance markers or DNA from a non-food-grade organism. Introduction of the DNA into the organism by natural means, such as conjugation or transduction, is currently acceptable.

Since many phage resistance genes reside on conjugal plasmids, conjugation has most often been chosen for food-grade introduction of plasmids into industrial strains. When phage resistance is not naturally encoded on a conjugal element, it may be possible to put the phage resistance gene on a conjugal plasmid using recombinant DNA techniques. Selection of transconjugants may be on the basis of phage resistance or another selectable or differential marker. Plasmids pNP40 *(69,112)* and pFK012 *(113),* for example, encode nisin resistance. Other markers available include fermentation of lactose and other carbohydrates and production of a bacteriocin *(73,114,115).*

Conjugation has been used to combine more than one phage resistance mechanism into one strain. Plasmids pCI829 (AbiA$^+$) and pCI751 (Lac$^+$, Abi$^+$) were conjugally transferred into a single strain where they provided complete resistance to small isometric- and prolate-headed phage tested *(114).* Plasmids pCI750 (Abi$^+$) and pCI528 (adsorption inhibition) were conjugally transferred into commercial starter cultures. These transconjugants were resistant to phage and were subsequently used in the Irish dairy industry *(3,71).* The combination of the conjugal plasmids pTR2030 (AbiA, R/M$^+$) and pTN20 (AbiC$^+$, R/M$^+$) resulted in complete resistance to a small isometric-headed phage, but only partial resistance to the prolate-headed phage c2 *(71).*

The previous examples demonstrate that phage resistance mechanisms can be combined in lactococci using food-grade strategies. Many naturally occurring resistance mechanisms are available for industrial use. Several genetically engineered mechanisms are also available, but not currently acceptable for industrial use. For many mechanisms, more studies will be necessary to understand the specific points where phage are inhibited. As more details are revealed, we will be able to combine phage resistance mechanisms logically on the basis of which step they inhibit during the phage infection cycle. Incorporation of strains containing multiple, complementary phage defense mechanisms into standard starter cultures or into a phage defense rotation strategy will play a key role in protection of dairy starter cultures from phage in the future.

Note Added in Proof

This paper was accepted for publication in January 1995, and since then, several works have been reported concerning phage resistance in lactococci. Moineua et al. (Moineau, S., Walker, S. A., Holler, B. J., Vedamuthu, E. R., and Vandenbergh, P. A. [1995] *Appl. Environ. Microbiol.* **61,** 2193–2202) have cloned and sequenced another R/M system from *Lactococcus lactis*, *Lla*II. LlaII was subsequently renamed according to standard nomenclature as *Lla*DCHI. Two genes encoding abortive infection mechanisms have been identified and sequenced. AbiD was reported by McLandsborough et al. (McLandsborough, L. A., Kolaetis, D. M., Requena, T., and McKay, L. L. [1995] *Appl. Environ. Microbiol.* **61,** 2023–2026), and AbiDl was reported by Anba et al. (Anba, J., Bidnenko, E., Hillier, A., Ehrlich, D., and Chopin, M.-C. [1995] *J. Bacteriol.* **177,** [3818–3823]. The predicted amino acid sequences of AbiD and AbiDl share 28% identity and 52% similarity. Bidnenko et al. have reported a phage operon involved in sensitivity to AbiDl (Bidnenko, E., Ehrlich, D., and Chopin, M.-C. [1995] *J. Bacteriol.* **177,** 3824–3829).

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References

1. Sanders, M. E. (1987) Bacteriophages of industrial importance, in *Phage Ecology* (Goyal, S. M., Gerba, C. P., and Bitton, G., eds.), Wiley Interscience, NY, pp. 211–244.
2. Hill, C. (1993) Bacteriophage and bacteriophage resistance in lactic acid bacteria. *FEMS Microbiol. Rev.* **12,** 87–108.
3. Klaenhammer, T. R. and Fitzgerald, G. F. (1994) Bacteriophages and bacteriophage resistance, in *Applied Genetics of Lactic Acid Bacteria* (Gasson, M. J. and de Vos, W., eds.), Backie and Son, UK, pp. 106–168.
4. King, W. R., Collins, E. B., and Barrett, E. L. (1983) Frequencies of bacteriophage-resistant and slow acid-producing variants of *Streptococcus cremoris*. *Appl. Environ. Microbiol.* **45,** 1481–1485.
5. Limsowtin, G. K. Y. and Terzaghi, B. E. (1976) Phage resistant mutants: their selection and use in cheese factories. *N.Z. J. Dairy Sci. Technol.* **11,** 251–256.
6. Marshall, R. J. and Berridge, N. J. (1976) Selection and some properties of phage-resistant starters for cheese-making. *J. Dairy Res.* **43,** 449–458.
7. Vlegels, P. A. P., Hazeleger, W. C., Helmerhorst, T. H., and Wouters, J. T. M. (1988) Phage resistance of *Streptococcus cremoris* due to low adsorption efficiency. *Neth. Milk Dairy J.* **42,** 195–206.
8. Valyasevi, R., Sandine, W. E., and Geller, B. L. (1990) The bacteriophage kh receptor of *Lactococcus lactis* subsp. *cremoris* KH is the rhamnose of the extracellular wall polysaccharide. *Appl. Environ. Microbiol.* **56,** 1882–1889.
9. Valyasevi, R., Sandine, W. E., and Geller, B. L. (1994) *Lactococcus lactis* ssp. *lactis* C2 bacteriophage sk1 receptor involving rhamnose and glucose moieties in the cell wall. *J. Dairy Sci.* **77,** 1–6.
10. Schäfer, A., Geis, A., Neve, H., and Teuber, M. (1991) Bacteriophage receptors of *Lactococcus lactis* subsp. "diacetylactis" F7/2 and *Lactococcus lactis* subsp. *cremoris* Wg2-1. *FEMS Microbiol. Lett.* **78,** 69–74.
11. Oram, J. D. and Reiter, B. (1968) The adsorption of phage to group N streptococci. The specificity of adsorption and the location of phage receptor substances in cell-wall and plasma-membrane fractions. *J. Gen. Virol.* **3,** 103–119.
12. Oram, J. D. (1971) Isolation and properties of a phage receptor substance from the plasma membrane of *Streptococcus lactis* ML3. *J. Gen. Virol.* **13,** 59–71.
13. Valyasevi, R., Sandine, W. E., and Geller, B. L. (1991) A membrane protein is required for bacteriophage c2 infection of *Lactococcus lactcis* subsp. *lactis* C2. *J. Bacteriol.* **173,** 6095–6100.
14. Geller, B. L., Ivey, R. G., Trempy, T. E., and Hettinger-Smith, B. (1993) Cloning of a chromosomal gene required for phage infection of *Lactococcus lactis* subsp. *lactis* C2. *J. Bacteriol.* **175,** 5510–5519.
15. Monteville, M. R., Ardestani, B., and Geller, B. L. (1994) Lactococcal bacteriophages require a host cell wall carbohydrate and a plasma membrane protein for adsorption and ejection of DNA. *Appl. Environ. Microbiol.* **60,** 3204–3211.
16. Gopal, P. K. and Crow, V. L. (1993) Characterization of loosely associated material from the cell surface of *Lactococcus lactis* subsp. *cremoris* E8 and its phage-resistant variant strain 398. *Appl. Environ. Microbiol.* **59,** 3177–3182.
17. Sanders, M. E. and Klaenhammer, T. R. (1983) Characterization of phage-sensitive mutants from a phage-insensitive strain of *Streptococcus lactis*: evidence for a plasmid determinant that prevents phage adsorption. *Appl. Environ. Microbiol.* **46,** 1125–1133.
18. de Vos, W. M., Underwood, H. M., and Davies, F. L. (1984) Plasmid encoded bacteriophage resistance in *Streptococcus cremoris* SK11. *FEMS Microbiol. Lett.* **23,** 175–178.
19. Sijtsma, L., Jansen, N., Hazeleger, W. C., Wouters, J. T. M., and Hellingwerf, K. J. (1990) Cell surface characteristics of bacteriophage-resistant *Lactococcus lactis* subsp. *cremoris* SK110 and its bacteriophage-sensitive variant SK112. *Appl. Environ. Microbiol.* **56,** 3230–3233.
20. Sijtsma, L., Sterkenburg, A., and Wouters, J. T. M. (1988) Properties of the cell walls of *Lactococcus lactis* subsp. *cremoris* SK110 and SK112 and their relation to bacteriophage resistance. *Appl. Environ. Microbiol.* **54,** 2808–2811.
21. Sijtsma, L., Wouters, J. T. M., and Hellingwerf, K. J. (1990) Isolation and characterization of lipoteichoic acid, a cell envelope component involved in preventing phage adsorption, from *Lactococcus lactis* subsp. *cremoris* SK110. *J. Bacteriol.* **172,** 7126–7130.
22. Lucey, M., Daly, C., and Fitzgerald, G. F. (1992) Cell surface characteristics of *Lactococcus lactis* harbouring pCI528, a 46 kb plasmid encoding inhibition of bacteriophage adsorption. *J. Gen. Microbiol.* **138,** 2137–2143.
23. Harrington, A. and Hill, C. (1992) Plasmid involvement in the formation of a spontaneous bacteriophage insensitive mutant of *Lactococcus lactis*. *FEMS Microbiol. Lett.* **96,** 135–142.
24. Watanabe, K., Ishibashi, K., Nadashima, Y., and Sakurai, T. (1984) A phage-resistant mutant of *Lactobacillus casei* which permits phage adsorption but not genome injection. *J. Gen. Virol.* **65,** 981–986.

25. Boussemaer, J. P., Schrauwen, P. P., Sourrouille, J. L., and Guy, P. (1980) Multiple modification/restriction systems in lactic streptococci and their significance in defining a phage-typing system. *J. Dairy Res.* **47,** 404–409.
26. Chopin, A., Chopin, M.-C., Moillo-Batt, A., and Langella, P. (1984) Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. *Plasmid* **11,** 260–263.
27. Froseth, B. R., Harlander, S. K., and McKay, L. L. (1988) Plasmid-mediated reduced phage sensitivity in *Streptococcus lactis* KR5. *J. Dairy Sci.* **71,** 275–284.
28. Gautier, M. and Chopin, M.-C. (1987) Plasmid-determined systems for restriction and modification activity and abortive infection in *Streptococcus cremoris*. *Appl. Environ. Microbiol.* **53,** 923–927.
29. Higgins, D. L., Sanozky-Dawes, R. B., and Klaenhammer, T. R. (1988) Restriction and modification activities from *Streptococcus lactis* ME2 are encoded by a self-transmissible plasmid, pTN20, that forms cointegrates during mobilization of lactose-fermenting ability. *J. Bacteriol.* **170,** 3435–3442.
30. Hill, C., Pierce, K., and Klaenhammer, T. R. (1989) The conjugative plasmid pTR2030 encodes two bacteriophage defense mechanisms in lactococci, restriction/modification (R^+/M^+) and abortive infection (Hsp^+). *Appl. Environ. Microbiol.* **55,** 2416–2419.
31. Josephsen, J. and Vogensen, F. K. (1989) Identification of three different plasmid-encoded restriction/modification systems in *Streptococcus lactis* subsp. *cremoris* W56. *FEMS Microbiol. Lett.* **59,** 161–166.
32. McKay, L. L., Bohanon, M. J., Polzin, K. M., Rule, P. L., and Baldwin, K. A. (1989) Localization of separate genetic loci for reduced sensitivity towards small isometric-headed bacteriophage sk1 and prolate-headed bacteriophage c2 on pGBK17 from *Lactococcus lactis* subsp. *lactis* KR2. *Appl. Environ. Microbiol.* **55,** 2702–2709.
33. Pearce, L. E. (1978) The effect of host controlled modification on the replication rate of a lactic streptococcal bacteriophage. *N.Z. J. Dairy Sci. Technol.* **13,** 166–171.
34. Sanders, M. E. and Klaenhammer, T. R. (1980) Restriction and modification in group N streptococci: Effect of heat on development of modified lytic bacteriophage. *Appl. Environ. Microbiol.* **40,** 500–506.
35. Sanders, M. E. and Klaenhammer, T. R. (1981) Evidence for plasmid linkage of restriction and modification in *Streptococcus cremoris* KM. *Appl. Environ. Microbiol.* **42,** 944–950.
36. Sanders, M. E. and Shultz, J. W. (1990) Cloning of phage resistance genes from *Lactococcus lactis* ssp. *cremoris* KH. *J. Dairy Sci.* **73,** 2044–2053.
37. Sing, W. D. and Klaenhammer, T. R. (1991) Characterization of restriction-modification plasmids from *Lactococcus lactis* ssp. *cremoris* and their effects when combined with pTR2030. *J. Dairy Sci.* **74,** 1133–1144.
38. Steenson, L. R. and Klaenhammer, T. R. (1986) Plasmid heterogeneity in *Streptococcus cremoris* M12R: effects on proteolytic activity and host-dependent phage replication. *J. Dairy Sci.* **69,** 2227–2236.
39. Fitzgerald, G. F., Daly, C., Broen, L. R., and Gingeras, T. R. (1982) *Scr*FI: a new sequence specific endonuclease from *Streptococcus cremoris*. *Nucleic Acids Res.* **10,** 8171–8179.
40. Nyengaard, N., Vogensen, F. K., and Josephsen, J. (1993) *Lla*AI and *Lla*BI, two type-II restriction endonucleases from *Lactococcus lactis* subsp. *cremoris* W9 and W56 recognizing, respectively, 5'-/GATC-3' and 5'-C/TRYAG-3'. *Gene* **136,** 371,372.
41. Mayo, B., Hardisson, C., and Brana, A. F. (1991) Nucleolytic activities in *Lactococcus lactis* subsp. *lactis* NCDO 497. *FEMS Microbiol. Lett.* **79,** 195–198.
41a. Solaiman, D. K. Y. and Somkuti, G. A. (1990) Isolation and characterization of a type II restriction endonuclease from *Streptococcus thermophilus*. *FEMS Microbiol. Lett.* **67,** 261–266.
41b. Solaiman, D. K. Y. and Somkuti, G. A. (1991) A type II restriction endonuclease of *Streptococcus thermophilus* ST117. *FEMS Microbiol. Lett.* **80,** 75–80.
41c. Benbadis, L., Garel, J.-R., and Hartley, D. L. (1991) Purification, properties, and sequence specificity of *Ssl*I, a new type II restriction endonuclease from *Streptococcus salivarius* subsp. *thermophilus*. *Appl. Environ. Microbiol.* **57,** 3677,3678.
42. Davis, R., van der Lelie, D., Mercenier, A., Daly, C., and Fitzgerald, G. F. (1993) *Scr*FI restriction-modification system of *Lactococcus lactis* subsp. *cremoris* UC503: cloning and characterization of two *Scr*FI methylase genes. *Appl. Environ. Microbiol.* **59,** 777–785.
43. Klaenhammer, T. R. and Sanozky, R. B. (1985) Conjugal transfer from *Streptococcus lactis* ME2 of plasmids encoding phage resistance, nisin resistance, and lactose fermenting ability: evidence for a high-frequency conjugal plasmid responsible for abortive infection of virulent bacteriophage. *J. Gen. Microbiol.* **131,** 1531–1541.
44. Hill, C., Miller, L. A., and Klaenhammer, T. R. (1991) In vivo genetic exchange of a functional domain from a type II A methylase between lactococcal plasmid pTR2030 and a virulent bacteriophage. *J. Bacteriol.* **173,** 4363–4370.
45. O'Sullivan, D. J. and Klaenhammer, T. R. (1995) C-*Lla*I is a bifunctional regulatory protein of the *llaI* restriction modification operon from *Lactococcus lactis*, in *Genetics of Streptococci, Enterococci, and Lactococci; Proceedings of the IVth International Conference on Streptococci Genetics* (Ferretti, J. J., Gilmore, M., Klaenhammer, T. R., and Brown, F., eds.), Dev. Biol. Stand., Karger, Basel, vol. 85, pp. 591–595.
46. O'Sullivan, D. J., Zagula, K., and Klaenhammer, T. R. (1995) *In vivo* restriction by *Lla*I is encoded by

three genes, arranged in an operon with *llaIM,* on the conjugative *Lactococcus* plasmid pTR2030. *J. Bacteriol.* **177,** 134–143.
47. Duckworth, D. H., Glenn, J., and McCorquodale, D. J. (1981) Inhibition of bacteriophage replication by extra-chromosomal elements. *Microbiol. Rev.* **45,** 52–71.
48. Klaenhammer, T. R. (1989) Genetic characterization of multiple mechanisms of phage defense from a prototype phage-insensitive strain, *Lactococcus lactis* ME2. *J. Dairy Sci.* **72,** 3429–3443.
49. Sing, W. D. and Klaenhammer, T. R. (1990) Plasmid-induced abortive infection in lactococci: a review. *J. Dairy Sci.* **73,** 2239–2251.
50. Sing, W. D. and Klaenhammer, T. R. (1986) Conjugal transfer of bacteriophage resistance determinants on pTR2030 into *Streptococcus cremoris* strains. *Appl. Environ. Microbiol.* **51,** 1264–1271.
51. Steenson, L. R. and Klaenhammer, T. R. (1985) *Streptococcus cremoris* M12R transconjugants carrying the conjugal plasmid pTR2030 are insensitive to attack by lytic bacteriophages. *Appl. Environ. Microbiol.* **50,** 851–858.
52. Jarvis, A. W. and Klaenhammer, T. R. (1986) Bacteriophage resistance conferred on lactic streptococci by the conjugative plasmid pTR2030: effects on small isometric-, large isometric-, and prolate-headed phages. *Appl. Environ. Microbiol.* **51,** 1272–1277.
53. Jarvis, A. W. and Klaenhammer, T. R. (1987) Bacteriophage resistance plasmid pTR2030 inhibits lytic infection of r_1t temperate bacteriophage but not induction of r_1t prophage in *Streptococcus cremoris* R1. *Appl. Environ. Microbiol.* **53,** 385–389.
54. Hill, C., Romero, D. A., McKenney, D. S., Finer, K. R., and Klaenhammer, T. R. (1989) Localization, cloning, and expression of genetic determinants for bacteriophage resistance (Hsp) from the conjugative plasmid pTR2030. *Appl. Environ. Microbiol.* **55,** 1684–1689.
55. Hill, C., Miller, L. A., and Klaenhammer, T. R. (1990) Nucleotide sequence and distribution of the pTR2030 resistance determinant *(hsp)* which aborts bacteriophage infection in lactococci. *Appl. Environ. Microbiol.* **56,** 2255–2258.
56. Coffey, A. G., Fitzgerald, G. F., and Daly, C. (1991) Cloning and characterization of the determinant for abortive infection of bacteriophage from lactococcal plasmid pCI829. *J. Gen. Microbiol.* **137,** 1355–1362.
57. Dinsmore, P. K. and Klaenhammer, T. R. (1994) Phenotypic consequences of altering the copy number of *abiA,* a gene responsible for aborting bacteriophage infections in *Lactococcus lactis. Appl. Environ. Microbiol.* **60,** 1129–1136.
58. Casey, J., Daly, C., and Fitzgerald, G. F. (1992) Controlled integration into the *Lactococcus* chromosome of the pCI829-encoded abortive infection gene from *Lactococcus lactis* subsp. *lactis* UC811. *Appl. Environ. Microbiol.* **58,** 3283–3291.
59. Hill, C., Massey, I. J., and Klaenhammer, T. R. (1991) Rapid method to characterize lactococcal bacteriophage genomes. *Appl. Environ. Microbiol.* **57,** 283–288.
60. Moineau, S., Durmaz, E., Pandian, S., and Klaenhammer, T. R. (1993) Differentiation of two abortive mechanisms by using monoclonal antibodies directed toward lactococcal bacteriophage capsid proteins. *Appl. Environ. Microbiol.* **59,** 208–212.
61. Cluzel, P.-J., Chopin, A., Erlich, S. D., and Chopin, M.-C. (1991) Phage abortive infection mechanism from *Lactococcus lactis* subsp. *lactis,* expression of which is mediated by an isoIS*S1* element. *Appl. Environ. Microbiol.* **57,** 3547–3551.
62. Durmaz, E., Higgins, D. L., and Klaenhammer, T. R. (1992) Molecular characterization of a second abortive phage resistance gene present in *Lactococcus lactis* subsp. *lactis* ME2. *J. Bacteriol.* **174,** 7463–7469.
63. Coffey, A., Costello, V., Daly, C., and Fitzgerald, G. (1991) Plasmid encoded bacteriophage insensitivity in members of the genus *Lactococcus,* with special reference to pCI829, in *Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci* (Dunny, G. M., Cleary, P. P., and McKay, L. L., eds.), American Society for Microbiology, Washington, DC, pp. 131–135.
64. Jarvis, A. W. (1992) Analysis of phage resistance mechanisms encoded by lactococcal plasmid pAJ2074. *Can. J. Microbiol.* **39,** 252–258.
65. Ward, A. C., Davidson, B. E., Hillier, A. J., and Powell, I. B. (1992) Conjugally transferable phage resistance activities from *Lactococcus lactis* DRC1. *J. Dairy Sci.* **75,** 683–691.
66. Durmaz, E. and Klaenhammer, T. R. (1995) A starter culture rotation strategy incorporating paired restriction/modification and abortive infection bacteriophage defenses in a single *Lactococcus lactis* strain. *Appl. Environ. Microbiol.* 1266–1273.
67. Josephsen, J. and Klaenhammer, T. R. (1990) Stacking of three different restriction and modification systems in *Lactococcus lactis* by contransformation. *Plasmid* **23,** 71–75.
68. Sing, W. D. and Klaenhammer, T. R. (1993) A strategy for rotation of different bacteriophage defenses in a lactococcal single-strain starter culture system. *Appl. Environ. Microbiol.* **59,** 365–372.
69. McKay, L. L. and Baldwin, K. A. (1984) Conjugative 40-megadalton plasmid in *Streptococcus lactis* subsp. *diacetylactis* DRC3 is associated with resistance to nisin and bacteriophage. *Appl. Environ. Microbiol.* **47,** 68–74.
70. Jarvis, A. W. (1988) Conjugal transfer in lactic streptococci of plasmid-encoded insensitivity to prolate- and small isometric-headed bacteriophages. *Appl. Environ. Microbiol.* **54,** 777–783.
71. Kelly, W., Dobson, J., Jorch-Remberg, D., Fitzgerald, G., and Daly, C. (1990) Introduction of bacterioph-

age resistance plasmids into commercial *Lactococcus* starter strains. *FEMS Microbiol. Rev.* **87,** 63.

72. Sanders, M. E., Leonhard, P. J., Sing, W. D., and Klaenhammer, T. R. (1986) Conjugal strategy for construction of fast acid-producing, bacteriophage-resistant lactic streptococci for use in dairy fermentations. *Appl. Environ. Microbiol.* **52,** 1001–1007.
73. Broadbent, J. R. and Kondo, J. K. (1991) Genetic construction of nisin-producing *Lactococcus lactis* subsp. *cremoris* and analysis of a rapid method for conjugation. *Appl. Environ. Microbiol.* **57,** 517–524.
74. Klaenhammer, T. R. (1987) Plasmid-directed mechanisms for bacteriophage defense in lactic streptococci. *FEMS Microbiol. Rev.* **46,** 313–325.
75. Romero, D. A. and Klaenhammer, T. R. (1990) Abortive phage infection and restriction/modification activities directed by pTR2030 determinants are enhanced by recombination with conjugal elements in lactococci. *J. Gen. Microbiol.* **136,** 1817–1824.
76. Romero, D. A. and Klaenhammer, T. R. (1990) Characterization of Gram-positive insertion sequence IS*946,* and iso-IS*S1* element, isolated from the conjugative lactococcal plasmid pTR2030. *J. Bacteriol.* **172,** 4151–4160.
77. Romero, D. A. and Klaenhammer, T. R. (1991) Construction of an IS*946*-based composite transposon in *Lactococcus lactis* subsp. *lactis. J. Bacteriol.* **173,** 7599–7606.
78. Polzin, K. M. and McKay, L. L. (1991) Identification, DNA sequence, and distribution of IS*981,* a new, high-copy number insertion sequence in lactococci. *Appl. Environ. Microbiol.* **57,** 734–743.
79. Kim, J. H. and Batt, C. A. (1991) Identification of a nucleotide sequence conserved in *Lactococcus lactis* bacteriophages. *Gene* **98,** 95–100.
80. Chung, D. K., Chung, S. K., and Batt, C. A. (1992) Antisense RNA directed against the major capsid protein of *Lactococcus lactis* subsp. *cremoris* bacteriophage 4-1 confers partial resistance to the host. *Appl. Microbiol. Biotechnol.* **37,** 79–83.
81. Jarvis, A. W. Relationships by DNA homology between lactococcal phages 7-9, P335, and New Zealand industrial lactococcal phages. *Intl. Dairy J.,* in press.
82. Hill, C., Miller, L. A., and Klaenhammer, T. R. (1990) Cloning, expression, and sequence determination of a bacteriophage fragment encoding bacteriophage resistance in *Lactococcus lactis. J. Bacteriol.* **172,** 6419–6426.
83. O'Sullivan, D. J., Hill, C., and Klaenhammer, T. R. (1993) Effect of increasing the copy number of bacteriophage origins of replication, in *trans,* on incoming-phage proliferation. *Appl. Environ. Microbiol.* **59,** 2449–2456.
84. Moineau, S., Pandian, S., and Klaenhammer, T. R. (1994) Evolution of a lytic bacteriophage via DNA acquisition from the *Lactococcus lactis* chromosome. *Appl. Environ. Microbiol.* **60,** 1832–1841.
85. Lenski, R. E. (1984) Coevolution of bacteria and phage: are there endless cycles of bacterial defenses and phage counterdefenses? *J. Theor. Biol.* **108,** 319–325.
86. Kruger, D. H. and Bickle, T. A. (1983) Bacteriophage survival: multiple mechanisms for avoiding the deoxyribonucleic acid restriction systems of their hosts. *Microbiol. Rev.* **47,** 345–360.
87. Sharpe, P. M. (1986) Molecular evolution of bacteriophages: evidence of selection against the recognition sites of host restriction enzymes. *Mol. Biol. Evol.* **3,** 75–83.
88. Moineau, S., Pandian, S., and Klaenhammer, T. R. (1993) Restriction/modification systems and restriction endonucleases are more effective on lactococcal bacteriophages that have emerged recently in the dairy industry. *Appl. Environ. Microbiol.* **59,** 197–202.
89. Braun, V., Hertwig, S., Neve, H., Geis, A., and Teuber, M. (1989) Taxonomic differentiation of bacteriophages of *Lactococcus lactis* by electron microscopy, DNA-DNA hybridization, and protein profiles. *J. Gen. Microbiol.* **135,** 2551–2560.
90. Coveney, F. A., Fitzgerald, G. F., and Daly, C. (1987) Detailed characterization and comparison of four lactic streptococcal bacteriophages based on morphology, restriction mapping, DNA homology, and structural protein analysis. *Appl. Environ. Microbiol.* **53,** 1439–1447.
91. Jarvis, A. W. and Meyer, J. (1986) Electron microscopic heteroduplex study and restriction endonuclease cleavage analysis of the DNA genomes of three lactic streptococcal bacteriophages. *Appl. Environ. Microbiol.* **51,** 1272–1277.
92. Kim, J. H. and Batt, C. A. (1991) Molecular characterization of *Lactococcus lactis* bacteriophage F4-1. *Food Microbiol.* **8,** 15–24.
93. Moineau, S., Fortier, F., Ackermann, H. W., and Pandian, S. (1992) Characterization of lactococcal bacteriophages in Quebec cheese plants. *Can. J. Microbiol.* **38,** 875–882.
94. Powell, I. A., Arnold, P. M., Hillier, A. J., and Davidson, B. E. (1989) Molecular comparison of prolate- and isometric-headed bacteriophages of lactococci. *Can. J. Microbiol.* **35,** 860–866.
95. Powell, I. B. and Davidson, B. E. (1986) Resistance to in vitro restriction of DNA from lactic streptococcal bacteriophage c6A. *Appl. Environ. Microbiol.* **51,** 1358–1360.
96. Prevots, F., Mata, M., and Ritzenthaler, P. (1990) Taxonomic differentiation of 101 lactococcal bacteriophages and characterization of bacteriophages with unusually large genomes. *Appl. Environ. Microbiol.* **56,** 2180–2185.
97. Shearman, C. A., Hertwig, S., Teuber, M., and Gasson, M. J. (1991) Characterization of the prolate-headed lactococcal bacteriophage vML3: location of the lysin gene and its DNA homology with other prolate-headed phages. *J. Gen. Microbiol.* **137,** 1285–1291.

98. Alatossava, T. and Klaenhammer, T. R. (1991) Molecular characterization of three small isometric-headed bacteriophages which vary in their sensitivity to the lactococcal phage resistance plasmid pTR2030. *Appl. Environ. Microbiol.* **57,** 1346–1353.
99. D'Amelio, G. (1994) MS thesis. North Carolina State University.
100. Bidnenko, E., Schouler, C., Ehrlich, S. D., and Chopin, M.-C. (1993) Identification of a phage fragment conferring resistance to abi-105 by complementation or recombination. *FEMS Microbiol. Rev.* **12,** P107.
101. Bidnenko, E., Valyasevi, R., Cluzel, P. J., Parreira, R., Hillier, A., Gautier, M., Anba, J., Ehrlich, S. D., Chopin, M.-C. (1993) Amélioration de la résistance des lactocoques aux bactériophages. *Lait* **73,** 199–205.
102. Parreira, R., Valyasevi, R., Ehrlich, S. D., and Chopin, M.-C. (1993) Identification of a phage DNA fragment conferring resistance to abi-416 by complementation. *FEMS Microbiol. Rev.* **12,** P107.
103. Lawrence, R. C., Thomas, T. D., and Terzaghi, B. E. (1976) Reviews in the progress of dairy science: cheese starters. *J. Dairy Res.* **43,** 141–193.
104. Lodics, T. A. and Steenson, L. R. (1993) Phage-host interactions in commercial mixed-strain dairy starter cultures: practical significance—a review. *J. Dairy Sci.* **76,** 2380–2391.
105. Sandine, W. E. (1989) Use of bacteriophage-resistant mutants of lactococcal starters in cheesemaking. *Neth. Milk Dairy J.* **43,** 211–219.
106. Jarvis, A. W. (1977) The serological differentiation of lactic streptococcal bacteriophage. *N.Z.J. Dairy Sci. Technol.* **12,** 176–181.
107. Klaenhammer, T. R. (1984) Interactions of bacteriophages with lactic streptococci. *Adv. Appl. Microbiol.* **30,** 1–29.
108. Graham, D. M. Parmelee, C. E., and Nelson, F. E. (1952) The carrier state of lactic *Streptococcus* bacteriophage. *J. Dairy Sci.* **35,** 813.
109. Tesfaigzi, J. and Sussmuth, R. (1989) Proportion of phage-insensitive and phage-sensitive cells within pure strains of lactic streptococci, and the influence of calcium. *J. Dairy Res.* **56,** 151–154.
110. de Vos, W. M. (1989) On the carrier state of bacteriophages in starter lactococci: an elementary explanation involving a bacteriophage resistance plasmid. *Neth. Milk Dairy J.* **43,** 221–227.
111. Moineau, S., Borkaev, M., Walker, S. A., Vedamuthu, E. R., and Vandenbergh, P. A. (1994) Novel starter rotation system based on phage species sensitivity. *J. Dairy Sci.* **77,** 18.
112. Harrington, A. and Hill, C. (1991) Construction of a bacteriophage-resistant derivative of *Lactococcus lactis* subsp. *lactis* 425A by using the conjugal plasmid pNP40. *Appl. Environ. Microbiol.* **57,** 3405–3409.
113. Hughes, B. F. and McKay, L. L. (1992) Deriving phage-insensitive lactococci using a food-grade vector encoding phage and nisin resistance. *J. Dairy Sci.* **75,** 914–923.
114. Coffey, A. G., Fitzgerald, G. F., and Daly, C. (1989) Identification and characterisation of a plasmid encoding abortive infection from *Lactococcus lactis* ssp. *lactis* UC811. *Neth. Milk Dairy J.* **43,** 229–244.
115. Murphy, M. C., Steele, J. L., Daly, C., and McKay L. L. (1988) Concomitant conjugal transfer of reduced-bacteriophage-sensitivity mechanisms with lactose- and sucrose-fermenting ability in lactic streptococci. *Appl. Environ. Microbiol.* **54,** 1951–1956.