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Review

Use of antisense RNA to confer bacteriophage resistance in dairy starter cultures

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SUMMARY

The strategy and implementation of a unique system for engineering bacteriophage resistant starter cultures of *Lactococcus lactis* employing antisense RNA is reviewed. As a necessary prerequisite for developing this system, we have cloned and sequenced a number of bacteriophage genes coding for minor and major structural proteins. In addition, we have also identified a series of genes whose function(s) is not known but their sequences appear to be conserved in a vast number of isolates. One of these latter sequences, designated *gp51C*, codes for a 51-kDa protein which is extremely charged and shares some homology with yeast translation intiation factor. Resistance to a broad class of isometric bacteriophages has been achieved by expression of an antisense RNA targeted against, for example, *gp51C*. In the best case, expression of the antisense *gp51C* RNA results is a greater than 99% reduction in the total number of plaque forming units. Additional antisense RNA constructs directed against other bacteriophage genes, including the major capsid protein, also appear effective at inhibiting infection from 40–55% suggesting that this approach may prove useful for engineering a set of truly isogenic strains to be used in a starter culture rotation plan.

BACTERIOPHAGE RESISTANCE AND DAIRY STARTER CULTURES

Bacteriophage which infect lactic starter cultures are encountered in the dairy industry, and they inhibit or totally prevent acidfication (Ref. 34; see review by Ref. 14). The true magnitude of the problem is difficult to accurately assess and many poor quality cheese fermentations (in terms of organoleptic evaluation and yield) may be due to sublethal bacteriophage infection. The ubiquity of bacteriophages in cheese plants has probably arisen, in part, due to the industrial scale up of the process, creating an accessible target population of organisms on which the bacteriophages can propagate. This coupled to the ability of bacteriophages to constantly evolve and the rapid succession of new infective variants forces a continual vigilance to prevent the consequences of their proliferation.

Several approaches have been proposed and implemented to develop bacteriophage resistant starter cultures. These include the direct screening for a 'bacteriophage resistant phenotype' as mediated by a variety of unknown mechanisms and/or the purposeful introduction of a plasmid-borne resistance(s). The plasmid-borne resistance mechanisms seem to involve either one or a combination of the following: restriction/modification, abortive infection or receptor alteration. Only some of these mechanisms have been characterized in detail culminating in the determination of their nucleotide sequence(s) as reported by Hill et al. [9,10]. Strains obtained by direct screening have been employed commercially, usually within the context of a starter rotation program [24,32]. The more directed, plasmid mediated approach has been demonstrated to be effective by conjugal transfer of a plasmid pTR2030 into both *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* [15]. Recently, however, bacteriophages have appeared which are no longer sensitive to these plasmid-borne mechanisms as developed by Alatossava and Klaenhammer [2]. These include isolates that may have acquired the appropriate methylase gene from the resistance-conferring plasmid, pTR2030 [11]. The ability of these bacteriophages to circumvent this restriction/modification mechanism suggests that these strategies will not completely solve the problem associated with bacteriophage infection

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in dairy starter cultures. In contrast to these efforts to harness endogenous resistance mechanisms that have apparently evolved naturally in some attempt to thwart bacteriophage infection, we have employed antisense RNA technology targeted against specific and, in some cases, conserved gene functions to effectively confer bacteriophage resistance.

IDENTIFICATION OF BACTERIOPHAGE GENES

Despite the economic ramifications of bacteriophage infection of lactic acid starters, only a limited amount of information is known about these bacteriophages, especially with respect to their genome organization and regulation of gene expression. Extensive taxonomical surveys have been conducted using electron microscopy and to a lesser extent immunological analysis. Studies on the genome of these bacteriophages have been mostly limited to restriction mapping and estimation of the genome size by electron microscopy (for example see Ref. 23). One of the most extensive analysis of the genome of a bacteriophage which can infect group N lactococci was reported by Lakshmidevi *et al.* [22] for a temperate bacteriophage of *L. lactis* subsp. *cremoris*. This bacteriophage has a circularly permuted genome, and the sites for integration (*att*) and packaging initiation (*pac*) have been located. In another study, the genome for the *L. lactis* subsp. *lactis* ϕvML3 bacteriophage has been mapped and location of the lysin gene determined [29]. There is, however, a great absence of information concerning the replication and gene regulation of these bacteriophages. Again an exception is the study by Lakshmidevi et al. [22] where a series of promoters and a putative regulatory gene have been discovered.

The classic approaches toward the identification of gene functions in bacteriophages include complementation of mutations which may be conditionally lethal or affect plaque morphology. These conditional lethal mutations would be generated and catalogued using a panel of host strains with various termination suppressors. Then through a series of recombination experiments, alleles are assigned to each individual bacteriophage mutation. Unfortunately, in the case of the lactic acid bacteria there are no established suppressor strains, in part, due to their rather fastidious nature which precludes the isolation of the requisite auxotrophic mutations. Therefore, all of the bacteriophage gene functions which have been identified, to date, in isolates that infect lactic acid bacteria have involved one of the following approaches: (i) screening libraries using antibodies raised against bacteriophage coat proteins; (ii) direct phenotypic expression of a particular trait (i.e., lysin) in a heterologous or homologous host; and (iii) nucleotide sequencing of regions targeted on the basis of presumed homology as predicted by restriction analysis/hybridization.

Only a limited number of genes from bacteriophage which infect lactic acid bacteria have been extensively characterized including the lysin gene from *L. lactis* subsp. *lactis* bacteriophage ML3 [28]. Our initial efforts focused on the identification of the major and minor capsid proteins of a bacteriophage which infects *L. lactis* subsp. *cremoris*. The bacteriophage selected for these studies is a small isometric isolate found in the cheese whey originally collected in 1984 and this bacteriophage was designated F4-1 [30]. Antiserum against intact bacteriophage F4-1 particles could be easily raised by subcutaneous injection of rabbits and after several immunizations, sufficient titers were obtained for all subsequent experiments. The resultant antiserum clearly reacted with a number of presumed coat proteins and immunoelectronmicroscopic studies document its reactivity with virtually all of the surface structures present on this bacteriophage (S.G. Kim, H. Neve and C.A. Batt, unpublished results). Libraries of bacteriophage genomic DNA were constructed by partial restriction endonuclease digestion and these libraries were propagated in *Escherichia coli* then screened for immunoreactive proteins [17]. A number of transformants which reacted with the rabbit anti-bacteriophage antiserum were obtained, and the largest insert observed consisted of a 7.6-kb *Eco*RI/*Hin*dIII fragment. To date, approx. 6.5-kb of this insert has been sequenced and a number of putative genes were located initally on the basis of deletion and open reading frame (ORF) analyses (Fig. 1). The major capsid protein (MCP) has been unambiguously identified on the basis of amino acid sequencing of the N-terminal of the MCP [5]. Flanking the MCP is a minor structural protein whose function is unknown and designated *gp*35 [18]. ORF analysis of the nucleotide sequence containing

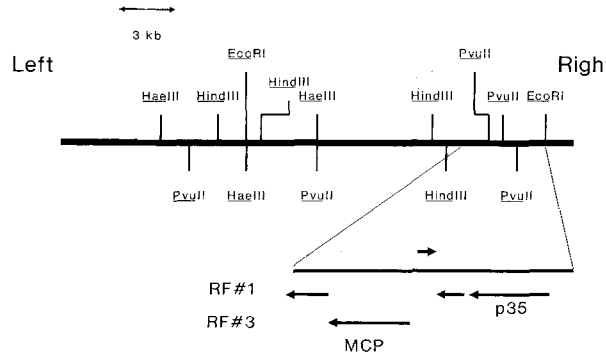

Fig. 1. Restriction map of *L. lactis* subsp. *lactis* bacteriophage F4-1. Arrows indicate position and orientation of open reading frames for gp35 and MCP. Unlabeled arrows denote additional open reading frames based solely on nucleotide sequence analysis. RF refers to reading frame.

the *gp*35 ORF revealed two in-frame translational starts which would generate both a 43-kDa and a 35-kDa protein. Each ORF was preceeded by a suitable ribosome binding site and both proteins were observed by Western blotting in *E. coli* and *L. lactis* subsp. *lactis* recombinants carrying this fragment. Evidence for the expression of the GP35 includes the immunoprecipitation of a 35-kDa ^{35}S-labeled protein in *L. lactis* subsp. *cremoris* infected with bacteriophage F4-1, suggesting that either the 43-kDa protein is an artifact or is rapidly processed to generate the GP35 protein [18].

The long-term success of a program to use antisense RNA to render dairy starter cultures resistant to bacteriophage infection would depend upon the distribution of the targeted gene within the bacteriophage population resident in a cheese manufacturing plant. Studies on lambdoid bacteriophages suggest that within their genome, blocks of sequences coding for specific gene functions evolve as discrete elements [4]. Therefore, it would be expected that essential gene functions could be initially located on the basis of a simple restriction enzyme analysis to identify fragments of the same size which appear in a wide number of isolates.

A genetic element that is conserved in the genome of numerous *L. lactis* subsp. *lactis* bacteriophages which were isolated over a 7-year period has been identified [19]. It is located within a 1.6-kb *Eco*RI fragment (isolates carrying this size fragment are designated group I), although one of these *Eco*RI sites is polymorphic and in certain isolates a corresponding 5.6-kb *Eco*RI fragment (isolates carrying this size fragment are designated group II) is observed (Fig. 2). Genetic variation in other regions of the genomes of these bacteriophages is exhibited by changes in the overall restriction pattern. Plaque hybridization of raw whey samples was performed to determine, in an unamplified population, the extent of bacteriophages which carried sequences homologous to the 1.6-kb *Eco*RI fragment. Approximately 93–97% of all the plaques observed positively hybridized to the probe suggesting that this sequence is ubiquitous in the populations examined (Table 1).

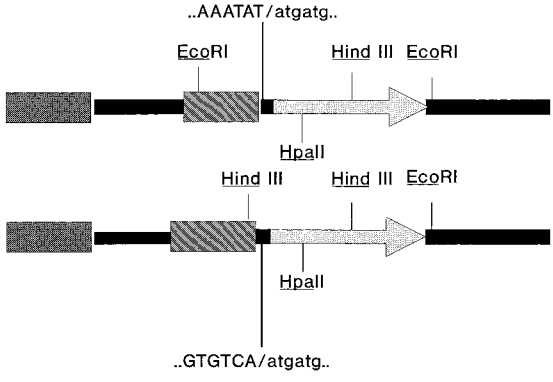

Fig. 2. Restriction map of region flanking *gp51C* ORF in group I (upper) and group II (lower) *L. lactis* subsp. *lactis* bacteriophage isolates. Stripped boxes (not to scale) are the variable sequence blocks which range in size from 68 to 708-bp which are observed in different isolates. Solid bars are conserved sequence blocks. Hatched arrow is the *gp51C* ORF. Nucleotide sequences are at the break points between one set of variable and conserved blocks.

The complete nucleotide sequence for this 1.6-kb region was determined from nine independent *L. lactis* subsp. *lactis* bacteriophage isolates (from group I and group II) and only five changes in the sequence were observed [19]. This region has a single large 1356-bp ORF coding for a 51-kDa protein (GP51C). Three out of the five changes occur within an 197-bp nontranslated region, 5′ to *gp51C* ORF. The two additional changes are found within the 1356-bp ORF but they are concurrent and result in two amino acid substitutions that do not, however, change the net charge of the protein. The encoded protein is extremely charged and shares some homology with yeast translation initiation factor. In addition, there is a potential zinc-binding domain within this protein, similar to those observed in genes from bacteriophages T4 and T7.

Comparison of the nucleotide sequence from the region upstream of the *gp51C* ORF between group I and group II isolates indicate that there is a region of approx. 100 to 300-bp (depending upon the isolate) whose sequence varies considerably and includes this polymorphic *Eco*RI site (Figs. 3 and 4). Further upstream of this variable region is a second block of 708-bp that is conserved among all isolates. Still further upstream is a region of between 68–294-bp that is again variable. This block motif of conserved and variable regions is similar to that observed in other bacteriophages, most notably lambdoid phages.

TABLE 1

Distribution of bacteriophages carrying 1.6-kb conserved region

Whey sample[a]	Total plaques	Positive[b]	Frequency (%)
1983a	46	44	96
1983b	183	176	96
1987a	41	39	95
1987b	46	43	93
1987c	42	40	95
1989a	113	109	96
1989b	25	24	96

[a] Year sample collected.

[b] Hybridized with 1.6-kb *Eco*RI fragment from *L. lactis* subsp. *lactis* bacteriophage 7–9.

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a          10          20          30          40          50          60
           |           |           |           |           |           |
1    CTTGTTAATG ATGTAGAACT CATCTCTTAT ACTGGAAATT TTGAACAAAT TAATACACTT
61   CTTGATTGTA CCCTAGATGA ATCTAATGCT AACGTTTCTG ATTTGGATGA TTTTAGAACA
121  CGCTTCGAAA AGTTATTGAC TAGAAAAGAC GTTGACACTT TAGGCCGAAT CGACATGAGT
181  GATTTAGCAG CGCTTGATAA AGCTTTGAAT TTACCAGAAT TGAGTGTCAa tgatg
                       HindIII

b          10          20          30          40          50          60
           |           |EcoRI      |           |           |           |
1    GAAAAGTTCT TCGGGTAGCG AATTCGAATA CATCAACAAA GTTTGGAATA ACTTTCAACT
61   TTGTGAGATG ACAATGGCGC TCATTGGGAG GCTGACTCCT GAAGAACTCG TACAAATATT
121  TCCTGCTGAA AAAATATatg atg
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Fig. 3. (a) Nucleotide sequence of variable region from bacteriophage isolates with 5-kb fragment without polymorphic *Eco*RI site. Lower case nucleotides are from the conserved region common to all bacteriophage isolates. (b) Nucleotide sequence of variable region from bacteriophage isolates that contain the 1.6-kb *Eco*RI fragment and the polymorphic *Eco*RI site. Lower case nucleotides are from the conserved region common to all bacteriophage isolates.

Furthermore, electron microscopic heteroduplex analysis of lactic acid bacteriophages reveals regions of extensive homology, interspersed with regions of low homology supporting our sequence information (Refs. 16, 22 and S.G. Kim, H. Neve and C.A. Batt, unpublished data). There are no significant ORFs or inverted repeats flanking these blocks in *L. lactis* subsp. *lactis* bacteriophage 7–9 or related isolates that would suggest a transposon-mediated event gave rise to this pattern. Furthermore, Southern hybridization using oligonucleotide probes derived from these variable regions do not hybridize with any other regions of the bacteriophage or host genome obviating any clues as to their origin.

We have recently identified and sequenced two additional ORFs that are also conserved in a majority of *L. lactis* subsp. *lactis* bacteriophage isolates (Fig. 4). They code for an 18-kDa (GP18C) and a 21-kDa (GP21C) protein, respectively, and they are arranged along with the previously identified *gp51C* in a tandem motif similar to other bacteriophages. The presence of *gp18C* and *gp21C* sequences in a number of bacteriophage isolates was confirmed by polymerase chain reaction (PCR) using primers specific for these regions (S.G. Kim, Y-C. Bor and C.A. Batt, unpublished data).

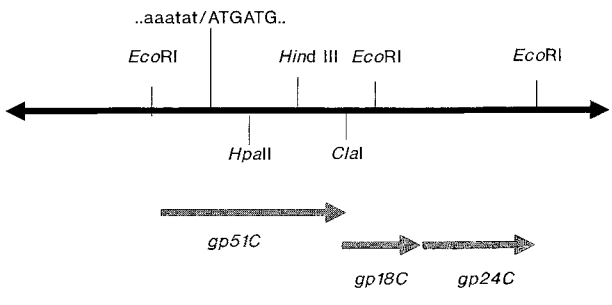


Fig. 4. Restriction map of region containing *gp51C*, *gp24C* and *gp18C*. Stripped arrows indicate the ORF for each gene. The nucleotide sequences in lower case and upper case represent the breakpoint in variable and conserved regions, respectively.

It is difficult to argue, in an absolute sense, that the regions which we have identified as conserved are truly found in all bacteriophages that infect lactic acid bacteria. A number of factors confuse the issue including the extremely diverse morphology of bacteriophages and the rapid succession of bacteriophages that is possible within a relatively short period of time. The bacteriophages that we have identified were the predominant population from 1983–1990, at two geographically distinct plants; they have persisted in these plants despite numerous starter culture rotations and in one case a total shutdown and renovation of the plant. Although within our sampling they are relatively ubiquitous, they do not fall within the seven taxonomical groups as defined by Braun et al. [3]. Collections of bacteriophages do not necessarily represent the true scope of a local population with respect to both diversity and relative numbers.

ANTISENSE RNA INHIBITION OF GENE EXPRESSION

Antisense RNA is the product of transcribing the DNA strand complementary to the strand which codes for the protein [27]. Although antisense RNA has been reported to block the expression of a targeted gene in a number of systems, the mechanism is not completely understood. Antisense RNA may act to block a targeted gene's expression by interfering with the translation of the mRNA. The assumption is that the complementary antisense RNA binds to the sense mRNA forming a duplex [1]. It is not clear if it acts to block ribosome binding or functions to enhance the degradation of the abnormal duplex RNA.

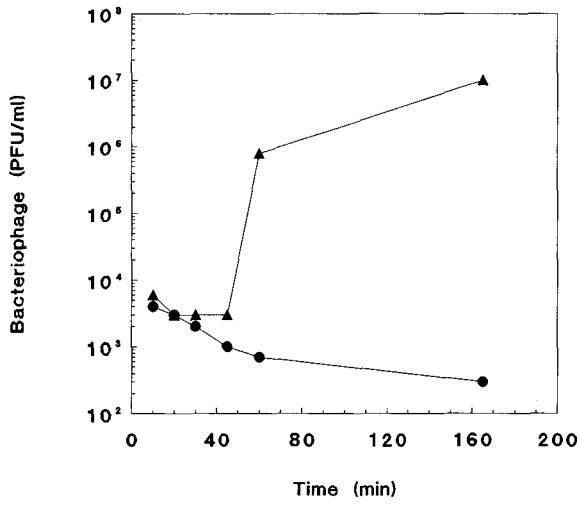


Fig. 5. Replication of *L. lactis* subsp. *lactis* bacteriophage 7–9 in *L. lactis* subsp. *lactis* CC9 alone and carrying pSGK1.5R., ▲, CC9 and ●, CC9 [pSGK1–5R].

There are at least four factors that would appear to modulate the level of inhibition by antisense RNA: (i) the transcriptional level of the targeted gene; (ii) the amount of antisense RNA produced; (iii) the length of the complementary region between the sense and antisense RNAs; and (iv) the spatial localization of the antisense RNA relative to the ribosome binding site on the sense mRNA. The first three factors appear to follow a logical progression; the greater the ratio of antisense to sense RNA the greater the inhibition of expression. Furthermore, as the length of the region complementary between the sense and antisense RNA increases, the level of inhibition increases, presumably due to the greater stability of the RNA duplex. The last factor the impact of the spatial position of the region targeted by the antisense RNA is not clear. Although it was first believed that only the ribosome binding site and the 5′ end of the targeted mRNA need be duplexed with an antisense RNA, a survey of the literature shows no apparent absolute pattern.

Antisense RNA has been employed to investigate the expression of bacterial [26], mammalian [13] and plant genes [8]. It may also normally regulate the expression of certain genes, for example the *E. coli opmF* gene [25]. The use of antisense RNA to block bacteriophage or viral infection has been reported for the Rous sarcoma virus [31] and the *E. coli* SP bacteriophage [7]. In this latter study, up to 96% inhibition of plaque-forming units was observed. This degree of inhibition may be conservative since no attempts were made to maximize the effect of the antisense RNA.

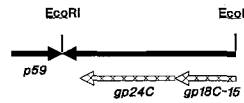
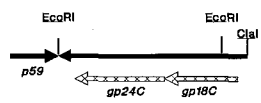
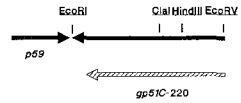
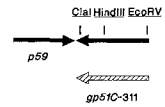
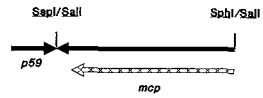
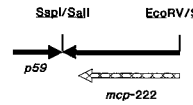
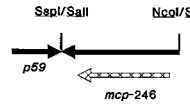
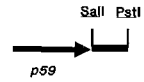
ANTISENSE INHIBITION OF BACTERIOPHAGE INFECTION IN LACTIC ACID BACTERIA

Resistance to a broad class of isometric bacteriophages which infect strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* has been engineered into a dairy starter culture by expression of antisense RNA targeted against a specific gene [6,20,21]. For example, we have obtained a high degree of protection using a 1654-bp fragment containing the entire *gp51C* coding sequence is positioned in the antisense orientation with respect to a promoter which functions in *L. lactis*. Similarly, antisense constructs have been assembled which carry portions of *gp24C*, *gp18C* (pSGK1.0R and pSGK1.5R) and *mcp* (pDC100, pDC101, pSC1) either individually or in combination (Table 2). Although some level inhibition is observed, in almost all cases the highest level is observed with pSGK1.6R, where a greater than 99% reduction of the total number of plaque forming units is observed [20]. A variety of truncated genes including the *gp51C* ORF expressed in the sense orientation fail to provide any significant measure of resistance as compared to the intact ORF (Table 2). Plaques that do form in strains carrying pSGK1.6R and pSGK1.5R are characterized by their relatively small size and irregular shape. In contrast, antisense constructs containing, for example, only the 25-bp including the ribosome binding site and the 5′ nontranslated region for *gp51C* fail to show any significant level of protection. Although it was initially anticipated that only a small region including the ribosome-binding site would be needed in order to confer an antisense RNA effect, it now appears that for these systems, a significantly longer RNA is required. Observations similar to ours have been made in a number of systems, and there appear to be no absolute rules for predicting which region will be most effective in inhibiting the targeted gene's expression [7,12].

Antisense RNA targeted against the *L. lactis* subsp. *cremoris* F4-1 MCP reduced bacteriophage replication up to 50% [6]. Antisense RNA directed against the MCP did not prove as effective perhaps due to the high level expression of the *mcp* gene and the possibility that it may not be a rate-limiting component of the replication cycle. For example, if the amount of MCP produced is 10-times higher than is required to package all of the copies of the genome produced during a single round of infection even a 90% reduction in MCP synthesis (due to the expression of an antisense *mcp* RNA) will not have any noticeable effect. The high level of MCP synthesis in *L. lactis* subsp. *cremoris* bacteriophage F4-1 may be a function of the stability and/or level of translation of the *mcp* mRNA rather than the strength of the promoter. Preliminary studies measuring the transcription of the *mcp* promoter by *cat86*

TABLE 2

Effect of various antisense RNA constructs on EOP of *L. lactis* subsp. *lactis* bacteriophages

Plasmid	Construct	EOP
None	none	1.0
SGK1.0R		0.69
pSGK1.5R		0.45
pSGK1.6R		0.004
pSGK1.0VR		1.0
pSGK0.8R		1.0
pDC100		0.58
pSC1		0.50
pDC101		0.77
pGKV259		1.0

fusions reveal it to be relatively weak as compared to the p59 promoter [33].

Antisense RNAs directed against different regions of the maturation protein, coat protein and the replicase gene of *E. coli* bacteriophage SP, all inhibited phage proliferation [7]. The antisense RNA complementary to a 240-bp region containing 24-bp from the noncoding region (including the ribosome binding site) and 216-bp from the coding region of the maturation gene inhibited bacteriophage replication more then antisense RNA directed against the coat protein and the replicase gene [12]. The maturation protein gene appears to be the best target because its product is present in only one copy in the mature phage. In contrast to our results, a 19-bp sequence encompassing the ribosome binding site but not the coding region of the *E. coli* bacteriophage SP maturation protein gene inhibited phage proliferation 94%.

L. lactis subsp. *lactis* strains harboring the antisense *gp51C* RNA construct, pSGK1.6R grow at a rate equivalent to the host strain alone suggesting that antisense expression of this sequence is not deleterious to normal cellular metabolism [20]. There were, however, some adverse effects on the host due to the antisense *mcp* RNA construct, pDC100 [6]. During stationary phase there was a loss in cell viability as exhibited by a decline in the optical density of the culture. In contrast, *L. lactis* subsp. *lactis* CC9 carrying pSGK1.6R has a rate of acidification which is equivalent to the host strain alone, even in the presence of bacteriophage at a multiplicity of infection of > 100 suporting our claims on the utility of these strains for use as starter cultures [20]. The bacteriophage do not replicate and there is actually a two log reduction in the total number of bacteriophages since those that infect the cells are effectively trapped. These strains, therefore, act as a sanitizing agent removing bacteriophage from the environment (Fig. 5).

ANTISENSE RNA BACTERIOPHAGE RESISTANT STRAINS IN A SURVEY/ROTATION PROGRAM

Bacteriophages in any ecological niche are dynamic and continually evolve due to a stringent selection process that dictates their survival. Their rapid replication rates and ability to generate hundreds of progeny from a single-cycle allows them to quickly evade host resistance mechanisms. Restriction/modification systems are particularly sensitive due to the ability of the bacteriophage, which evades restriction, to generate progeny with the immune methylation pattern. Although our antisense RNA based system is by nature difficult to circumvent, we would envision a system where the bacteriophage population in a plant is monitored to quickly ascertain its genetic makeup. A critical diagnostic tool would be a polymerase chain

reaction assay to characterize the class of conserved elements within the bacteriophage population. The manipulation and analysis of nucleic acids has realized a quantum leap with the advent of DNA amplification using the thermostable DNA polymerase isolated from *Thermus aquaticus* (*taq* polymerase). PCR involves the selected amplification of a region of DNA as delineated by a set of oligonucleotide primers. By successively cycling at different temperatures for different times, a series of annealing, extension and dissociation steps can be carried out with the net result of exponentially amplifying the sequences flanked by these primers. In approx. 30 cycles, with a total time on the order of 2–4 h, a million-fold amplification of the targeted DNA sequence can be realized. By amplifying a specific region of DNA over the rest of the chromosome, there is a great increase in the signal to noise ratio obtained as compared to most nucleic acid based detection systems. PCR detection can be accomplished in 2–4 h without the need to purify the bacteriophage particles from the whey sample. Due to its extreme sensitivity, PCR can identify a potential problem well in advance of the buildup in bacteriophage numbers that might inhibit starter culture activity. It can be used, for example, to quickly establish whether a bacteriophage population is developing that carries the *gp51C* sequence. As a corrective action we would then install a strain that expresses the antisense *gp51C* RNA to help reduce this bacteriophage population. By careful monitoring, the appropriate strains could be used well in advance of any significant buildup of a specific bacteriophage population that might manifest itself in a fermentation failure. It is well recognized that our system cannot completely exclude the replication of all bacteriophages and in fact no system is perfect. If 'new' bacteriophage isolates appear which do not carry a conserved region within the current repertoire, additional antisense RNA expressing strains can be developed in a rational manner.

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