

Expression of Antisense RNA Targeted against *Streptococcus thermophilus* Bacteriophages†

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Antisense RNA complementary to a putative helicase gene (*hel3.1*) of a *cos*-type *Streptococcus thermophilus* bacteriophage was used to impede the proliferation of a number of *cos*-type *S. thermophilus* bacteriophages and one *pac*-type bacteriophage. The putative helicase gene is a component of the Sfi21-type DNA replication module, which is found in a majority of the *S. thermophilus* bacteriophages of industrial importance. All bacteriophages that strongly hybridized a 689-bp internal *hel3.1* probe were sensitive to the expression of antisense *hel3.1* RNA. A 40 to 70% reduction in efficiency of plaquing (EOP) was consistently observed, with a concomitant decrease in plaque size relative to that of the *S. thermophilus* parental strain. When progeny were released, the burst size was reduced. Growth curves of *S. thermophilus* NCK1125, in the presence of variable levels of bacteriophage κ 3, showed that antisense *hel3.1* conferred protection, even at a multiplicity of infection of approximately 1.0. When the *hel3.1* antisense RNA cassette was expressed in *cis* from the κ 3-derived phage-encoded resistance (PER) plasmid pTRK690::ori3.1, the EOP for bacteriophages sensitive to PER and antisense targeting was reduced to between 10^{-7} and 10^{-8} , beyond the resistance conferred by the PER element alone (less than 10^{-6}). These results illustrate the first successful applications of antisense RNA and explosive delivery of antisense RNA to inhibit the proliferation of *S. thermophilus* bacteriophages.

Lactic acid bacteria represent a heterogeneous family of nondifferentiating, gram-positive eubacteria that derive metabolic energy from the fermentation of carbohydrates to lactate. The dairy industry has utilized extensively species from the genera *Lactococcus*, *Lactobacillus*, and *Streptococcus*, as starter cultures or culture adjuncts for use in the manufacture of a variety of fermented dairy products. Bacteriophages specific for dairy starter cultures, notably lactococci and, recently *Streptococcus thermophilus*, have long been recognized as a significant problem for the dairy industry. The problem became more severe as cheese plants increased in size and product throughput became more mechanized (4). Pasteurized milk and lysogenic starter cultures serve as continuous reservoirs for virulent bacteriophages capable of disrupting product manufacture (6, 33). Loss of fermentative capacity associated with starter culture lysis can significantly retard or halt batch fermentations, thereby causing significant losses of time and production capital to the dairy industry each year.

In recent years, an increased incidence of bacteriophage-related problems have been observed for *S. thermophilus* strains, which are an essential component of starter systems for yogurt and Italian cheese varieties. Our understanding of *S. thermophilus* bacteriophages has progressed rapidly since the release of six bacteriophage whole-genome sequences: those for DT1 (42), ϕ O1205 (40), Sfi11 (24), Sfi19 (25), Sfi21 (25), and ϕ 7201 (41). In contrast to bacteriophages that infect *Lac-*

tococcus species, *S. thermophilus* bacteriophages are closely related, at both the genetic and morphological levels, making differentiation difficult. Electron microscopy studies revealed that *S. thermophilus* bacteriophages, both temperate and lytic, are nearly identical and that all belong to the *Siphoviridae* family (morphotype B1), having small isometric heads, long non-contractile tails, and genomes comprised of double-stranded DNA (31). It has recently been shown that *S. thermophilus* strains are attacked by two groups of highly related bacteriophages that differ in their mechanism of genome encapsidation: *cos* type and *pac* type (23). These bacteriophages can be identified and differentiated by examination of their distinct capsid protein profiles or through the detection of the genes that encode those structural proteins (23).

Studies on native bacteriophage defense systems remain limited. *S. thermophilus* strains have been found to possess both chromosomal and plasmid-borne restriction and modification (R-M) systems (2, 15, 37, 38, 39). At this juncture, however, these native R-M systems have not yet been exploited as means of augmenting the intrinsic level of resistance of industrial starter strains. The plasmid-borne expression of *LlaDCHI* (formerly *LlaII*), a heterologous R-M system derived from *Lactococcus lactis*, does confer broad-range bacteriophage resistance in various strains of *S. thermophilus* (32). In addition, efforts to construct strains of *S. thermophilus* with passive resistance properties are also under way. Lucchini et al. (27) recently described the use of pG⁺host9::ISS1-mediated insertional mutagenesis to identify four distinct host-encoded loci involved in bacteriophage sensitivity. A putative transmembrane protein (Orf394) was discovered and proposed to be functionally analogous to the lactococcal Pip protein, which is essential for infection of *L. lactis* by c2-type bacteriophages (13).

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The application of molecular biology and modern functional genomics is accelerating the development of novel bacteriophage resistance mechanisms through genetic engineering. Sequence analysis can be used to interpret the encoded genetic information, determine genetic features that are common to a variety of bacteriophages, and target potentially sensitive events in bacteriophage development. Recently, comparative genomic analyses have revealed that the genomes of *S. thermophilus* bacteriophages are molecular mosaics assembled upon a relatively simple scaffold consisting of four independently evolving segments (5, 26). The bacteriophage genome replication functions are clustered on the same genomic segment. To date, two distinct DNA replication modules have been identified among the various *S. thermophilus* bacteriophages: Sfi21 type and ϕ 7201 type. Hybridization studies have demonstrated that the Sfi21-type module, which is present in five of the six completely sequenced bacteriophages (i.e., DT1, ϕ O1205, Sfi11, Sfi19, and Sfi21), is also present in a majority of industrial bacteriophage isolates (3, 8). Computational analysis of the Sfi21-type replication module predicts a single origin of DNA replication (*ori*) and several open reading frames that encode a putative helicase, a putative primase, and a number of other proteins of undetermined function. This module is highly conserved at the nucleotide level, suggesting a recent acquisition via horizontal gene transfer followed by rapid dissemination (5). The replication module from bacteriophage ϕ 7201, on the other hand, includes two distinct *oris* and encodes a probable single-stranded DNA binding protein, a putative replication protein, a putative DnaC homologue, and a number of other proteins of undetermined function (41).

Origin-conferred phage-encoded resistance (PER) was first reported to be effective in *L. lactis* (16, 28), and has since been demonstrated to be effective in *S. thermophilus* (12, 41). When a bacteriophage *ori* is provided in *trans* on a recombinant plasmid, the origin acts as a molecular decoy that competes for and titrates away bacteriophage-specific replication factors. The result is a reduction in the number of bacteriophage genomes replicated and a dramatic increase in plasmid copy number. The efficacy of PER is bacteriophage specific, and the conferred level of resistance correlates with the copy number of the false origin presented in *trans* (29, 34). Further, when an antisense RNA expression cassette was linked, in *cis*, to a PER vector, the result was the expression of an explosive dose of antisense RNA that effectively inhibited a lactococcal bacteriophage (43).

Genome replication functions in *S. thermophilus* bacteriophages are perhaps the most obvious targets for gene silencing by antisense RNA, since they are highly conserved among industrial bacteriophages and expressed early and transiently during the lytic life cycle. Recently, antisense RNAs, which were designed to target genes involved in DNA replication, have been found to be extremely effective at inhibiting a number of related lactococcal bacteriophages (29). In this study, the expression of a putative helicase, which is a component of the highly conserved Sfi21-type DNA replication module, was targeted for disruption by antisense RNA.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. All bacterial stocks were maintained at -80°C in

fresh culture medium supplemented with 10% glycerol. All bacteriological media and components were purchased from Difco Laboratories (Detroit, Mich.). *Escherichia coli* strains were grown at 37°C with constant aeration in Luria-Bertani broth. Unless otherwise indicated, *S. thermophilus* derivatives were propagated aerobically at 42°C in Elliker broth supplemented with 1% (wt/vol) beef extract (Elliker-B). Chloramphenicol was added at $2.5\ \mu\text{g/ml}$ for both *E. coli* and *S. thermophilus* when appropriate. For solid media, Bacto Agar was added at final concentrations of 1.5% (wt/vol) for base agar and 0.75% (wt/vol) for soft agar.

Plasmids, bacteriophages, and propagation assays. The plasmids and bacteriophages used in this study are listed in Table 1. Bacteriophages described in this study were isolated from mozzarella whey. Bacteriophages were propagated in Elliker-B broth supplemented with 10 mM CaCl_2 (Elliker-BC) at 42°C and diluted in $0.1\times$ Elliker-B broth supplemented with 10 mM CaCl_2 . For plaque assays, 20 ± 1 ml of M17-G base agar supplemented with 10 mM CaCl_2 was dispensed using a Bellco Biotechnology (Vineland, N.J.) automatic medium dispenser to limit volume-dependant variations in plaque size and incubated aerobically at 37°C for 18 h prior to analysis. The efficiency of plaquing (EOP) was calculated by dividing the bacteriophage titer, in PFU per milliliter, of the test strain by the bacteriophage titer of the parental strain. Bacteriophages were characterized as *cos*- or *pac*-type bacteriophages as described by Le Marrec et al. (23). Lysis-in-broth assays were performed in Elliker-BC medium as described previously, except that samples were taken every 15 min for a period of 4 h (41).

Enzymes and reagents. Restriction enzymes, *Taq* DNA polymerase, and deoxynucleoside triphosphates were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). T4 DNA ligase, the SuperScript reverse transcription kit, and DNA molecular weight markers were obtained from Gibco-BRL Life Technologies, Inc. (Gaithersburg, Md.). *Pwo* DNA polymerase was obtained from Roche (Indianapolis, Ind.), and all enzymes were used according to the manufacturer's specifications. All other chemicals were of analytical grade and obtained from Sigma Chemical Company (St. Louis, Mo.).

Bacterial transformation. All electroporations were performed using a Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.) apparatus configured to 25 μF , 2.5 kV, and 200 Ω . Preparation of electrocompetent *E. coli* strain MC1061 (18) was conducted as described by Sambrook et al. (36), and electrocompetent *S. thermophilus* strains were prepared by a method modified from that of Holo and Nes (17). A stationary-phase culture of *S. thermophilus* was diluted 100-fold into 42°C Elliker-B broth, incubated at 42°C , and allowed to reach an optical density at 600 nm of 0.2 prior to the addition of 1/10 volume of 42°C 15% (wt/vol) glycine and 1/10 volume of 42°C $2\times$ Elliker-B broth. Cells were harvested by centrifugation at an optical density at 600 nm of between 0.6 and 0.8, washed three times with 2 volumes of sterile deionized water, washed once with 2 volumes of SG buffer (0.5 M sucrose and 10% glycerol), resuspended in 0.003 volume of SG buffer, and incubated on ice prior to use. Plasmid DNA (1 μg) was mixed with 40 μl of cells in a chilled Gene Pulser 0.2-cm cuvette. Following electroporation, cells were immediately resuspended in 960 μl of recovery medium (Elliker-B broth supplemented with 20 mM MgCl_2 and 2 mM CaCl_2) and incubated for 2 h at 42°C , before being spread onto Elliker-B base agar supplemented with chloramphenicol (5.0 $\mu\text{g/ml}$).

Preparation of plasmid and genomic DNAs. Small-scale preparations of plasmid DNA were isolated from *E. coli* (36) and *S. thermophilus* (35) as described previously. Large-scale preparations of plasmid DNA were isolated using a Midi Kit (Qiagen, Chatsworth, Calif.) according to the manufacturer's instructions. PCR products were purified by using the Qiagen PCR Purification Kit prior to further manipulation. When required, DNA was extracted from agarose gels by using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

Southern hybridizations. Bacteriophage genomic DNA was isolated using the Qiagen Lambda Kit according to the manufacturer's instructions. Alkaline transfer of *Hind*III-digested genomic DNA fragments from electrophoresed 0.8% agarose gels to 0.45- μm -pore-size Magnacharge nylon membranes (Micron Separations, Inc., Westborough, Mass.) was performed as described by Sambrook et al. (36). A 689-bp internal *hel3.1* fragment was amplified by PCR in the presence of digoxigenin (DIG)-11-UTP (Roche Molecular Biochemicals) using primers JMSp4 and JMSp5 (Table 2) and was used as a hybridization probe. Southern hybridizations (with 30% formamide and at 42°C) were performed using the Roche Molecular Biochemicals DIG-based nonradioactive nucleic acid labeling and detection system according to the manufacturer's instructions.

PCR and DNA sequencing. PCR was performed in a Hybaid Ltd. (Middlesex, United Kingdom) PCR Express thermal cycler with either *Taq* or *Pwo* DNA polymerase. DNA primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa). When appropriate, restriction endonuclease recognition sites were incorporated into the 5' ends of DNA primers to facilitate the cloning

TABLE 1. Bacterial strains, bacteriophages, and plasmids

Bacterial strain, bacteriophage, or plasmid	Relevant characteristic(s) ^a	Source or reference
<i>S. thermophilus</i> strains		
NCK1434	Industrial isolate; sensitive to κ 1; Cm ²	This study
NCK1125	Industrial isolate; sensitive to κ 3, κ 4, κ 5, κ 6, κ 9, κ 10, and κ 12; Cm ⁸	This study
NCK1435	Industrial isolate; sensitive to κ 2; Cm ⁸	This study
NCK1124	Industrial isolate; sensitive to κ 13; Cm ⁸	This study
SMQ495	Industrial isolate; sensitive to DT1; Cm ⁸	42
<i>E. coli</i> MC1061	Transformation host	18
Bacteriophages		
κ 1	<i>pac</i> -type bacteriophage	This study
κ 2	No information available	This study
κ 3	<i>cos</i> -type bacteriophage	This study
κ 4	<i>cos</i> -type bacteriophage	This study
κ 5	<i>cos</i> -type bacteriophage	This study
κ 6	<i>pac</i> -type bacteriophage	This study
κ 9	<i>cos</i> -type bacteriophage	This study
κ 10	<i>cos</i> -type bacteriophage	This study
κ 12	<i>pac</i> -type bacteriophage	This study
κ 13	No information available	This study
DT1	<i>cos</i> -type bacteriophage	42
Plasmids		
pNZ123	2.8 kb; high-copy-number shuttle vector; Cm ^r	9
pTRK686	2.4 kb; deletion derivative of pNZ123; Cm ^r	This study
pTRK687	3.0 kb; pTRK686 containing the high-expression P6 promoter	This study
pTRK688:: <i>hel3.1</i> -S	4.5 kb; pTRK687 containing 1.4-kb sense <i>hel3.1</i> cassette	This study
pTRK689:: <i>hel3.1</i> -AS	4.5 kb; pTRK687 containing 1.4-kb antisense <i>hel3.1</i> cassette	This study
pTRK690:: <i>ori3.1</i>	3.7 kb; pTRK687 containing 0.7-kb <i>ori3.1</i> cassette; Per ⁺	This study
pTRK691:: <i>ori3.1</i> :: <i>hel3.1</i> -AS	5.1 kb; pTRK690 containing 1.4-kb antisense <i>hel3.1</i> cassette	This study

^a Abbreviations: Cm^r, resistant to chloramphenicol; Cm^s, sensitive to chloramphenicol; Per⁺, origin-conferred phage-encoded resistance.

of PCR products. Primers utilized in this study are listed in Table 2. Cycle sequencing reactions and DNA sequence determination were performed by the University of California-Davis automated DNA sequencing facility, using an ABI Prism 377 DNA sequencer with a 96-lane upgrade (Applied Biosystems, Foster City, Calif.). DNA sequences were analyzed using the Genetics Computer Group, Inc. (Madison, Wis.) sequence analysis package version 10.0 and Clone Manager version 6.0 (Scientific and Educational Software, Durham, N.C.). Searches for nucleic acid (BLASTN) and protein (BLASTX) homology were performed with the basic local alignment search tool (1), using the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>).

RNA isolation and strand-specific RT-PCR. RNA was isolated from *S. thermophilus* at various times during the bacteriophage infection cycle by using the TRIzol reagent (Gibco-BRL) according to the procedure described by Dinsmore and Klaenhammer (10). Strand-specific reverse transcription-PCR (RT-PCR) was performed using the SuperScript reverse transcriptase kit (Gibco-BRL).

Nucleotide sequence accession numbers. The DNA sequences for the κ 3-derived putative helicase (*hel3.1*), κ 3-derived origin of replication (*ori3.1*) region, and bacteriophage DT1 are available through GenBank under nucleotide sequence accession numbers AF442520, AF442521, and AF085222, respectively.

RESULTS

Amplification of *hel*-containing fragments from *S. thermophilus* bacteriophages. The nucleic acid sequences for the DNA replication modules of bacteriophages DT1 (GenBank accession number NC_002072), ϕ O1205 (U88974), Sfi11 (NC_002214), Sfi18 (AF158601), Sfi19 (NC_000871), and Sfi21 (NC_000872) were aligned, and a consensus sequence was generated (data not shown). *Pst*I-tagged primers JMSp1 and JMSp2, designed upstream and downstream of the consensus putative helicase gene, respectively, were used to amplify the putative helicase genes (*hel*) from the *S. thermophilus* bacteriophages listed in Table 1, using DT1 genomic DNA as a positive

control. The expected 1.4-kb fragment, which contained the upstream putative ribosome binding site (RBS), was successfully amplified from DT1, κ 1, κ 3, κ 4, κ 5, κ 9, and κ 10 but failed to be amplified from κ 2, κ 6, κ 12, and κ 13. These results indicated that the local syntony and nucleotide sequence proximal to the nested primer sites are highly conserved among the *cos*- and *pac*-type bacteriophages that encode the Sfi21-type DNA replication module.

Sequence and phylogenetic analyses of the κ 3-derived *hel3.1* cassette. The 1,431-bp PCR fragment amplified from bacteriophage κ 3 was sequenced and revealed a single open reading frame of 1,331 bp (GenBank accession number AF442520). This open reading frame, designated *hel3.1*, begins with a 5'-ATG-3' translation initiation codon, ends with a 5'-TAA-3' stop codon, and is preceded by a putative RBS (5'-AAATTTGGTGA-3'). The putative Hel3.1 protein is 443 amino acids

TABLE 2. Primers used in this study

Primer name	Nucleic acid sequence (5' nucleotide position) ^a
JMSp15'-AAACTGCAGGCTTGCAAGATTGAAGACC-3' (25911)
JMSp25'-AAACTGCAGCCGCTCTTTGATAGATCCG-3' (27341)
JMSp35'-GGAGCGTGATTTTATGG-3'
JMSp45'-GTTAAAGCTAAGACCTACC-3' (26275)
JMSp55'-CCCTTAGTGACCATTCACGG-3' (26963)
JMSp65'-GGAATTCAGTTAGGTTCTGTGG-3' (29810)
JMSp75'-GGAATTCGCCATAATCTTCGTCGGTCC-3' (30486)

^a *Pst*I (5'-CTGCAG-3') and *Eco*RI (5'-GAATTC-3') restriction sites are underlined. The position of the 5' nucleotide (in boldface) relative to the DT1 genomic sequence is indicated when appropriate.

long and has a predicted molecular mass of 50.5 kDa. Conserved structural motifs that are characteristic of ATP-dependent helicases were found in the deduced primary sequence, including the nucleoside triphosphate binding (SPPRS₃GKT), nucleoside triphosphate hydrolysis (DEAH), and variant zinc finger (CDECYATFW₃SAERICPLC) motifs (14).

The coding region of *hel3.1* was compared to the putative helicase genes from *S. thermophilus* bacteriophages DT1, ϕ O1205, Sfi11, Sfi18, Sfi19, and Sfi21. BLASTN sequence analysis revealed the coding region of the bacteriophage κ 3 putative helicase gene to have 99% sequence similarity to that of bacteriophage DT1 and 90% similarity to those of bacteriophages Sfi11, Sfi18, Sfi19, Sfi21, and ϕ O1205. Therefore, *hel3.1* was more closely related to the helicase gene from bacteriophage DT1 than to the helicase alleles from the other bacteriophages. Over the entire length of the proteins, BLASTX analysis indicated the following amino acid similarities: DT1, 99%; ϕ O1205, 98%; Sfi11, 97%; Sfi18, 97%; Sfi19, 97%; and Sfi21, 97%.

Southern hybridization. A 689-bp internal *hel3.1* fragment generated by PCR using primers JMSp4 and JMSp5 was used as a probe during Southern hybridization experiments to confirm the conservation of the helicase gene among the bacteriophages listed in Table 1. Genomic DNAs were isolated from bacteriophages DT1, κ 1, κ 2, κ 3, κ 5, κ 6, κ 9, κ 10, κ 12, and κ 13; digested with *Hind*III; and probed under low-stringency conditions (Fig. 1). The 5' ends of primers JMSp4 and JMSp5 were designed 346 bp upstream and 337 bp downstream, respectively, of the *Hind*III site located near the midpoint of the *hel3.1* gene. Two strong bands of *hel3.1* hybridization (7.4 and 2.0 kb) were present in the *cos*-type bacteriophages DT1, κ 3, κ 5 (data not shown), κ 9, and κ 10. The *pac*-type bacteriophage κ 1 also showed two strong hybridization bands (4.3 and 2.0 kb). Three bacteriophages (i.e., the *pac*-type bacteriophages κ 6 and κ 12 and the uncharacterized bacteriophage κ 2) failed to hybridize the internal *hel3.1* probe. These results indicated that the putative helicase gene was present in both *cos*- and *pac*-type *S. thermophilus* bacteriophages but is not universally conserved. The 2.0-kb *hel3.1*-hybridizing fragment present in bacteriophages DT1, κ 1, κ 3, κ 5 (data not shown), κ 9, and κ 10 is a component of the Sfi21-type DNA replication module that is highly conserved among the *S. thermophilus* bacteriophages (5, 8). In contrast, bacteriophage κ 13 (Fig. 1, lanes 6) showed a single, weaker band of homology (8.0 kb) and loss of an internal *Hind*III site. This suggested that the κ 13-derived fragment was divergent from the κ 3-derived *hel3.1* allele. These results indicated that a majority of the bacteriophages listed in Table 1 possessed the Sfi21-type DNA replication module.

Construction of a basal antisense RNA expression vector (pTRK687). Of the vector systems tested in our laboratory to date, only those that replicate via a rolling-circle mechanism are transformable into strains of *S. thermophilus* (unpublished observations). As a result, antisense RNA expression systems were assembled on a stable derivative of the high-copy-number plasmid pNZ123 (9). This vector, which was recovered from *E. coli* as a deletion derivative and designated pTRK686, was completely sequenced (2,410 bp). This plasmid transforms *S. thermophilus* NCK1124, which is plasmidless, and NCK1125, which contains two native plasmids, at frequencies of between 10^4 and 10^5 transformants per μ g of supercoiled plasmid DNA.

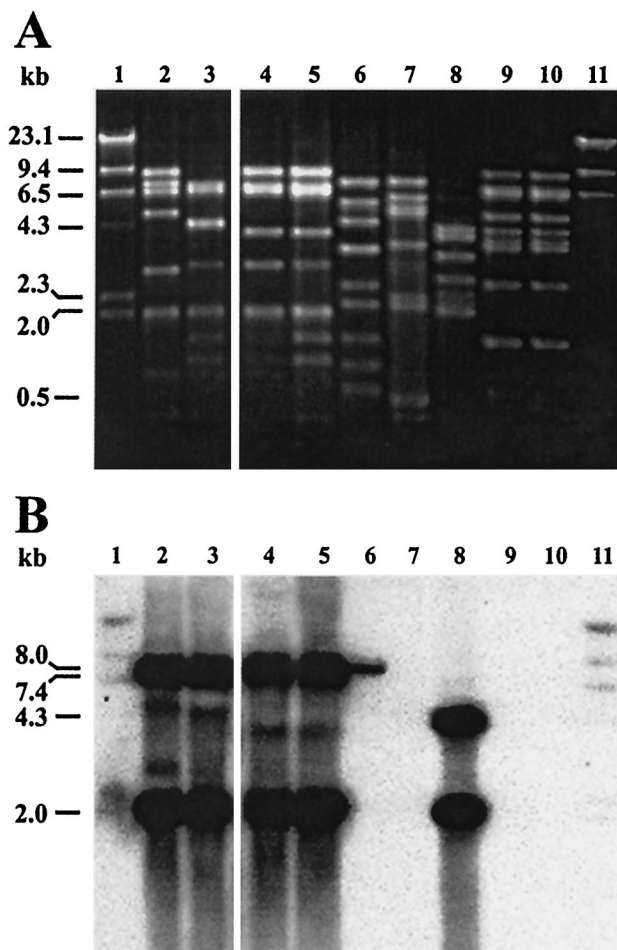


FIG. 1. (A) Restriction profiles of *Hind*III-restricted bacteriophage genomic DNAs. (B) Southern hybridization using an internal *hel3.1* probe. Lanes 1 and 11, DIG-labeled λ DNA molecular size marker; lanes 2, DT1; lanes 3, κ 3; lanes 4, κ 9; lanes 5, κ 10; lanes 6, κ 13; lanes 7, κ 2; lanes 8, κ 1; lanes 9, κ 6; lanes 10, κ 12.

The 0.6-kb *Bgl*III expression cassette from pTRK593 (43), containing the *Lactobacillus acidophilus* ATCC 4356 P6 promoter (11) and a mini-multiple cloning site with a downstream coliphage T7 transcriptional terminator, was cloned into the *Sau*3AI site of pTRK686. The resulting 3,018-bp plasmid, designated pTRK687, was used as a basal RNA expression vector.

Construction of a DNA helicase-based antisense RNA expression system. The 1.4-kb *hel3.1*-containing fragment amplified from *cos*-type bacteriophage κ 3 was digested with *Pst*I and cloned, in either orientation (i.e., sense or antisense) relative to the P6 promoter, into the *Pst*I site of pTRK687. The resulting sense (*hel3.1*-S) and antisense (*hel3.1*-AS) constructs, designated pTRK688::*hel3.1*-S and pTRK689::*hel3.1*-AS, respectively (Fig. 2), were electroporated into *S. thermophilus* NCK1124, NCK1125, and NCK1434 to determine their impact on bacteriophage infection during standard plaque assays. The plasmid pTRK688::*hel3.1*-S was included in this study as a sense RNA control to exclude the possibility that any observed drop in EOP or plaque size might be attributed to the increased metabolic burden associated with RNA expression from these high-copy-number expression vectors.

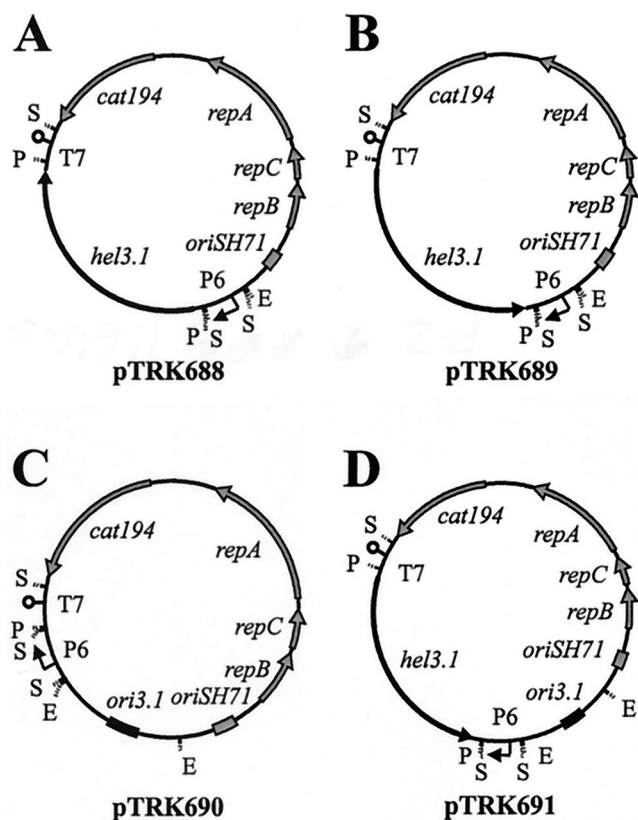


FIG. 2. (A) Sense RNA control plasmid pTRK688::*hel3.1-S*; (B) antisense RNA plasmid pTRK689::*hel3.1-AS*; (C) PER plasmid pTRK690::*ori3.1*; (D) explosively replicated antisense RNA expression plasmid pTRK691::*ori3.1*::*hel3.1-AS*. Abbreviations: T7, coliphage T7 transcription terminator; P6, *L. acidophilus* P6 promoter; *repBCA*, genes encoding plasmid replication factors; *cat194*, chloramphenicol resistance gene; *oriSH71*, plasmid origin of DNA replication; *ori3.1*, bacteriophage $\kappa 3$ -derived origin of DNA replication; *hel3.1*, bacteriophage $\kappa 3$ -derived putative helicase. Restriction endonuclease recognition sites: E, *EcoRI*; S, *Sau3AI*; P, *PstI*.

Effect of antisense *hel3.1* expression on EOP and plaque size. *S. thermophilus* NCK1125, NCK1434, and their derivatives harboring both sense and antisense constructs were challenged with *cos*- or *pac*-type bacteriophages during standard plaque assays (Table 3). Antisense *hel3.1* expression consistently caused a 40 to 70% reduction in EOP with a concomitant decrease in plaque size (relative to the parent strains) when challenged with bacteriophages that harbored strong bands of *hel3.1* hybridization (i.e., bacteriophages $\kappa 1$, $\kappa 3$, $\kappa 4$, $\kappa 5$ [data not shown], $\kappa 9$, and $\kappa 10$). Bacteriophages picked from plaques formed on NCK1125(pTRK689::*hel3.1-AS*) and NCK1434(pTRK689::*hel3.1-AS*) were seemingly unchanged and remained equally sensitive upon reinfection of antisense *hel3.1*-expressing hosts.

The expression of antisense *hel3.1* by NCK1125(pTRK689::*hel3.1-AS*) failed to affect bacteriophages that lacked fragments of *hel3.1* hybridization, (i.e., the *pac*-type bacteriophages $\kappa 6$ and $\kappa 12$) (Table 3). When expressed from NCK1124(pTRK689::*hel3.1-AS*), antisense *hel3.1* was also ineffective against bacteriophage $\kappa 13$, which weakly hybridized the *hel3.1*-internal probe (Table 3).

When *cos*-type bacteriophages $\kappa 3$, $\kappa 4$, $\kappa 5$ (data not shown), $\kappa 9$, and $\kappa 10$ were plaqued on vector control strains, including pTRK687 and pTRK688::*hel3.1-S*, they generally gave rise to slightly enlarged plaques and did not exhibit large reductions in EOP (Table 3). The plaque size of *pac*-type bacteriophages $\kappa 6$ and $\kappa 12$ was similarly affected by the presence of the control plasmids.

The bacteriophage sensitivity data correlated well with the data obtained from both the *hel3.1*-specific Southern hybridization and JMSp1- and JMSp2-derived PCR amplification experiments. Bacteriophages sensitive to the antisense technology hybridized the *hel3.1*-specific probe and gave rise to *hel*-containing amplicons during PCR. Conversely, bacteriophages that failed to generate *hel*-containing amplicons and either failed to hybridize or weakly hybridized the *hel3.1*-specific probe were insensitive to antisense *hel3.1* RNA.

Lysis-in-broth assays. The growth curves of *S. thermophilus* NCK1125 and the antisense *hel3.1* construct were evaluated in the presence and absence of bacteriophage $\kappa 3$ at various multiplicities of infection (MOI) (i.e., 0 [negative control], ≈ 5 , ≈ 1 , and ≈ 0.1) (Fig. 3). The control strains, NCK1125, NCK1125 (pTRK687) (data not shown), and NCK1125(pTRK688::*hel3.1-S*) (data not shown), were all lysed within 120 min at all experimental MOIs tested. Expression of antisense *hel3.1* from the plasmid pTRK689::*hel3.1-AS* conferred significant protection from $\kappa 3$ -mediated lysis. Bacteriophage $\kappa 3$ failed to lyse the antisense-expressing culture at initial MOIs of approximately 1 and 0.1, although the rate of growth and accumulated cell mass were slightly below those of the unchallenged parent strain at the higher MOI.

Strand-specific RT-PCR. Nonquantitative, strand-specific RT-PCR was performed to confirm that *hel3.1* antisense RNA was expressed in the appropriate strains. Primer JMSp4 was used during first-strand (RT) synthesis of cDNA, and primers JMSp4 and JMSp5 were used during the subsequent PCR amplification (Fig. 4). The expected 0.7-kb fragment was amplified only from total RNA isolated from NCK1125 (pTRK689::*hel3.1-AS*), indicating that (i) antisense *hel3.1* RNA was being expressed from pTRK689::*hel3.1-AS* and (ii) antisense *hel3.1* RNA was not expressed in strains that did not harbor the plasmid.

A second experiment was carried out to ensure the absence of residual plasmid DNA during the confirmation of antisense expression. The primer JMSp1, which is located 346 bp 5' of JMSp4, was substituted for JMSp4 during PCR amplification (while still using JMSp4 for first-strand cDNA synthesis). In this case, a 1.0-kb fragment was obtained only when higher concentrations of RNA were used for cDNA synthesis (i.e., concentrations of more than 1 μ g of total RNA per 20- μ l reaction mixture). These results demonstrated that P6 promoter-driven transcription from pTRK689::*hel3.1-AS* yields a population of multimeric RNA transcripts. In all cases, the PCR control reactions performed on DNase-treated, non-reverse-transcribed samples failed to generate an amplification product, indicating the absence of detectable levels of plasmid DNA in the total RNA preparations.

Construction of PER and explosive antisense RNA vectors. *EcoRI*-tagged primer JMSp6 was designed from the DT1-, ϕ O1205-, Sfi11-, Sfi19-, and Sfi21-derived consensus region upstream of the putative origin of DNA replication (*ori*) con-

TABLE 3. Effects of antisense *hel3.1* RNA and *ori*-conferred PER on various *S. thermophilus* bacteriophages

Bacteriophage isolate	Strain (construct)	EOP (mean \pm SD)	Δ PS ^a	Bacteriophage isolate	Strain (construct)	EOP (mean \pm SD)	Δ PS ^F
κ 1	NCK1434	1.0	—	κ 9	NCK1125	1.0	—
	NCK1434(pTRK687)	0.8 \pm 0.2	SE		NCK1125(pTRK687)	0.9 \pm 0.1	SE
	NCK1434(<i>hel3.1</i> -S)	0.8 \pm 0.2	SE		NCK1125(<i>hel3.1</i> -S)	1.0 \pm 0.1	SE
	NCK1434(<i>hel3.1</i> -AS)	0.3 \pm 0.2	PP		NCK1125(<i>hel3.1</i> -AS)	0.5 \pm 0.2	PP
	NCK1434(<i>ori3.1</i>)	NT ^b	NT		NCK1125(<i>ori3.1</i>)	<10 ⁻⁶	IP
	NCK1434(<i>ori3.1</i> :: <i>hel3.1</i> -AS)	NT	NT		NCK1125(<i>ori3.1</i> :: <i>hel3.1</i> -AS)	<10 ⁻⁸	IP
κ 3	NCK1125	1.0	—	κ 10	NCK1125	1.0	—
	NCK1125(pTRK687)	0.9 \pm 0.2	SE		NCK1125(pTRK687)	0.9 \pm 0.2	SE
	NCK1125(<i>hel3.1</i> -S)	0.9 \pm 0.2	SE		NCK1125(<i>hel3.1</i> -S)	1.1 \pm 0.2	SE
	NCK1125(<i>hel3.1</i> -AS)	0.5 \pm 0.1	PP		NCK1125(<i>hel3.1</i> -AS)	0.4 \pm 0.1	PP
	NCK1125(<i>ori3.1</i>)	<10 ⁻⁶	IP		NCK1125(<i>ori3.1</i>)	<10 ⁻⁶	IP
	NCK1125(<i>ori3.1</i> :: <i>hel3.1</i> -AS)	<10 ⁻⁸	IP		NCK1125(<i>ori3.1</i> :: <i>hel3.1</i> -AS)	<10 ⁻⁸	IP
κ 4	NCK1125	1.0	—	κ 12	NCK1125	1.0	—
	NCK1125(pTRK687)	1.1 \pm 0.1	SE		NCK1125(pTRK687)	0.8 \pm 0.1	SE
	NCK1125(<i>hel3.1</i> -S)	1.2 \pm 0.1	SE		NCK1125(<i>hel3.1</i> -S)	0.8 \pm 0.2	SE
	NCK1125(<i>hel3.1</i> -AS)	0.6 \pm 0.2	PP		NCK1125(<i>hel3.1</i> -AS)	0.9 \pm 0.2	SE
	NCK1125(<i>ori3.1</i>)	<10 ⁻⁶	IP		NCK1125(<i>ori3.1</i>)	0.8 \pm 0.2	SE
	NCK1125(<i>ori3.1</i> :: <i>hel3.1</i> -AS)	<10 ⁻⁷	IP		NCK1125(<i>ori3.1</i> :: <i>hel3.1</i> -AS)	0.8 \pm 0.2	SE
κ 6	NCK1125	1.0	—	κ 13	NCK1124	1.0	—
	NCK1125(pTRK687)	0.8 \pm 0.1	SE		NCK1124(pTRK687)	0.8 \pm 0.2	SE
	NCK1125(<i>hel3.1</i> -S)	0.9 \pm 0.1	SE		NCK1124(<i>hel3.1</i> -S)	0.8 \pm 0.2	SE
	NCK1125(<i>hel3.1</i> -AS)	0.8 \pm 0.1	SE		NCK1124(<i>hel3.1</i> -AS)	0.9 \pm 0.2	SE
	NCK1125(<i>ori3.1</i>)	0.9 \pm 0.2	SE		NCK1124(<i>ori3.1</i>)	NT	SE
	NCK1125(<i>ori3.1</i> :: <i>hel3.1</i> -AS)	1.0 \pm 0.1	SE		NCK1124(<i>ori3.1</i> :: <i>hel3.1</i> -AS)	NT	SE

^a Δ PS, change in plaque size. —, reference plaque size; SE, slightly enlarged; PP, pinpoint; IP, irregularly shaped pinpoint plaques.

^b NT, not tested.

sensus sequence, while *Eco*RI-tagged primer JMSp7 was designed exclusively from the DT1 genomic sequence. Regions downstream of the putative *ori* diverged among these bacteriophages, so preference was given to DT1 because it was derived from North American cheese fermentations. Primers JMSp6 and JMSp7 were used to amplify the putative *ori* from the *S. thermophilus* bacteriophages listed in Table 1, except that κ 1 was not tested. The expected 0.7-kb amplicon was generated only from bacteriophages DT1, κ 3, and κ 5. The 677-bp κ 3-derived fragment, designated *ori3.1*, was sequenced (GenBank accession number AF442521), digested with *Eco*RI, and cloned into the *Eco*RI site of pTRK687, which is located upstream of the P6 promoter. The resulting PER plasmid, designated pTRK690::*ori3.1* (Fig. 2), served as a base vector for the construction of an explosive antisense RNA expression system. The *hel3.1* fragment described above was subsequently cloned into the *Pst*I site of pTRK690::*ori3.1* to yield pTRK691::*ori3.1*::*hel3.1*-AS (Fig. 2). The plasmids pTRK690::*ori3.1* and pTRK691::*ori3.1*::*hel3.1*-AS were then electroporated into *S. thermophilus* NCK1125 to determine their impact on the infection of bacteriophages κ 3, κ 4, κ 6, κ 9, κ 10, and κ 12 during standard plaque assays (Table 3). The presence of the κ 3-derived origin alone on the PER plasmid pTRK690::*ori3.1* had a significant impact on the proliferation of bacteriophages κ 3, κ 4, κ 9, and κ 10 but did not affect the replication of κ 6 or κ 12. The pTRK690::*ori3.1* construct reduced the EOP of sensitive bacteriophages to less than 10⁻⁶ relative to that of the NCK1125 parental strain and gave rise to irregularly shaped pinpoint plaques. The addition of the antisense *hel3.1* cassette to the PER plasmid further impeded bacteriophage κ 3 replication significantly beyond the level with the PER parent plas-

mid alone (Table 3). NCK1125(pTRK691::*ori3.1*::*hel3.1*-AS) lowered the EOP of sensitive bacteriophages to less than 10⁻⁷ and 10⁻⁸ and gave rise to irregularly shaped pinpoint plaques, often with faint halos, that were difficult to enumerate. Bacteriophages picked from these plaques failed to propagate to detectable levels, even after multiple propagations on NCK1125. No antisense *hel3.1* RNA-resistant bacteriophages have been isolated to date.

DISCUSSION

In this study, comparative computational analyses of the genomes of six *S. thermophilus* bacteriophages were used to choose genetic targets suitable for the construction of antisense RNA and explosive RNA expression strategies. When the bacteriophage κ 3-derived putative helicase gene (*hel3.1*) was cloned in the antisense orientation behind the strong, *L. acidophilus* P6 promoter and expressed from a high-copy-number vector, *hel3.1* antisense RNA consistently mediated a 50% reduction in EOP and reduction in plaque size against bacteriophage κ 3. The proliferation of other *hel*-containing *S. thermophilus* bacteriophages, (i.e., κ 1, κ 4, κ 5 [data not shown], κ 9, and κ 10) was similarly impeded by the expression of *hel3.1* antisense RNA, causing a 40 to 70% reduction in EOP with a concomitant reduction in plaque size. Antisense *hel3.1* failed to affect the proliferation of bacteriophages κ 13 or κ 6 and κ 12, which either weakly hybridized or failed to hybridize a *hel3.1*-specific probe during Southern hybridization experiments, respectively.

The magnitude of bacteriophage inhibition via antisense RNA expression is similar to results reported previously for *L.*

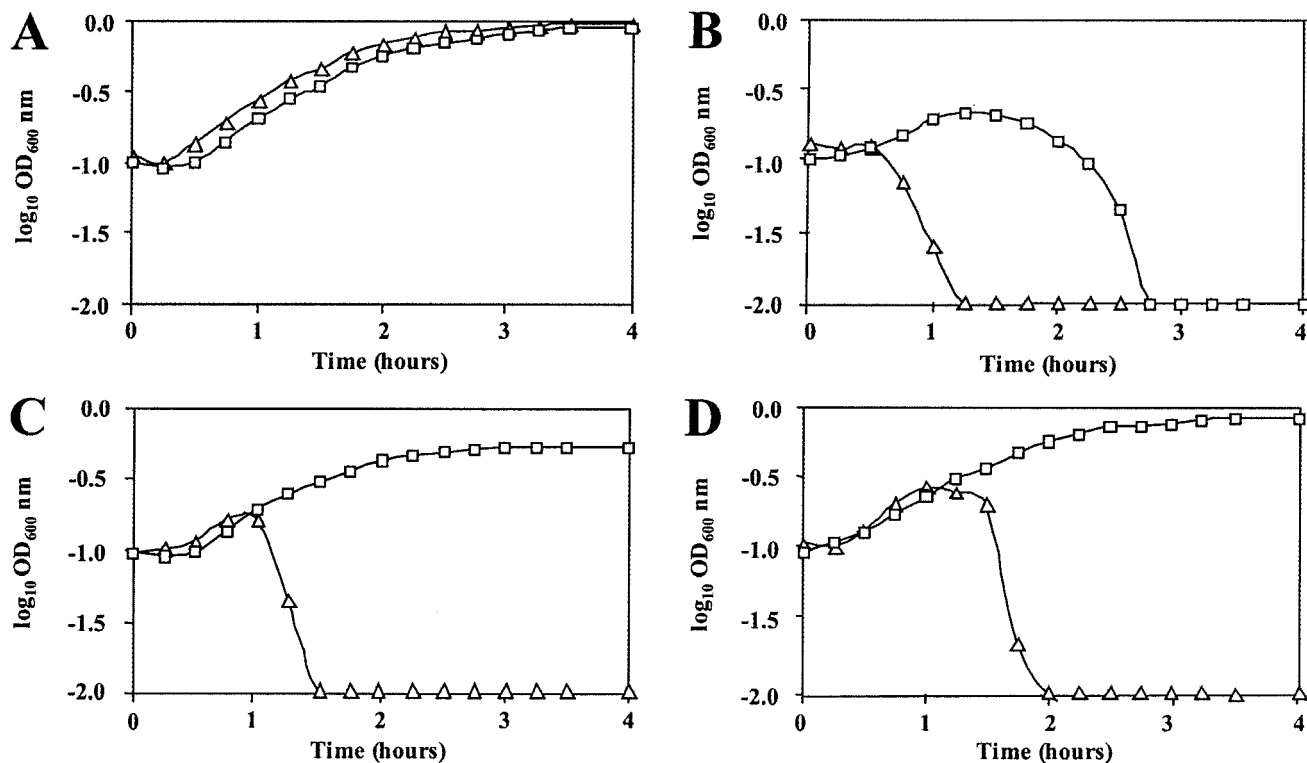


FIG. 3. Effect of bacteriophage $\kappa 3$ on the growth of NCK1125 (Δ) and NCK1125(pTRK688::*hel3.I-AS*) (\square) at various MOI. (A) Growth in the absence of bacteriophage $\kappa 3$ (i.e., MOI = 0). (B to D) Growth in the presence of bacteriophage $\kappa 3$ at MOIs of ≈ 5 (B), ≈ 1 (C), and ≈ 0.1 (D). OD₆₀₀, optical density at 600 nm.

lactis. Kim et al. (21) found that antisense expression of two polycistronic open reading frames, designated *gp18C* and *gp24C*, inhibited the P335-type bacteriophage $\phi 7-9$, as measured by a 55% reduction in EOP. Interestingly, the reduction in EOP dropped to 30% if the RBS and coding region for the first 15 amino-terminal residues of Gp18C were omitted from the antisense construct. In both cases, the plaque size was also reduced by approximately 10-fold. Chung et al. (7) obtained variable reductions in EOP, which ranged between 0.5 and 0.8, as they expressed different lengths of the $\phi F4-1$ major coat protein (*mcp*) gene. Kim and Batt (20) found that antisense expression of the full-length, bacteriophage $\phi 7-9$ -derived *gp15C* mediated a reduction in EOP of $\phi 7-9$ (and other *gp15C*-containing bacteriophages) to 10^{-2} . More recently, McGrath et al. (29) targeted DNA replication functions and found a 50% to a 10^{-6} -log-unit reduction in EOP, depending on the targeted gene and bacteriophage tested.

The results from these and other studies clearly indicate that certain genes are better targets than others for silencing by antisense RNA. Polzin et al. (K. M. Polzin, L. J. Collins, M. W. Lubbers, and A. W. Jarvis, Abstr. 5th Symp. Lactic Acid Bacteria, abstr. F2, 1996) found that the antisense expression of four early open reading frames (including *e5*, encoding a putative subunit of DNA polymerase; *e12*, encoding a putative transcription regulator; and *e15*, encoding a putative recombinase) and of four late open reading frames (including *l7*, encoding a major tail protein, and *l12*, encoding a putative terminase) was in all cases ineffective in inhibiting the replication of the *L. lactis* prolate-headed bacteriophage $c2$, regardless of

the gene dosage tested. In addition, Walker and Klaenhammer (43) found that two middle-expressed open reading frames, including *orf1* and *orf2*, and four late-expressed open reading frames, *orf3* through *orf6*, were also ineffective at inhibiting the *L. lactis* P335-type bacteriophage $\phi 31$ when expressed from the *L. acidophilus* P6 promoter on the high-copy-number vector pTRKH2. In order to increase the ratio of antisense RNA to sense RNA, those authors cloned the aforementioned antisense expression cassettes into pTRK360, a low-copy-number vector containing the bacteriophage $\phi 31$ putative origin of DNA replication (*ori31*). Following bacteriophage $\phi 31$ invasion, the expression of bacteriophage-derived DNA replication

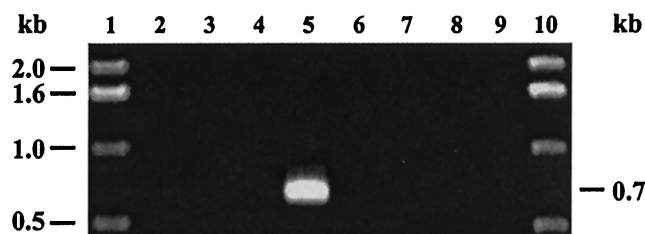


FIG. 4. DNase-treated total RNA preparations were subjected to RT-PCR (lanes 2 to 5) and control PCRs (without cDNA synthesis; lanes 6 to 9) in order to detect *hel3.I* antisense RNA expression in NCK1125 (lanes 2 and 6), NCK1125(pTRK687) (lanes 3 and 7), NCK1125(pTRK688::*hel3.I-S*) (lanes 4 and 8), and NCK1125(pTRK689::*hel3.I-AS*) (lanes 5 and 9). Lanes 1 and 10, 1-kb ladder (Gibco-BRL Life Technologies).

factors triggered the explosive replication of pTRK360 from *ori31* and produced inhibiting levels of antisense RNA during the later stages of the lytic cycle.

Mechanistically, antisense RNA hybridizes to the sense RNA strand and creates a translationally inactive double-stranded RNA (dsRNA) molecule (19). Formation of the dsRNA duplex molecule silences gene expression through the cooperative action of one or more intermolecular mechanisms. If the antisense RNA includes sequences complementary to the RBS, then the formation of dsRNA may mask the RBS, preventing efficient ribosome loading and reducing translation of the gene of interest. Formation of dsRNA downstream of the RBS may also interfere with translation by sterically impeding, to some degree, the procession of the mRNA through the ribosome. In addition, the formation of dsRNA may destabilize the sense mRNA by promoting the action of dsRNA-specific ribonucleases. Lastly, if the gene of interest is transcribed on a polycistronic mRNA, then antisense targeting may also negatively affect the expression of translationally coupled genes located downstream, causing pleiotropic effects that might further inhibit bacteriophage proliferation.

The variation in efficacy of antisense RNA-mediated gene silencing raises questions about the key characteristics of an ideal target gene or locus. Essentiality of the target RNA(s) for the replication, maturation, or release of progeny bacteriophages is perhaps the most obvious criterion. Unfortunately, essentiality must be derived empirically, cannot be garnered from the analysis of genomic data, and, often, should not be extrapolated from prior observations in heterologous systems. Analysis of a bacteriophage's transcriptome could provide additional insights into the choice of targets. In theory, optimal candidates will be genes that are transiently expressed, expressed at a very low level, and/or coded for by unstable, inefficiently translated mRNA species. In addition, the secondary structure(s) of potential mRNA species may also be examined and should be able to form structures that are conducive to recognition of the expressed antisense RNA molecule(s).

With regard to the practical efficacy of antisense cassettes in the dairy environment, identification of target genes that are effective against a variety of industrially relevant bacteriophages is of utmost importance. In this case, the use of nucleic acid hybridization in conjunction with the genomic analyses was employed to identify potential targets, although these processes cannot guarantee antisense RNA functionality. We found that the genes associated with the Sfi21-type DNA replication module are excellent candidates for targeting with antisense RNA. Five of the six model bacteriophages presently in the database (i.e., DT1, Sfi11, Sfi19, Sfi21, and ϕ O1205) encode the 2.0-kb *Hind*III fragment. According to the consensus sequence of the DT1-, ϕ O1205-, Sfi11-, Sfi19-, and Sfi21-derived replication modules, the conserved 2.2-kb *Hind*III hybridization signal (fragment B) of bacteriophage Sfi21, reported by Desiere et al. (8), actually corresponds to a conserved 2,027-bp *Hind*III restriction fragment. This 2.0-kb fragment is part of a larger, Sfi21-type DNA replication module that is highly conserved among *S. thermophilus* bacteriophages of industrial importance (8).

When using nucleotide sequence similarity as an indication of evolutionary descent, it was clear that the putative helicase genes of *S. thermophilus* bacteriophages can be divided into

two groups based on either (i) the fermented product (i.e., yogurt versus cheese) or (ii) geographic location (i.e., North America versus Europe). Phylogenetically, one group contained bacteriophages isolated from North American cheese plants (DT1 and κ 3), while the other included bacteriophages that were isolated from European yogurt plants (Sfi11, Sfi19, Sfi21, and ϕ O1205).

Multiplex PCR strategies have been used to identify 936, c2, and P335 species of lactococcal bacteriophages, the three principal species encountered in worldwide dairy fermentations (22). While this technology has not yet been applied to *S. thermophilus* bacteriophages, composite oligonucleotide primers (Table 2) derived from a consensus DNA replication module were initially used in this study to amplify the putative helicase genes from a heterogeneous collection of bacteriophages. In other systems, molecular beacons have been used successfully in PCR to provide real-time, direct detection of pathogenic *E. coli* O157:H7 in food products (30). The combination of these two technologies would result in a real-time multiplex PCR strategy that could detect the presence or absence of antisense-targeted bacteriophage genes or loci, such as the putative helicase gene, among a heterogeneous bacteriophage population. If such a technology was applied to fermentation substrates prior to the addition of starter cultures, it would allow for the rapid design of culture rotation strategies that allow operators to choose which antisense RNA-expressing strains to deploy in order to maximize the integrity and quality of the fermentation.

In conclusion, we have exploited the conservation of the Sfi21-type DNA replication module and constructed effective antisense RNA expression strategies that are effective against *S. thermophilus* bacteriophages. Both *cos*- and *pac*-type *S. thermophilus* bacteriophages that attack two different strains of *S. thermophilus* are sensitive to these technologies. The combination of a bacteriophage origin of replication with a high-expression antisense RNA cassette provided significant protection from specific bacteriophages at levels higher than for each mechanism alone. Work is continuing to target other components of the putative DNA replication module.

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