

Antisense RNA Targeting of Primase Interferes with Bacteriophage Replication in *Streptococcus thermophilus*

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The putative primase gene and other genes associated with the Sfi21-prototype genome replication module are highly conserved in *Streptococcus thermophilus* bacteriophages. Expression of antisense RNAs complementary to the putative primase gene (*pri3.1*) from *S. thermophilus* phage κ 3 provided significant protection from κ 3 and two other Sfi21-type phages. Expression of *pri3.10-AS*, an antisense RNA that covered the entire primase gene, reduced the efficiency of plaquing (EOP) of κ 3 to 3×10^{-3} and reduced its burst size by 20%. Mutant phages capable of overcoming antisense inhibition were not recovered. Thirteen primase-specific antisense cassettes of different lengths (478 to 1,512 bp) were systematically designed to target various regions of the gene. Each cassette conferred some effect, reducing the EOP to between 0.8 and 3×10^{-3} . The largest antisense RNAs (1.5 kb) were generally found to confer the greatest reductions in EOP, but shorter (0.5 kb) antisense RNAs were also effective, especially when directed to the 5' region of the gene. The impacts of primase-targeted antisense RNAs on phage development were examined. The expression of *pri3.10-AS* resulted in reductions in target RNA abundance and the number of phage genomes synthesized. Targeting a key genome replication function with antisense RNA provided effective phage protection in *S. thermophilus*.

Strains of lactic acid bacteria are used in starter cultures or culture adjuncts during the manufacture of a variety of fermented dairy products. Phage contamination during product manufacture can result in significant loss of starter culture activity and remains the leading cause of failed batch fermentations. These losses are particularly severe when highly specialized strains, which are themselves a valuable product of scientific discovery and product development, become susceptible to phage attack. In this case, costs committed for strain development will not be recovered if the expected lifetime of a new, highly specialized strain is diminished by the appearance of lytic phages capable of attacking it. The crux of the problem is that the dairy environment and fermentation substrate provide a continuous reservoir for the influx of new virulent phages (7, 25), while existing phages adapt by mutation and recombination (4, 13). Together, these events enable the appearance of subpopulations of phages capable of subverting previously resistant cultures and necessitate the development of strains of lactic acid bacteria with enhanced phage resistance properties.

Novel and more efficacious phage defense strategies continue to be developed, including the expression of antisense RNA targeted against phage-encoded transcripts. These antisense RNAs have been constitutively expressed by starter strains (reviewed in references 2, 24, 32, and 34) or triggered in response to phage infection through the use of phage-encoded promoters and/or origins of replication (32, 34). Regardless of the delivery strategy employed, antisense RNAs act to interfere with phage development by promoting the degradation of

mRNA transcripts or inhibiting the translation of phage-encoded genes necessary for normal development, albeit at markedly variable and often poor efficiencies (18).

Currently, 6 *Streptococcus thermophilus*-, 11 *Lactococcus*-, and 3 *Lactobacillus*-specific phage genomes have been sequenced completely and subjected to extensive comparative genomic analyses (5, 10), especially between *S. thermophilus* phages (23). The developmental pathways encoded by these genomes reveal susceptibilities to engineered phage defense systems, including antisense RNA. When used in conjunction with comparative hybridization studies, these analyses enable the elimination of poorly conserved targets in silico, while facilitating the identification of well-conserved targets present in a wide variety of phages (32). This advantage is of great importance for industrial applications, where defense strategies ideally confer resistance against broad groups of related phages.

Among *S. thermophilus* phages, genome replication functions are catalyzed by two distinct but likely interchangeable clusters of nonorthologous genes, which are exemplified by the phage Sfi21- and 7201-derived prototype modules (23). For several reasons, the genes associated with the Sfi21-type genome replication module were found to be among the best-conserved targets for the expression of phage-inhibitory antisense RNAs (32). First, gross comparisons revealed that six of the seven sequenced phages encoded variants of the Sfi21-type module, while only the remaining phage, 7201, did not. Second, hybridization studies against unsequenced phages have demonstrated that variants of the Sfi21-type module are found in the majority of problematic industrial isolates, suggesting that this module may confer a competitive advantage over phages encoding the 7201-type module (6, 32). Finally, fine-scale comparisons between Sfi21-type module-containing phages revealed that nucleotide sequence similarity dropped

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TABLE 1. Bacterial strains, bacteriophages and plasmids

Strain, bacteriophage, or plasmid	Relevant characteristic(s) ^a	Source or reference
<i>Streptococcus thermophilus</i>		
NCK1125	Industrial isolate; sensitive to phages κ3, κ5, κ6, κ9; Cm ^s	32
SMQ495	Industrial isolate; sensitive to phage DT1; Cm ^s	33
<i>Escherichia coli</i> MC1061	Transformation host	17
<i>Lactococcus lactis</i> NCK203	Sensitive to phage φ31	30
Bacteriophage		
κ3	Encodes Sfi21-type replication module; <i>cos</i> -type encapsidation module	32
κ5	Encodes Sfi21-type replication module; <i>cos</i> -type encapsidation module	32
κ6	Encodes Sfi21-type replication module; <i>pac</i> -type encapsidation module	32
κ9	Encodes Sfi21-type replication module; <i>cos</i> -type encapsidation module	32
DT1	Encodes Sfi21-type replication module; <i>cos</i> -type encapsidation module	33
φ31	P335-type lactococcal phage	1
Plasmid		
pTRK686	2.4 kb; deletion derivative of pNZ123; Cm ^r	32
pTRK687	3.0 kb; pTRK686 containing the P6 promoter in α-orientation	32
pTRK696	3.0 kb; pTRK686 containing the P6 promoter in β-orientation	This study
pTRK787:: <i>pri3.4</i> -AS	3.6 kb; pTRK687 containing the 587-bp <i>pri3.4</i> antisense cassette	This study
pTRK788:: <i>pri3.7</i> -AS	4.0 kb; pTRK687 containing the 1,023-bp <i>pri3.7</i> antisense cassette	This study
pTRK789:: <i>pri3.8</i> -AS	3.5 kb; pTRK687 containing the 519-bp <i>pri3.8</i> antisense cassette	This study
pTRK790:: <i>pri3.10</i> -AS	4.5 kb; pTRK687 containing the 1,512-bp <i>pri3.10</i> antisense cassette	This study
pTRK791:: <i>pri3.11</i> -AS	4.0 kb; pTRK687 containing the 1,008-bp <i>pri3.11</i> antisense cassette	This study
pTRK792:: <i>pri3.12</i> -AS	3.5 kb; pTRK687 containing the 504-bp <i>pri3.12</i> antisense cassette	This study
pTRK793:: <i>pri3.13</i> -AS	4.5 kb; pTRK687 containing the 1,506-bp <i>pri3.13</i> antisense cassette	This study
pTRK794:: <i>pri3.14</i> -AS	4.5 kb; pTRK687 containing the 1,486-bp <i>pri3.14</i> antisense cassette	This study
pTRK795:: <i>pri3.15</i> -AS	4.0 kb; pTRK687 containing the 982-bp <i>pri3.15</i> antisense cassette	This study
pTRK796:: <i>pri3.16</i> -AS	3.5 kb; pTRK687 containing the 487-bp <i>pri3.16</i> antisense cassette	This study
pTRK797:: <i>pri3.18</i> -AS	4.0 kb; pTRK687 containing the 1,008-bp <i>pri3.18</i> antisense cassette	This study
pTRK798:: <i>pri3.19</i> -AS	3.5 kb; pTRK687 containing the 504-bp <i>pri3.19</i> antisense cassette	This study
pTRK799:: <i>pri3.21</i> -AS	3.5 kb; pTRK687 containing the 504-bp <i>pri3.21</i> antisense cassette	This study
pTRK800:: <i>pri3.12</i> -AS	3.5 kb; pTRK696 containing the 504-bp <i>pri3.12</i> antisense cassette	This study

^a Cm^r, encodes chloramphenicol resistance; Cm^s, sensitive to chloramphenicol.

off sharply outside the module's boundaries, while the replication modules themselves shared striking sequence conservation, exhibiting greater than 99.9% sequence similarity between variants (5). The Sfi21-type replication module is comprised of a single origin of DNA replication (*ori*) and several open reading frames that encode a putative primase, a putative helicase, and a number of other proteins of undetermined function (5).

In this study, the putative primase gene, which is a component of the Sfi21-type genome replication module encoded by phage κ3 (*pri3.1*), was targeted for antisense RNA-mediated gene silencing in *S. thermophilus*. Regions responsible for antisense efficacy were determined through the characterization of a variety of constructs engineered to constitutively express antisense RNA complementary to various structural or putative regulatory regions of *pri3.1*. The effects of primase-targeted antisense RNA on phage development were also examined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Unless otherwise indicated, bacteria were propagated as described previously (32).

Bacteriophages and propagation assays. The phages used in this study are listed in Table 1. Bacteriophages were propagated as described previously (32). The efficiency of plaquing (EOP) was calculated by dividing the plaque-forming

units per milliliter (PFU/ml) on the test strain by the PFU/ml on the control strain, *S. thermophilus* NCK1125, harboring the base vector used for antisense RNA constructs. The diameters of phage plaques were measured by using a caliper, and values represent the averages of the measurements of 60 random plaques chosen over three independent experiments. Center-of-infection assays were performed as described previously, and the efficiency of center of infection formation (ECOI) was calculated by dividing the number of infective centers on the test strain by the number of infective centers on NCK1125(pTRK687) (31). Adsorption assays were performed as described previously, except that Elliker-BC medium was used (29). Lysis-in-broth assays were performed in Elliker-BC medium in the presence or absence of individual phage isolates at a multiplicity of infection (MOI) of 0.1% ± 20%, as described previously (32). Burst size determinations were performed at 42°C in Elliker-BC medium but otherwise were performed as described previously (21). *Lactococcus lactis* phages were propagated and enumerated as described previously (13).

PCR and DNA sequencing. PCR and DNA sequencing and sequence analyses were all performed as described previously (32). When appropriate, restriction endonuclease recognition sites were incorporated into the 5' ends of oligonucleotide primers to facilitate the cloning of PCR products. The primers used in this study are listed in Table 2.

Plasmid construction and bacterial transformation. The plasmids used in this study are listed in Table 1. Unless otherwise indicated, antisense RNA expression vectors were constructed from pTRK687 (Fig. 1). The orientation of cloned PCR products was confirmed by restriction digestion and PCR amplification by using the primer P6 with either of the two primers used to amplify the PCR fragment (Table 2). Electrocompetent *Escherichia coli* (28), *L. lactis* (16), and *S. thermophilus* were prepared and electroporated as described previously (32).

Plasmid, phage, and genomic DNA preparations. Plasmid DNAs were isolated from *E. coli* (28) and *S. thermophilus* (27) as described elsewhere. Phage genomic DNA was isolated from cells infected with phage κ3 at a MOI of 1% ± 20% or

TABLE 2. Primers used in this study

Primer ^a	Nucleic acid sequence ^b	Position ^c
S1	5'-AA <u>ACTGCAG</u> CAGAGAACAATTGCAAGC-3'	28,575
S2	5'-AA <u>ACTGCAG</u> CAACACCCAAGGCC-3'	28,466
S3	5'-AA <u>ACTGCAG</u> TAAAGGAGGATTGGACTTGAC-3'	28,534
S4	5'-AA <u>ACTGCAG</u> TTGACAACGATTGATTTTCG-3'	28,549
S5	5'-AA <u>ACTGCAG</u> ATTAATTTTAGTACCATTG-3'	29,053
S6	5'-AA <u>ACTGCAG</u> GGTACATATCGACGTATCG-3'	29,557
S7	5'-AA <u>ACTGCAG</u> TAGCTATATATGATCCAG-3'	28,787
S8	5'-CCCAAGAGCCTTTGGGCAATAAGG-3'	28,471
A1	5'-AA <u>ACTGCAG</u> GTTCGAATAACCTGCCG-3'	30,080
A2	5'-AA <u>ACTGCAG</u> GTGAGTAACCATAACCAC-3'	30,060
A3	5'-AA <u>ACTGCAG</u> AAACTTATGGTCAAACGATAG-3'	29,052
A4	5'-AA <u>ACTGCAG</u> TTTGACTTATTCTTAAACAC-3'	29,556
A5	5'-AA <u>ACTGCAG</u> CTTCCCATTTCGAGGG-3'	28,786
A6	5'-CTAAGTAACTAAAGCAACCGAACCC-3'	30,135
P6	5'-GGAGCGTGATTTTATGG-3'	— ^d
T7	5'-GCTTCCGGCTCGTATGTTGTGTGG-3'	—

^a S and A primers are derived from the sense and antisense strands, respectively.

^b *Pst*I (5'-CTGCAG-3') restriction sites are underlined when appropriate.

^c The 5' nucleotide position relative to the DT1 sequence is shown in bold when appropriate.

^d —, not applicable.

from uninfected control cultures as described previously by using the Lambda kit (QIAGEN) (14). DNAs were purified after PCR and extracted from agarose gels as described previously (32).

RNA isolation and RNA-RNA slot blot hybridizations. Using the Lign'Scribe promoter addition kit (Ambion, Austin, Tex.), DNA adapters containing coliphage T7 promoters were ligated to a 504-bp *pri3.12* PCR fragment amplified from the 5' region of the putative primase gene of phage κ 3 by using primers S4 and A3 (Fig. 2; Table 2). Double-stranded DNA templates used during downstream

in vitro transcription reactions were generated by PCR by using the primer T7, which was specific to the T7 promoter adapter, and either primer S4 or A3. The T7-S4 and T7-A3 templates were used to generate probes *detect-S* and *detect-AS*, which detected the phage-encoded sense mRNA and plasmid-encoded antisense RNA, respectively. In vitro transcription was carried out in the presence of [α -³²P]UTP (NEN, Boston, Mass.) by using the high-yield MEGAscript transcription kit (Ambion). Radiolabeled RNA probes were purified by using Nuc-Trap probe purification columns (Stratagene, La Jolla, Calif.). Total RNAs were isolated (i) from cultures 10, 20, 30, and 60 min after infection with phage κ 3 at a MOI of 1% \pm 20% and (ii) from uninfected control cultures grown in parallel. RNAs were isolated by using the TRIZOL reagent (GIBCO-BRL) as described previously (11). RNA-RNA slot hybridizations were performed by using the Bio-Dot-SF apparatus and Zeta-probe membranes (Bio-Rad Laboratories, Richmond, Calif.) according to the manufacturer's instructions.

Nucleotide sequence accession numbers. The DNA and deduced protein sequences for phage DT1 (33) and the κ 3-derived putative primase have been submitted to the GenBank database under the nucleotide accession numbers AF085222 and AY196178, respectively.

RESULTS

Base RNA expression vectors. When present in *S. thermophilus*, the base vector pTRK687 resulted in an increased plaque size but did not impact the EOP of any of the phages tested, as observed previously (32) (Fig. 3A). In contrast, the presence of pTRK696 (Fig. 1) resulted in a 20% reduction in EOP without altering plaque size. Due to the artificial reduction in phage EOP associated with pTRK696 replication, plasmid pTRK687 was used as the basal antisense RNA expression vector in this study unless indicated otherwise. As a result, all EOP and plaque size comparisons were made relative to the strain NCK1125(pTRK687).

Amplification and sequence characteristics of the primase gene. Using a consensus sequence generated from the alignment of the Sfi21-type genome replication modules from six *S. thermophilus* phages (32), primers S8 and A6 were used to amplify primase-containing fragments from the *S. thermophilus* phages listed in Table 1. The expected 1.7-kb fragment, which spanned the entire consensus primase open reading frame, was successfully amplified from the positive control, phage DT1, and other Sfi21-type phages, including κ 3, κ 4, and κ 9 (data not shown). The 1.7-kb fragment was not generated from the negative control, phage κ 6, which encodes a variant of the 7201-type genome replication module.

The 1.7-kb PCR fragment amplified from phage κ 3 was sequenced and revealed a single open reading frame of 1,515 bp (GenBank accession number AY196178). This open reading frame, designated *pri3.1*, begins with a 5'-TTG-3' translation initiation codon, ends with a 5'-TAA-3' stop codon, and is preceded by a putative ribosome binding site (RBS) (5'-AGG AGG-3'). The deduced Pri3.1 protein is 504 amino acids long and has a predicted molecular mass of 59.0 kDa. A BlastP (3) search for conserved domains detected a conserved Parvo_NS1 domain (pfam01057) within the Pri3.1 primary amino acid sequence. Both DNA helicase and ATPase activities are associated with this domain, which is required for genome replication in double-stranded DNA parvoviruses (26, 35).

Primase-targeted antisense RNA expression. The 1.5-kb fragment designated *pri3.10* (Fig. 2) was amplified by PCR from phage κ 3 by using primers S4 and A1, digested with *Pst*I, and cloned in the antisense (-AS) orientation into the *Pst*I site of pTRK687, which is downstream from the *Lactobacillus aci-*

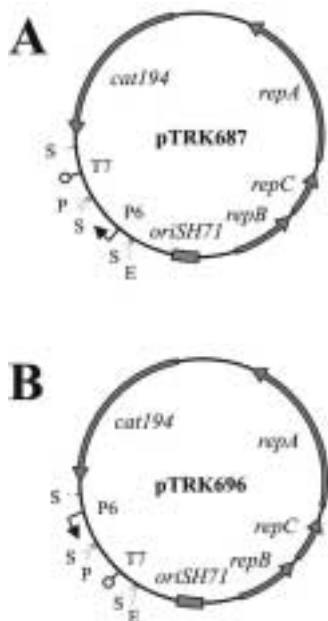


FIG. 1. The antisense RNA expression vectors used in this study that contain the P6 promoter cassette cloned in opposite orientations relative to the *cat194* gene. (A) pTRK687; (B) pTRK696. Abbreviations: T7, coliphage T7 transcription terminator; P6, *L. acidophilus* P6 promoter; *repBCA*, genes encoding plasmid replication factors; *cat194*, chloramphenicol resistance gene; *oriSH71*, plasmid origin of DNA replication; *pri3.1*, phage κ 3-derived putative primase. Restriction endonuclease recognition sites: E, *Eco*RI; S, *Sau*3AI; P, *Pst*I.

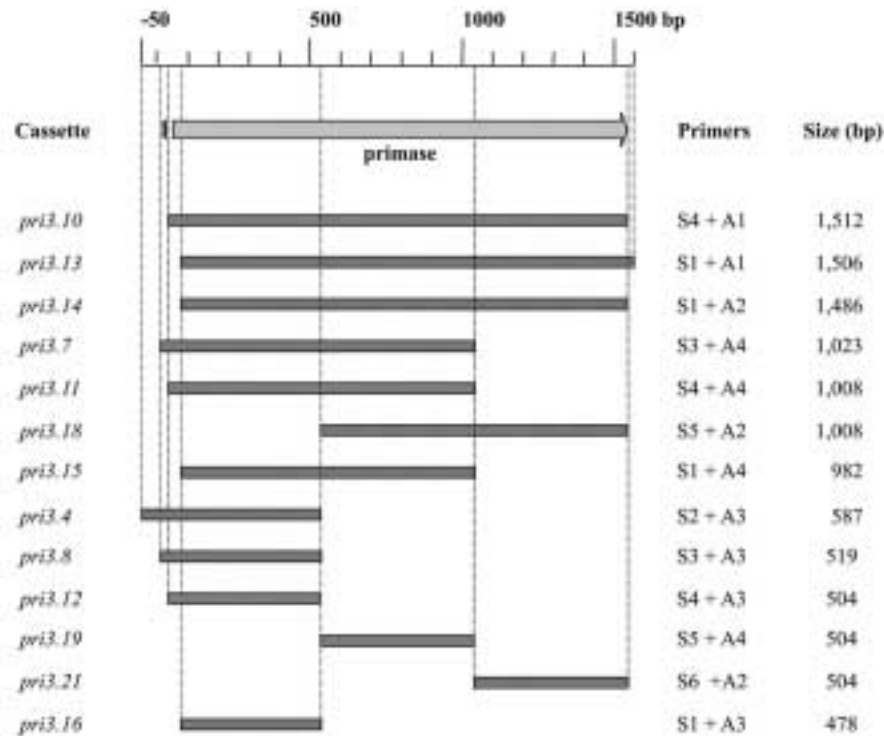


FIG. 2. Schematic of the *pri3.1* gene (arrow) and upstream putative ribosome binding site (small vertical black bar). The boundaries of the subcloned fragments are shown as bars demarcated with dotted lines. Base pair (bp) coordinates are numbered relative to the 5' position of the primase translation initiation codon.

dophilus P6 promoter (12). The resulting vector, pTRK790::*pri3.10-AS*, was electroporated into NCK1125 and tested for its ability to impede phage replication. The growth of the *pri3.10-AS*-expressing strain and the parental strain, NCK1125 (pTRK687), was evaluated over time in the presence and absence of Sfi21-type phages $\kappa 3$, $\kappa 4$, and $\kappa 9$ and 7201-type phage $\kappa 6$ at a MOI of $0.1\% \pm 20\%$ (Fig. 4). The control strain NCK1125(pTRK687) was lysed within 120 min by all phages tested. The *pri3.10-AS*-expressing strain was similarly lysed by 7201-type phage $\kappa 6$ but grew nearly unimpeded when challenged with Sfi21-type phages $\kappa 3$, $\kappa 4$, and $\kappa 9$.

Since the antisense RNA cassette was generated from phage $\kappa 3$, the effects of *pri3.10-AS* expression on phage $\kappa 3$ development were further characterized. The expression of *pri3.10-AS* resulted in a 2.7-log cycle reduction in the EOP and reduced the average plaque size by 85%. Phages recovered from plaques formed on the antisense-expressing hosts remained sensitive to *pri3.10-AS* inhibition. No mutant phages insensitive to antisense inhibition were recovered. The expression of *pri3.10-AS* also lowered the ECOI to 0.5 and reduced the burst size per infective center by 20%. No significant difference in phage adsorption was observed between the strain expressing *pri3.10-AS* and the parent strain, NCK1125(pTRK687) (data not shown).

Identification of regions critical for antisense RNA efficacy. The importance of antisense RNA length and the region targeted were then examined. Twelve DNA cassettes ranging from 478 to 1,506 bp in length were amplified from the phage $\kappa 3$ -derived putative primase gene (Fig. 2). These cassettes

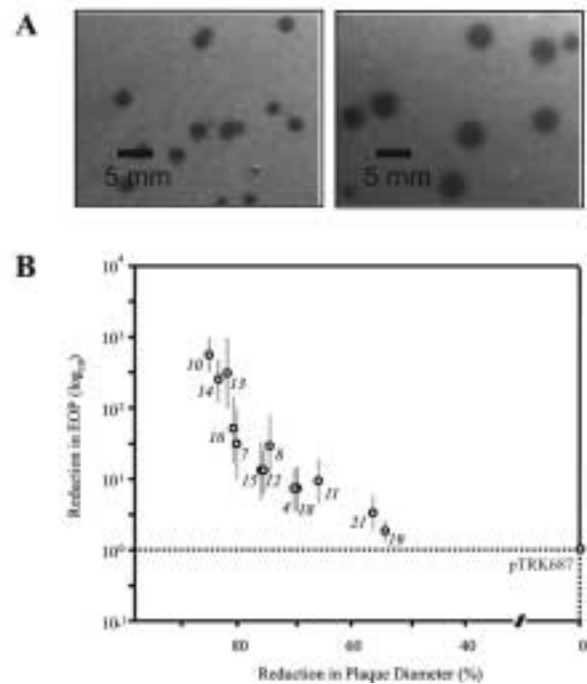


FIG. 3. (A) Plaque size of phage $\kappa 3$ when titered on NCK1125 (left) and NCK1125(pTRK687) (right). (B) Comparison of primase-derived antisense RNA constructs on the EOP and plaque size of phage $\kappa 3$ relative to that of the plasmid control, pTRK687. Horizontal and vertical dotted lines represent an EOP of 1.0 and a plaque size of 4 mm, respectively. The error bars for EOP were calculated from the results of three independent experiments.

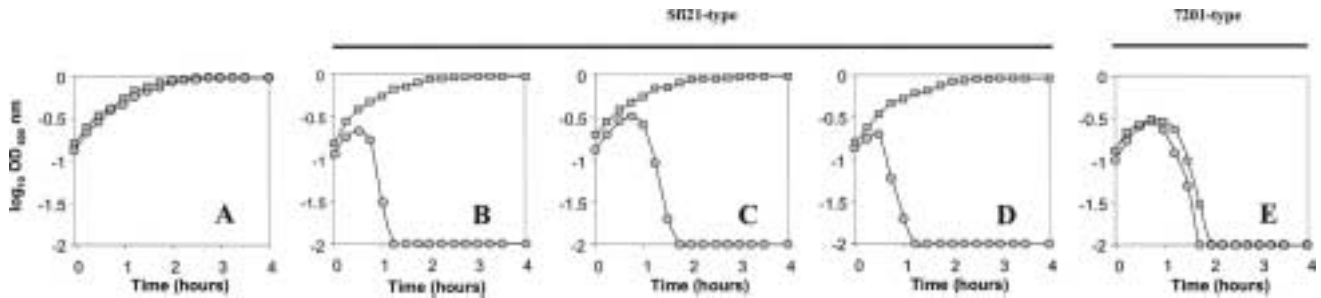


FIG. 4. The effect of various phages on the growth of NCK1125(pTRK687) (●) and NCK1125 (pTRK790::*pri3.10-AS*) (■). (A) Growth in the absence of phage. Growth in the presence of phage $\kappa 3$ (B); $\kappa 5$ (C); $\kappa 9$ (D); and $\kappa 6$ (E). The multiplicities of infection were $0.1\% \pm 20\%$ for all phages.

were cloned in an antisense orientation into the *Pst*I site of pTRK687. The antisense constructs were then electroporated into NCK1125, and the resulting transformants were evaluated for their sensitivity to the Sfi21-type phage $\kappa 3$. The effects of these constructs on both the EOP and average plaque size of phage $\kappa 3$ are illustrated in Fig. 3B. In most cases, a strong correlation between plaque size and EOP was observed. The largest antisense RNAs (e.g., *pri3.10-AS*, *pri3.13-AS*, and *pri3.14-AS*) were generally found to confer the most significant and consistent reductions in EOP; however, correlations between fragment size and the magnitude of EOP reduction were not observed as the length of the antisense fragments was systematically reduced (e.g., compare *pri3.10-AS*, *pri3.11-AS*, and *pri3.12-AS*). Reductions in EOP mediated by *pri3.12-AS*, *pri3.19-AS*, and *pri3.21-AS*, three 504-bp antisense cassettes designed to target different, nonoverlapping regions of the gene, exhibited significant differences in their ability to reduce

the EOP. In general, antisense constructs that contained sequences complementary to the putative RBS, which included *pri3.4-AS* (587 bp), *pri3.7-AS* (1,023 bp), and *pri3.8-AS* (519 bp), reduced the EOP below the level of similarly sized constructs that lacked sequences complementary to the RBS; however, this was not always the case (e.g., compare *pri3.7-AS* and *pri3.15-AS*).

None of the antisense RNA cassettes were found to negatively impact the replication of *S. thermophilus* phage $\kappa 6$, which encodes a variant of the heterologous 7201-type genome replication module. The lactococcal P335-type phage $\phi 31$ encodes a primase orthologue (AF208055) that exhibits short patches of local nucleotide sequence similarity to the phage $\kappa 3$ -encoded *pri3.1* (13). Given this finding, the *pri3.10-AS* construct was also transformed into *L. lactis* NCK203 and tested for its ability to impede the replication of phage $\phi 31$. This antisense construct, however, had no effect on $\phi 31$.

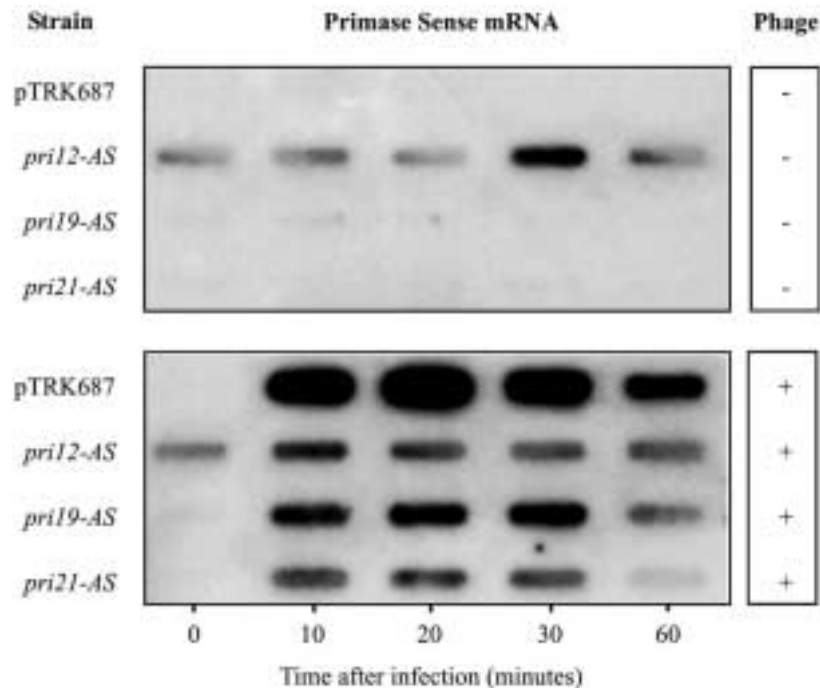


FIG. 5. RNA-RNA slot blot hybridization of three antisense RNA-expressing constructs and the vector control strain NCK1125(pTRK687) with *detect-S*, a *pri3.12*-derived single-stranded RNA probe that was complementary to the sense strand of the phage $\kappa 3$ -encoded primase mRNA (Fig. 2). RNA was isolated from cells in the absence (top panel) or presence (bottom panel) of phage $\kappa 3$.

Monitoring primase sense mRNA expression during phage infection. The impact of antisense RNA expression on transcript abundance over the course of a phage $\kappa 3$ lytic infection was monitored by RNA-RNA slot blot hybridization (Fig. 5). The single-stranded RNA probes *detect-S* and *detect-AS* were used to detect the phage $\kappa 3$ -encoded primase sense (-S) mRNA and the plasmid-encoded antisense RNA (-AS), respectively. Both probes were specific for the region comprised by the *pri3.12* PCR fragment (Fig. 2). In the absence of phage $\kappa 3$ infection, no primase mRNA was detected from NCK1125(pTRK687) or from strains expressing *pri3.19-AS* or *pri3.21-AS*. A weak primase mRNA-specific hybridization signal was observed only in the *pri3.12-AS*-expressing strain, indicating that a basal level of transcription occurred through the coliphage T7 transcriptional terminator (Fig. 1).

It was postulated that expression of the *pri3.12-S* transcript might have led to decreased levels of *pri3.12-AS* by means of the antisense effect. In order to address this, the *pri3.12* cassette was cloned in the antisense orientation into pTRK696, a pTRK686 derivative containing the P6 promoter cassette cloned in the opposite orientation relative to the *cat194* gene found in pTRK687 (Fig. 1). The resultant construct, designated pTRK800::*pri3.12-AS*, was electroporated into NCK1125 and tested for its ability to inhibit phage replication. The strain harboring pTRK800::*pri3.12-AS* failed to reduce the EOP of phage $\kappa 3$ beyond the level conferred by pTRK792::*pri3.12-AS*. These results indicated that the basal level of *pri3.12-S* expressed from pTRK792::*pri3.12-AS* did not result in a significant reduction in the efficacy of the expressed *pri3.12-AS* antisense RNA.

After infection with phage $\kappa 3$ and throughout the lytic cycle, a robust primase mRNA-specific signal was observed from NCK1125(pTRK687) (Fig. 5). The signal intensity weakened 60 min postinfection, correlating with culture lysis. When strains expressing *pri3.12-AS*, *pri3.19-AS*, and *pri3.21-AS* were infected with phage $\kappa 3$, the observed signal intensities from each strain were significantly weaker at every time point tested, relative to that of the NCK1125(pTRK687) control. Of these, the phage-infected strain expressing *pri3.19-AS* yielded the strongest sense mRNA signal, while those of infected strains expressing *pri3.12-AS* and *pri3.21-AS* were weaker. Notably, *pri3.12-AS*, which was most effective in reducing the EOP of phage $\kappa 3$ (Fig. 3B), appeared to have the greatest impact on lowering sense mRNA, especially if the background level of vector-derived *pri3.12-S* sense mRNA is considered.

Monitoring primase antisense RNA. Total RNAs from NCK1125(pTRK687) and the strain expressing *pri3.12-AS* were probed with *detect-AS*. As expected, primase-specific antisense RNAs were detected in the strain expressing *pri3.12-AS*. When NCK1125(pTRK792::*pri3.12-AS*) was infected with phage $\kappa 3$, the intensity of the primase antisense RNA-specific signal was significantly weaker at every time point tested relative to that of the uninfected control culture, which suggests its probable interaction with the sense mRNA expressed by the infecting phage. Primase-specific antisense RNAs were not detected in the presence or absence of phage $\kappa 3$ in NCK1125(pTRK687).

Interference with intracellular bacteriophage DNA replication. NCK1125(pTRK687) and strains expressing *pri3.8-AS* and *pri3.10-AS* were infected with phage $\kappa 3$ at a MOI of 1% \pm

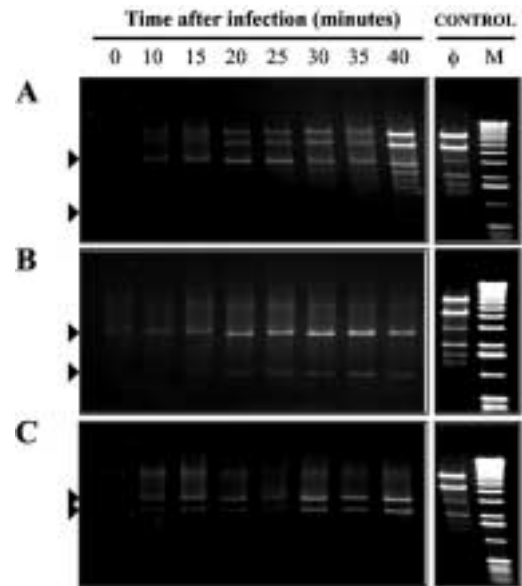


FIG. 6. The impact of antisense RNA expression on the in vivo accumulation of *Hind*III-digested phage $\kappa 3$ -specific DNA fragments over time. (A) Vector control strain NCK1125(pTRK687); (B) NCK1125(pTRK789::*pri3.8-AS*); (C) NCK1125(pTRK790::*pri3.10-AS*). Each plasmid encoded two *Hind*III sites; plasmid-specific DNA bands are demarcated with black triangles. Abbreviations: ϕ , purified phage $\kappa 3$ genomic DNA; M, 1-kb molecular size marker (GIBCO-BRL).

20%. Total genomic DNAs were isolated from infected cells over the course of the lytic cycle and digested with *Hind*III. The *Hind*III-digested DNA fragments were then subjected to agarose gel electrophoresis in order to determine if antisense RNA expression retarded the accumulation of phage-specific DNA bands over time (Fig. 6). Relative to that for NCK1125(pTRK687), the accumulation of phage-specific DNA fragments over time was greatly diminished in both antisense RNA-expressing strains.

DISCUSSION

Comparative genomics was successfully used to identify conserved and early-expressed genes in *S. thermophilus* phages that could be targeted by antisense RNA-based phage defense strategies. Antisense RNAs directed against the conserved putative primase gene, which is a component of the Sfi21-type genome replication module, retarded phage genome replication, significantly reduced the EOP, and severely limited the number of progeny phages released from an infected cell. The expression of 13 different antisense RNA constructs targeting specific regions of the *S. thermophilus* phage $\kappa 3$ -encoded putative primase gene was also evaluated in an attempt to approximate key regions that are more or less sensitive to antisense targeting. The expression of antisense RNA that covered the entire gene (*pri3.10-AS*) was the most effective of the constructs, resulting in an 85% reduction in plaque size, a 2.7-log cycle reduction in EOP, and a 50% reduction in the ECOI formation. Thus, only one of every two phage-infected cells released progeny phage, while those that released progeny phages exhibited a 20% reduction in the burst size. Overall,

this is among the strongest levels reported for inhibition of phage via antisense RNA.

Primase-targeted expression of *pri3.10-AS* antisense RNA also provided significant protection in broth lysis experiments from all Sfi21-type phages tested, including κ 3, κ 4, and κ 9—three phages that were each isolated from different dairy facilities across the continental United States. In contrast, phage κ 6, which encodes a heterologous 7201-type genome replication module, was not inhibited. Together, these results illustrate that the antisense targeting of the highly conserved, early-expressed putative primase is broadly effective against *S. thermophilus* phages that encode the conserved Sfi21-type replication module. It was further noted that mutant phages insensitive to primase antisense RNA were not recovered after numerous attempts to select or enrich for phage derivatives. This result was not expected in light of the prior work with RNA coliphage SP that demonstrated the appearance of antisense-insensitive phages by point mutation (8).

Given the effectiveness of *pri3.10-AS* expression on phage κ 3 development, efforts were made to identify regions that were more or less important for optimal efficacy *in vivo*. To address this, the *pri3.10-AS* region was systematically reduced through the construction of 12 additional subclones that spanned various structural or putative regulatory regions of the primase gene. The expression of all 13 antisense constructs resulted in statistically significant reductions in EOP that ranged from 0.2 to 2.7 log cycles. In general, the largest antisense RNAs (1.5 kb) were found to confer the largest reductions in EOP; however, shorter (478 bp) antisense RNAs designed to the 5' region of the gene retained much of the inhibitory function. The superior efficacy of larger antisense RNAs may stem from the fact that they have more opportunities over their length to maximize intermolecular base pairing and thus exert their inhibitory effects, perhaps through multiple associations within the target RNA (15, 22). Alternatively, larger antisense RNAs may simply exhibit decreased stability when bound to the target RNA. Considering both the length of antisense RNA and the potential for multiple associations, we suspect that these factors could limit the ease with which phages might overcome antisense inhibition via point mutation(s).

As the length of the antisense fragments was reduced, however, a correlation between fragment size and the magnitude of EOP reduction was not observed. For instance, reductions in EOP mediated by *pri3.12-AS*, *pri3.19-AS*, and *pri3.21-AS*, three antisense cassettes of equal length (504 bp) designed to target different, nonoverlapping regions of the entire gene, exhibited significant differences in their ability to reduce the EOP of phage κ 3. The observed variation in the effectiveness of these antisense RNAs may result from differences in the primary nucleotide sequence of the expressed antisense RNAs, which dictates the formation of higher-order intramolecular structures, and/or intrinsic, regional differences in the phage-encoded transcript that were strategically targeted (e.g., 5' or 3' regions). RNA-RNA slot blot analysis indicated that the expression of *pri3.12-AS*, *pri3.19-AS*, and *pri3.21-AS* resulted in marked decreases in the abundance of the sense, phage-encoded primase transcript (Fig. 5). These three antisense RNAs generally reduced the abundance of the target transcript in a manner consistent with the observed reductions in EOP (Fig. 3). Further, expression of antisense RNAs that strongly inhib-

ited plaque formation, as measured by EOP, also resulted in the synthesis of fewer phage genomes over time, indicating a correlation between the lowered abundance of primase transcripts, lowered levels of genome replication, and interference with progeny phage development.

In general, antisense constructs that contained sequences complementary to the putative ribosome-binding site reduced the EOP below the level of similarly sized constructs that lacked sequences complementary to the RBS; however, this was not always the case (e.g., compare *pri3.4-AS* and *pri3.16-AS*). This phenomenon is believed to be due to the formation of a double-stranded RNA over the length of the RBS, thus preventing efficient ribosome loading and reducing translation of the targeted gene (18), which may not only affect the translation of the targeted gene but may also result in the polar expression of translationally coupled genes located downstream from the target.

A previous study revealed that a 1.5-kb antisense RNA complementary to the complete *S. thermophilus* phage κ 3-derived putative helicase gene (*hel3.1-AS*) inhibited the proliferation of Sfi21-type phages, mediating a phage-specific 40 to 70% reduction in the EOP with a concomitant reduction in plaque size (32). In *L. lactis*, Kim et al. (20) found that antisense expression of two polycistronic open reading frames, designated *gp18C* and *gp24C*, inhibited the P335-type phage ϕ 7-9, as measured by a 55% reduction in EOP. 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. (9) obtained variable reductions in EOP, which ranged between 0.5 and 0.8, as they expressed different lengths of the ϕ F4-1 major coat protein (*mcp*) gene. Kim and Batt (19) found that antisense expression of the full-length, phage ϕ 7-9-derived *gp15C* mediated a 100-fold reduction in the EOP of ϕ 7-9 and other *gp15C*-containing phages.

More recently, six genes putatively involved in lactococcal P335-type phage genome replication were targeted with antisense RNA (24). The targeted genes were *orf14* (encoding a putative topoisomerase), *orf15* (encoding a putative single-stranded DNA binding protein), *orf16* (encoding a putative replisome organizer), *orf18* (encoding a putative methylase), and two *orfs* encoding proteins of undetermined function (i.e., *orf17* and *orf19*). For each gene, the expressed antisense RNAs were complementary to the complete open reading frame, including its upstream putative RBS. When challenged with four different P335-type phages, the authors found that the expression of antisense RNAs specific for *orf14*, *orf15*, and *orf18* each reduced the EOP of phage Tuc2009 10-fold but did not have any effect on phage Q30, Q33, or ul36. In contrast, the expression of *orf16* and *orf17* conferred significant but highly variable resistance to all four phages, as measured by 0.5- to 10^{-6} -log reductions in EOP. Antisense RNA specific for *orf19* failed to inhibit any of the four phages.

In general, antisense RNAs targeting early-expressed genes involved in genome replication (24, 32) have been more effective targets than genes expressed later in the lytic cycle (24, 34). Note, however, that some genes involved in genome replication are not effective targets. 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* (putative transcription regulator), and *e15* (putative recombinase), was ineffective in inhibiting the replication of the lactococcal prolate-headed phage ϕ 2, regardless of the gene dosage tested.

In these and other studies, the effectiveness of antisense RNA-based phage defense strategies has been highly variable, exhibiting both target- and phage-specific differences. The collective observations thus far do suggest some key characteristics of an ideal antisense RNA target. Genes that are transiently expressed, expressed at a very low level, and/or coded for by unstable, inefficiently translated mRNAs should make excellent candidates for antisense RNA targeting. From a practical standpoint, the target RNA must be essential for phage development or at least be critical to the synthesis or maturation of virulent progeny phages. In this study, selection of a vital, early gene target was very effective at restricting phage development and further limiting the appearance of mutant phages insensitive to antisense inhibition. In addition, expression of a sufficient dose of antisense RNA at the appropriate time during the lytic cycle is important to the effectiveness of antisense RNA-based phage resistance strategies. Walker and Klaenhammer (34) found that two middle-expressed open reading frames, including *orf1* and *orf2*, and four late-expressed open reading frames, *orf3* through *orf6*, were ineffective at inhibiting the *L. lactis* P335-type phage ϕ 31 when expressed from the high-copy-number vector pTRKH2. In order to increase the ratio of antisense RNA to sense RNA during the later stages of the lytic infection, the authors cloned the aforementioned antisense expression cassettes into a low-copy-number vector containing the ϕ 31 putative origin of DNA replication (*ori31*) and a phage-inducible promoter. Following ϕ 31 infection, the expression of phage-derived DNA replication factors triggered both the expression of antisense RNA and explosive replication of the plasmid replicon, thereby elevating levels of antisense RNA later in the lytic cycle (34).

When phage-encoded resistance systems are engineered, there are potential benefits in implementing comparative genomics as an initial screen for choosing potential targets, as described here. These analyses expedited the identification of well-conserved genes and *cis* regulatory elements shared between the various genomes, while conversely enabling the elimination of poorly conserved targets *in silico*. These advantages are of critical importance when engineering defense strategies intended for use in large-scale industrial settings, where protection is required against both the residing and potentially emerging phage populations.

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