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Antisense RNA directed against the major capsid protein of *Lactococcus lactis* subsp. *cremoris* bacteriophage 4-1 confers partial resistance to the host

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Summary. Antisense RNA targeted against the major capsid protein (MCP) of *Lactococcus lactis* subsp. *cremoris* bacteriophage F4-1 reduced bacteriophage replication by up to 50%. The region containing the *mcp* gene was oriented to transcribe the antisense strand using a *L. lactis* subsp. *cremoris* Wg2 promoter. The size of the *mcp* insert transcribed affected the level of bacteriophage inhibition and the greatest level of inhibition was achieved using a 301-bp fragment from the 5′ end of the *mcp*. Antisense *mcp* RNA constructs were stable and did not alter the endogenous plasmid profile in the host, *L. lactis* subsp. *cremoris* F4-1. There were, however, some adverse effects on the host during the stationary phase as exhibited by a decline in cell density.

Introduction

Bacteriophages that infect lactic starter cultures remain a problem in the dairy industry, since they inhibit or totally prevent acidification (Whitehead and Cox 1936; for review see Jarvis 1989). 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 industrial scale-up of the process. This has resulted in an accessible target population (starter cultures) in which the bacteriophages can propagate.

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 genomic organization and regulation of gene expression. An analysis of the genome of a bacteriophage that can infect group N streptococci was reported by Lakshmidevi et al. (1988) for a temperate bacteriophage of *L. lactis* ssp. *cremoris*. This bacteriophage has a circularly permuted genome and the sites for integration (*att*) and packaging initiation (*pac*) were located. There is, however, a lack of information concerning the replication and gene regulation for these bacteriophages. Only a limited number of genes from bacteriophages that infect lactic acid bacteria have been extensively characterized, including the lysin gene from *L. lactis* subsp. *lactis* bacteriophage ML3 (Shearman et al. 1989) and an endolysin from *Lactobacillus bulgaricus* bacteriophage mvl (Boizet et al. 1991). To date, we have identified and characterized bacteriophage genes coding several minor structural proteins (Kim and Batt 1991a, b), a major capsid protein (Chung et al. 1991) and several proteins of unknown but essential function that may be related to replication or transcription (Kim and Batt 1991c).

Several different approaches have been proposed and implemented to develop bacteriophage-resistant starter cultures. These include the classical direct screening for a "bacteriophage-resistant phenotype" as mediated by a variety of unknown mechanisms (Marshall and Berridge 1976; Thunell et al. 1981) and/or the purposeful introduction of plasmid-borne resistances including a host restriction/modification system and a heat-sensitive phage resistance (Hill et al. 1990a, b). Recently, however, bacteriophages resistant to these plasmid-borne defense mechanisms as developed by Klaenhammer and co-workers have arisen (Alatossava and Klaenhammer 1991; Hill et al. 1991). Although each system has merit, the use of antisense RNA to block infection represents a totally different approach to the problem.

Antisense RNA is the product of transcribing the DNA strand complementary to the strand that codes for the protein. 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 has been employed to investigate the expression of bacterial genes (Pestka et al. 1984), mammalian genes (Izant and Weintraub 1984) and plant genes (Ecker and Davis 1986). It may also regulate the expression of certain genes, for example,

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the *Escherichia coli ompF* gene (Mizuno et al. 1984). The use of antisense RNA to block bacteriophage or viral infection has been reported for the Rouse sarcoma virus (Stephenson and Zameknik 1978) and the *E. coli* SP bacteriophage (Coleman et al. 1985). *E. coli* F-specific bacteriophage SP, is a single-stranded positive phage and antisense RNAs directed against different regions of the maturation protein, coat protein and the replicase gene of bacteriophage SP, all blocked the formation of plaques and phage proliferation. Coleman et al. (1985) showed that the antisense RNA directed against the 5′ end region of coat protein (*micA*) and replicase (*micB*) inhibited phage production more than the antisense RNA directed against the 3′ end region of replicase (*micC*). In addition, a gene dosage effect was demonstrated by a triple antisense RNA construct having micA:B:C, which showed the greatest inhibition of phage production (96%). We have previously reported on the ability of antisense RNA to inhibit bacteriophage replication, when targeted against a conserved gene function (Kim and Batt 1991d). This approach has now been extended to target a bacteriophage capsid protein that, although only partially effective, further demonstrates the utility of antisense RNA for conferring bacteriophage resistance.

Materials and methods

Bacterial strains and media. L. lactis subsp. *cremoris* starter culture F4-1 and its virulent *L. lactis* subsp. *cremoris* bacteriophage F4-1 were isolated from cheese whey samples obtained from two commercial plants in New York State (Kim and Batt 1991a, b). *E. coli* JM105 (*Δlac, pro, thi, strA, endA, sbcB*15, *hsdR*4, [F′*traD*36, *proAB*$^+$, *lacI*^{q}, *ZM15*]) was used as a host for initial cloning and sequence analysis. The vectors used were pUC12/13 (Vieira and Messing 1982) pGKV259 (from J. Kok, University of Groningen; van der Vossen et al. 1987) and pBluescript (Stratagene, La Jolla, Calif., USA).

LB medium was used for the propagation of *E. coli* from which plasmid DNA was purified (Maniatis et al. 1982). M17 medium with 0.5% glucose (M17G) was used for propagation of *L. lactis* subsp. *cremoris* strains (Terzaghi and Sandine 1975). Where noted, agar (15 g/l), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; 40 μg/ml), chloramphenicol (Cm; 25 μg/ml), erythromycin (Em; 4 μg/ml for *L. lactis* subsp. *cremoris*; 200 μg/ml for *E. coli*), and isopropyl-β-D-thiogalactopyranoside (IPTG; 2 mM) were added.

Bacteriophage purification and quantification. The number of plaque-forming units (PFU) were quantified by standard plaque assays employing an overlay culture of *L. lactis* subsp. *cremoris* in M17 medium with 0.7% agar. Plates were incubated for 24 h at 30° C and the resulting plaques were counted.

DNA isolation and manipulation. Plasmid DNA was isolated from *E. coli* by the boiling method for small-scale preparation and by a modification of the alkaline lysis protocol for large scale cesium chloride ethidium bromide density gradient preparations (Maniatis et al. 1982). Plasmid DNA was isolated from *L. lactis* subsp. *cremoris* by the method of Anderson and McKay (1983). All restriction and other modifying enzymes were purchased from New England Biolabs (Beverly, Mass., USA), or U.S. Biochemical Co. (Cleveland, Ohio, USA) and used according to their instructions.

Bacterial transformations. E. coli JM105 was transformed by the method described by Hanahan (1983). *L. lactis* subsp. *cremoris* F4-1 was transformed by the method described by Kok et al. (1984) and transformants were selected on SM17G (25% sucrose added as an osmotic stabilizer) agar with Em (4 μg/ml).

Polymerase chain reaction (PCR). The *mcp* gene was cloned into pGKV259 vector by creating new restriction sites by PCR (Scharf 1990). Two primers were constructed to create *Pst*I restriction sites 35-bp before the start codon and 3-bp after the stop codon for the *mcp* gene. The 3′ PCR primer was 30 nucleotide (nt) (5′-GTTGCGT**CTGCA**GTCTTATGAATGGTCAGT-3′; *Pst*I site underlined; altered bp in bold letters). The 5′ PCR primer was also 30 nt (5′-ATGCCAA**CTGCA**GGAAATTATAGAAAAGAG-3′). A 100 μl reaction was prepared in a 0.5 ml microfuge tube as follows (Innis and Gelfand 1990); 100 ng of pJK3.3, 1 μl (50 pmol) of each primer, 5 μl of 1 M KCl, 0.67 μl of TRIS-HCl (pH 8.8), 1.5 μl of 0.1 M $MgCl_2$, 1 μl of bovine serum albumin (New England Biolabs, 10 μg/μl), 5 μl of each 1 mM deoxynucleotide triphosphates, 2 units of *Taq* DNA polymerase (United States Biochemical Corp.). After adding 75 μl mineral oil on the top of the reaction mixture, a total of 32 cycles were performed with a Hybaid thermal reactor (Hybaid, Teddington, Middlesex, UK) using the following conditions: denaturation 96° C at 15 s; annealing 55° C at 30 s; extension 72° C at 90 s. After amplification, the PCR product was extracted with phenol:chloroform, ethanol precipitated and restricted with *Pst*I.

Results

Construction of antisense mcp RNA expression vectors

The 905-bp *mcp* for *L. lactis* subsp. *cremoris* F4-1 is located within a 3.3-kb *Hin*dIII/*Spe*I fragment (Chung et al. 1991). It codes for a 35-kDa protein that accounts for approximately 80% of the total protein found in mature bacteriophage particles (Kim and Batt 1991a). Nucleotide and amino terminal sequence analysis further localized the *mcp* open reading frame (ORF) to within a 1.2-kb *Sph*I/*Ssp*I fragment (Chung et al. 1991).

To orient the entire *mcp* to express the antisense strand, two PCR primers were designed. These PCR primers uniquely delineated the *mcp* and amplified a 926-bp fragment, (from nt-461 to nt-1387, Chung et al. 1991) which included both the *mcp* ORF and the putative ribosome binding site. A *Pst*I restriction site was designed into both the 5′ and 3′ primers by incorporating 4-bp and 2-bp mismatches respectively into the primer sequence. The PCR product was digested with *Pst*I and ligated into pGKV259. pGKV259 is derivative of pGKV210, an *E. coli*/*Lactococcus* shuttle vector that carries a promoterless *cat*-86 cassette and a erythromycin resistance gene for selection in either *E. coli* or *L. lactis* (van der Vossen et al. 1987). pGVK259 carries an *L. lactis* subsp. *cremoris* Wg2 promoter-like sequence that we previously used to express other antisense RNA targeted against bacteriophage genes. Insertion of the 926-bp *mcp*-containing fragment disrupts the expression of the *cat*-86 resistance in pGVK259 and the resulting *E. coli* transformants were Em-resistant (Em^r) but Cm-sensitive (Cm^s). The pGVK259 derivative carrying the *mcp* in the antisense orientation was designated pDC100.

Additional antisense *mcp* RNA constructs were created to truncate the size of the *mcp* ORF from the 3′ end. pDC100 was digested with *Sal*I/*Nco*I and *Sal*I/

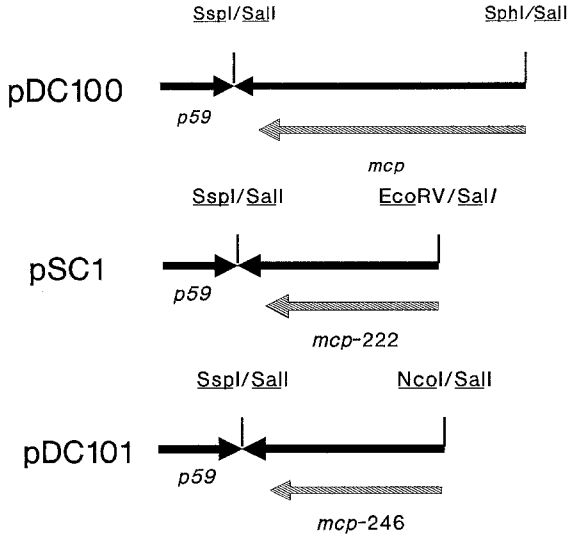

Fig. 1. Restriction map of antisense major capsid protein gene (*mcp*) RNA vectors. Regions shown are only for the p59 promoter and the *mcp* fragment. The balance of the vector is pGKV259 (van der Vossen et al. 1987)

*Eco*RV separately and then the termini rendered flush with Klenow. After self-ligation pDC100 derivatives with a 666-bp (from the *SalI/Eco*RV digest) and a 740-bp (from the *SalI/Nco*I digest) deletion from the 3′ end of the *mcp* were obtained. The vector carrying the 5′ 301-bp of the *mcp* was designated pSC1, while the other carrying 227 bp was designated pDC101 (Fig. 1).

Effect of antisense mcp RNA on bacteriophage replication

The parental vector, pGVK259 and its derivatives carrying various fragments of the *mcp* gene were transformed into *L. lactis* subsp. *cremoris* F4-1 selecting for Em^r. Individual transformants carrying these various constructs were then challenged with *L. lactis* subsp. *cremoris* bacteriophage F4-1 and the efficiency of plaquing used as a measure of inhibition. A strain carrying pGVK259 displayed a slightly lower level of plaquing efficiency as compared to the host strain alone. The various antisense *mcp* RNA constructs, however, all conferred a significantly higher degree of inhibition ranging from 23–50% (Table 1). The highest level of inhibition was observed with pSC1 which carries a truncated form of the *mcp* gene. The lowest level of inhibition was observed with pDC101, which is further deleted for an additional 74 bp as compared to pSC1.

The plaques observed on all strains carrying the antisense *mcp* RNA constructs were similar in size and morphology to those observed on the host strain alone. The effect of pDC100 on the burst size of the bacteriophage (as defined by the increase in the pfu per input bacteriophage particle) was also determined (Fig. 2). There was a 54% reduction in the rate at which bacteriophage particles were produced in strains harboring pDC100. In addition, the total number of bacteriophages produced was reduced by 37% as compared to the wild-type strain, a level similar to that observed for the reduction in the efficiency of plaquing (EOP).

Table 1. Efficiency of plaquing (EOP) of *Lactococcus lactis* subsp. *cremoris* bacteriophage F4-1 on *L. lactis* subsp. *cremoris* F4-1 carrying pGVK259, pDC100, pSC1 or pDC101

Plasmid	Inhibition[a] (%)
pGVK259	6.6±5.9
pDC101	23.2±6.6
pSC1	50.5±4.9
pDC100	42.1±4.5

Cultures were plated in an overlay on M17G agar with approximately 500 plaque-forming units/ml (using the host strain alone) and the number of resulting plaques quantified after 24 h
[a] Calculated relative to the bacteriophage titer using *L. lactis* subsp. *cremoris* F4-1 host alone

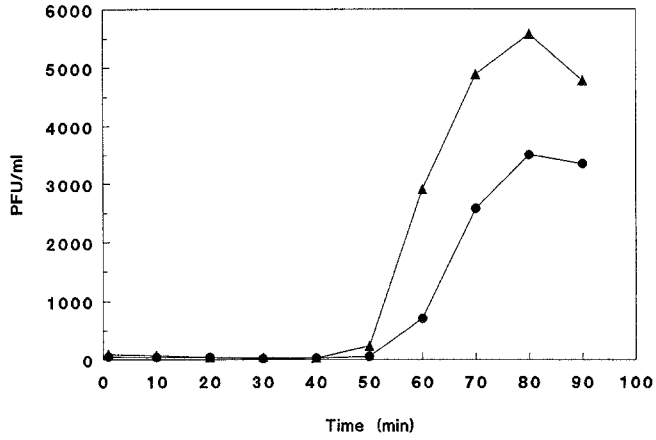

Fig. 2. Effect of antisense *mcp* RNA construct pDC100 on the replication of *Lactococcus lactis* subsp. *cremoris* bacteriophage F4-1. *L. lactis* subsp. *cremoris* F4-1 alone and carrying pDC100 was grown in M17G medium at 30° C and the bacteriophage added at a multiplicity of infection moi of 0.01. At various times, the plaque-forming units (pfu)/ml were determined using a standard overlay assay with *L. lactis* subsp. *cremoris* F4-1: ▲, *L. lactis* subsp. *cremoris* F4-1; ●, *L. lactis* subsp. *cremoris* F4-1 carrying pDC100

Effect of antisense mcp RNA on host plasmid profile and viability

L. lactis subsp. *cremoris* F4-1 carries a number of resident plasmids at least one of which appears to code for lactose metabolism (J. H. Kim and C. A. Batt, unpublished results). In certain cases due to incompatibility, resident plasmids are lost upon introduction of another 'foreign' plasmid. There is no apparent change in the plasmid profile as a consequence of pDC100, even after ten successive passages consisting of greater than 56 generations. Furthermore, pDC100 is of the expected size (5.9 kb) suggesting that it too is stably maintained. Similarly both pSC1 and pDC101 did not appear to alter the endogenous plasmid profile upon introduction (data not shown).

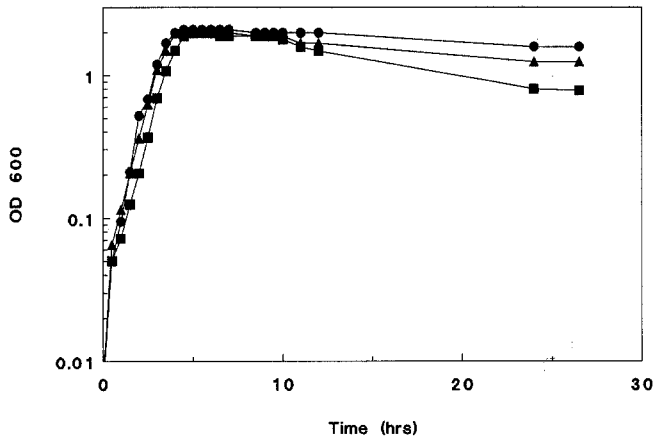

Fig. 3. Growth, measured as optical density at 600 nm (OD 600), of *L. lactis* subsp. *cremoris* F4-1 alone and carrying either pGKV259 or pSC1 in M17G medium at 30° C: ●, *L. lactis* subsp. *cremoris* F4-1; ▲, *L. lactis* subsp. *cremoris* F4-1 carrying pGVK259; ■, *L. lactis* subsp. *cremoris* F4-1 carrying pSC1

The viability of the host carrying the antisense *mcp* constructs was monitored by measuring the optical density with time of cultures grown in M17G medium (Fig. 3). There was a slight increase in the generation time in strains carrying the antisense *mcp* constructs. For example, *L. lactis* subsp. *cremoris* F1-4 carrying pSC1 grew with a generation time of 86 min as compared to the host alone, which had a generation time of 81 min. All of the antisense mcp constructs reduced the apparent cell viability as exhibited by a decline in the optical density as the cells entered the stationary phase. The vector pGVK259 itself reduced the cell viability but to a lesser degree compared to either pDC100, pDC101 or pSC1.

Discussion

We have demonstrated the limited utility of antisense *mcp* RNA to inhibit the replication of *L. lactis* subsp. *cremoris* bacteriophage F4-1. The maximum inhibition observed was 50% as measured either by the EOP or the number of bacteriophage particles produced from a single round to replication. This highest level of inhibition was obtained using a 301-bp fragment from the *mcp*. The level of inhibition observed with this *mcp*-based system is far less than the 99% inhibition that we previously obtained with a construct expressing the antisense *gp51C* RNA (Kim and Batt 1991d). GP51C is a 51-kDa cryptic protein, the gene for which is highly conserved in a number of different *L. lactis* subsp. *lactis* bacteriophage isolates.

The mechanism by which antisense mRNA inhibits the targeted gene expression is not clear but may involve the formation of a RNA duplex (Andersen et al. 1989). The duplex formed between an antisense RNA and its target RNA is affected by several factors including secondary structure, temperature, concentration, and cellular components (Inouye 1988). Antisense RNA directed against the MCP did not prove as effective, perhaps due to the high level expression of the *mcp* gene and the fact that it is probably not a rate-limiting component of the replication cycle. If the amount of MCP produced is, for example, ten times higher than is necessary to package all of the replicated progeny genomes, even a 90% reduction in MCP production (due to the expression of an antisense *mcp* RNA) will not have any noticeable effect. The high level of MCP expression 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. Our preliminay characterization of the *mcp* promoter using fusions to the *cat86* cassette reveal it to be relatively weak in comparison with the p59 promoter of pGVK259. The stability of the *mcp* mRNA may limit the efficiency of the antisense RNA inhibition. Hirashima et al. (1986) reported that the best targets for the antisense RNA immune system are the genes for minor viral proteins such as the maturation gene (present in only one molecule per phage), but not genes for major species such as coat protein and replicase. Antisense RNA against the maturation gene exhibited 98% inhibition of plaque formation for bacteriophage SP, whereas antisense RNA against replicase and coat protein gave only 42% and 69% inhibition, respectively.

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