Inhibition of bacteriophage replication in *Streptococcus thermophilus* by subunit poisoning of primase

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Invariant and highly conserved amino acids within a primase consensus sequence were targeted by site-specific mutations within the putative primase of *Streptococcus thermophilus* phage κ 3. PCR products containing the desired mutation(s) within putative ATPase/helicase and/or oligomerization domains of the κ 3-encoded primase gene were cloned into a high-copy-number vector and expressed in *S. thermophilus* NCK1125. The majority of the plasmid constructs failed to alter phage sensitivity; however, four of the constructs conferred strong phage resistance upon the host. Expression of the K238(A/T) and RR340-341AA mutant proteins *in trans* suppressed the function of the native phage primase protein in a dominant negative fashion via a proposed subunit poisoning mechanism. These constructs completely inhibited phage genome synthesis and reduced the efficiencies of plaquing and centre of infection formation by more than 9 and 3.5 logs, respectively. Amber mutations introduced upstream of the transdominant RR340-341AA and K238(A/T) mutations restored phage genome replication and sensitivity of the host, indicating that translation was required to confer phage resistance. Introduction of an E437A mutation in a putative oligomerization domain located downstream of the transdominant K238T mutation also completely suppressed phage resistance. This study appears to represent the first use of transdominant proteins to inhibit phages that are disruptive to cultures used in industrial fermentations.

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INTRODUCTION

Strains of the thermophilic lactic acid bacterium *Streptococcus thermophilus* are incorporated into starter cultures used during the manufacture of a variety of fermented dairy products. Despite the development of a variety of countermeasures, including culture rotation, improved sanitation strategies and the use of bacteriophage-resistant starter strains, phage contamination during manufacture continues to be the leading cause of failed or retarded batch fermentations. The problem endures because the dairy environment, non-sterile fermentation substrates, and even lysogenic starter cultures are all consistent sources of phage contamination (Bruttin *et al.*, 1997; Moineau *et al.*, 1996). In addition, existing phage populations can evolve resistance to phage defence systems by mutation and recombination (Bouchard & Moineau,

2000; Durmaz & Klaenhammer, 2000). Together, these selective pressures necessitate the continued development of starter cultures with enhanced phage-resistance properties.

A greater understanding of phage genomics and physiology has accelerated the development of novel and more efficacious phage-resistance systems. This information has enabled researchers to develop recombinant derivatives of starter strains through the expression of engineered phage resistance systems not previously found in nature (for a recent review, see Sturino & Klaenhammer, 2006). Interestingly, the functional components of these engineered systems have largely been phage-derived. Recently, it has been demonstrated in *S. thermophilus* that the utility of these techniques can be enhanced when they are directed by comparative genomic analyses (Sturino & Klaenhammer, 2002, 2004). Identification of highly conserved phage-encoded genes, pathways or processes can contribute to new defence systems effective against a wider variety of phage strains, which is of critical importance when designing defence systems for industrial applications.

S. thermophilus phages are excellent candidates for the development of such systems since commercial isolates are relatively homogeneous with regard to morphology and genomic organization (Desiere *et al.*, 2002). All phages for

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Abbreviations: COI, centre of infection; ECOI, efficiency of centre of infection; EOP, efficiency of plaquing; SOE PCR, splicing by overlap extension PCR.

A supplementary table of primers is available with the online version of this paper.

this species discovered to date belong to the *Siphoviridae* family (morphotype B1) of viruses, having small isometric heads, long, non-contractile tails, and genomes comprised of double-stranded DNA (dsDNA). Comparative bioinformatic, hybridization and genetic analyses have found that the genome replication functions of *S. thermophilus* phages are catalysed by two distinct clusters of non-orthologous genes, which are exemplified by the phage Sfi21- and 7201-derived prototype modules (Brüssow *et al.*, 1994; Lucchini *et al.*, 1999). For several important reasons, the genes associated with the Sfi21-type genome replication module were previously found to be well suited for engineered phage-inhibitory defence strategies (Sturino & Klaenhammer, 2002). First, they have a high frequency of distribution in industrial isolates: the Sfi21-type gene cluster is found in the majority of industrial *S. thermophilus* phage isolates (Sturino & Klaenhammer, 2002; Brüssow & Desiere, 2001). Second, independently isolated variants exhibit striking sequence conservation (i.e. >99.9%) at the nucleic acid level in this region. Lastly, genome replication functions have an intrinsic strategic importance since they are expressed early in the lytic cycle and may allow for the recovery of the host after the infection is aborted (Sturino & Klaenhammer, 2002).

The Sfi21-type replication module comprises a single origin of DNA replication (*ori*) and several ORFs that encode a putative primase, a putative helicase and a number of other proteins of undetermined function (Brüssow & Desiere, 2001). The putative primase is known to be essential for the replication of genomes composed of dsDNA (Frick & Richardson, 2001). Primases are DNA-dependent RNA polymerases, which are often hexameric, that catalyse the *de novo* synthesis of short oligoribonucleotide primers at sequence-specific loci located across the lagging strand. In this study, invariant and highly conserved amino acids within a consensus primase sequence were targeted by site-specific mutations in an effort to produce non-functional subunits of the oligomeric *S. thermophilus* phage κ 3-encoded putative primase. These mutant primases were then expressed *in trans* and their effect on phage replication was examined.

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 (Sturino & Klaenhammer, 2002).

Table 1. Bacterial strains, bacteriophages and plasmids

Bacterial strain, phage or plasmid	Relevant characteristic(s)*	Source or reference
<i>Streptococcus thermophilus</i>		
NCK1125	Industrial isolate; sensitive to phages κ 3, κ 4, κ 6, κ 9, κ 10; Cm ^S	Sturino & Klaenhammer (2002)
<i>Escherichia coli</i>		
MC1061	Transformation host	Huynh <i>et al.</i> (1985)
Bacteriophages		
κ 3, κ 4, κ 9, κ 10	Encode Sfi21-type replication module; <i>cos</i> -type encapsidation module	Sturino & Klaenhammer (2002)
κ 6	Encodes 7201-type replication module; <i>pac</i> -type encapsidation module	Sturino & Klaenhammer (2002)
Plasmids		
pTRK686	2.4 kb; deletion derivative of pNZ123; Cm ^R	Sturino & Klaenhammer (2002)
pTRK687	3.0 kb; pTRK686 containing the P6 promoter in α -orientation	Sturino & Klaenhammer (2002)
pTRK809	4.5 kb; pTRK687 encoding native protein; TTG start; ϕ^S	This study
pTRK810	4.5 kb; pTRK809 encoding N151am truncated wild-type protein; TTG start; ϕ^S	This study
pTRK811	4.5 kb; pTRK809 encoding E437D protein; ATG start; ϕ^S	This study
pTRK812	4.5 kb; pTRK809 encoding E437A protein; TTG start; ϕ^S	This study
pTRK813	4.5 kb; pTRK809 encoding E437A protein; ATG start; ϕ^S	This study
pTRK814	4.5 kb; pTRK809 encoding K238T protein; ATG start; ϕ^R	This study
pTRK815	4.5 kb; pTRK809 encoding K238T protein; TTG start; ϕ^R	This study
pTRK816	4.5 kb; pTRK809 encoding N326A protein; TTG start; ϕ^S	This study
pTRK817	4.5 kb; pTRK809 encoding RR340-341AA protein; ATG start; ϕ^R	This study
pTRK818	4.5 kb; pTRK817 encoding N151am truncated RR340-341AA protein; ATG start; ϕ^S	This study
pTRK819	4.5 kb; pTRK815 encoding K238T::E437A protein; ATG start; ϕ^S	This study
pTRK820	4.5 kb; pTRK809 encoding K238A protein; TTG start; ϕ^R	This study
pTRK822	4.5 kb; pTRK815 encoding K238T::E437A protein; TTG start; ϕ^S	This study
pTRK823	4.5 kb; pTRK815 encoding N151am truncated K238T protein; TTG start; ϕ^S	This study

*Abbreviations: Cm^S, sensitive to chloramphenicol; Cm^R, encodes chloramphenicol resistance; ϕ^S , sensitive to phages encoding variants of the Sfi21-type replication module; ϕ^R , confers resistance to phages encoding variants of the Sfi21-type replication module.

Bacteriophages and propagation assays. Phages used in this study are listed in Table 1. They were all independently isolated from different dairy facilities across the continental United States and were propagated as described previously (Sturino & Klaenhammer, 2002). The EOP was calculated by dividing the p.f.u. ml⁻¹ on the test strain by the p.f.u. ml⁻¹ on the control strain, *S. thermophilus* NCK1125(pTRK687). The diameters of phage plaques were measured using a caliper and the values reported represent the mean of 60 random plaques chosen over three independent experiments. Centre of infection (COI) assays were performed as described previously and the efficiency of centre of infection (ECOI) was calculated by dividing the number of infective centres on the test strain by the number of infective centres on NCK1125(pTRK687) (Sing & Klaenhammer, 1990).

Site-directed mutagenesis and plasmid construction. The plasmids and primers used in this study are listed in Table 1, and Supplementary Table S1 (available with the online version of this paper), respectively. Conventional PCR reactions, DNA sequencing and sequence analyses were all performed as described previously (Sturino & Klaenhammer, 2002). When appropriate, *Pst*I restriction endonuclease recognition sites were incorporated into the 5' ends of oligonucleotide primers to facilitate the cloning of PCR products. Primer-directed point mutations within specific functional protein domains or motifs were introduced by gene splicing by overlap extension (SOE) PCR as described elsewhere (Horton, 1995). All point mutations were confirmed by sequencing. Electrocompetent *Escherichia coli* and *S. thermophilus* were prepared and electroporated as described previously (Sturino & Klaenhammer, 2002).

Southern hybridizations and alkaline gel electrophoresis. Genomic DNAs were isolated from (i) cultures 10, 20, 30, and 60 min after infection with phage κ 3 at an m.o.i. of $1 \pm 20\%$ and (ii) uninfected control cultures grown in parallel as described previously (Hill *et al.*, 1991). Alkaline transfer of *Hind*III-digested genomic DNA fragments and subsequent Southern hybridizations were performed as described previously (Sturino & Klaenhammer, 2002). The digoxigenin-11-dUTP labelled probes used during hybridization studies were derived from phage κ 3 genomic DNA and purified using NucTrap Probe Purification columns (Stratagene).

Protein sequence manipulation and GenBank accession numbers. The nucleic acid and deduced amino acid sequences for the κ 3-encoded putative primase proteins can be found in the GenBank database under the nucleotide accession number AY196178 (Sturino & Klaenhammer, 2004). Conserved domains were detected using the BLAST (Altschul *et al.*, 1997) and clusters of orthologous groups (COG) database tools (Tatusov *et al.*, 2001) available at the NCBI website (<http://www.ncbi.nlm.nih.gov/>). Multiple alignments were performed using CLUSTAL_X (Thompson *et al.*, 1997).

RESULTS

Overexpression of the native putative primase protein

The *S. thermophilus* phage κ 3-derived primase is encoded by a single ORF of 1515 bp. The boundaries of this ORF are demarcated by an alternative 5'-TTG-3' translation initiation codon and a 5'-TAA-3' translation termination codon. The deduced protein sequence consists of 504 amino acids and has a predicted molecular mass of 59 kDa. The ORF is preceded by a consensus putative ribosome-binding site (RBS) (5'-AGGAGG-3') and is located

upstream of an iteron-rich intergenic region, which was previously shown to contain the putative origin of DNA replication (Sturino & Klaenhammer, 2002).

A 1.5 kb fragment containing the native primase gene was amplified without its native promoter using primer set L001L (Supplementary Table S1), digested with *Pst*I, and cloned into the *Pst*I site of pTRK687 in the sense orientation relative to and downstream from the *Lactobacillus acidophilus* P6 promoter (Djordjevic *et al.*, 1997). The resulting vector, designated pTRK809, was electroporated into *S. thermophilus* NCK1125 and tested for its ability to interfere with phage replication. As measured by efficiency of plaquing (EOP), the expression of the native protein did not confer resistance to phage κ 3 (Fig. 1) or any of the other Sfi21-type phages tested (data not shown). In addition, the expression of the parental-type primase protein did not alter the growth of NCK1125 in broth (data not shown). During standard plaque assays, replication of the pTRK687 base vector resulted in an increased plaque size relative to the NCK1125 native strain but did not affect the EOP of any of the phages tested, as was observed previously (Sturino & Klaenhammer, 2002).

Identification of protein domains and motifs common to prokaryotic primases and ATPases and site-directed mutagenesis

Using BLASTP in conjunction with the COG database, a COG3378-type predicted ATPase domain (*E*-value 2×10^{-59}) and a constituent conserved Parvo_NS1 domain (pfam01057) (*E*-value 3×10^{-3}) were detected within the primary amino acid sequence of the putative phage κ 3 primase (Fig. 2b, c). Both DNA helicase and ATPase activities are associated with the pfam01057 domain, which is required for genome replication in dsDNA parvoviruses (Nuesch & Tattersall, 1993; Wang *et al.*, 1998). This domain was found to contain a signature glycine-rich phosphate-binding loop of sequence GNGNDGKGT near the centre of the protein (Notarnicola *et al.*, 1995). In order to identify highly conserved residues potentially important for enzyme specificity, catalysis and/or structure, the top BLAST hits having cutoff *E*-values of less than 10^{-3} were aligned using the CLUSTAL_X program (Thompson *et al.*, 1997). Regions of strong sequence conservation were identified (Fig. 2). The locations of these motifs relative to the conserved domains described above are shown in Fig. 2(a).

Thirteen mutant primase alleles derived from the native, primase-encoding plasmid pTRK809 were constructed by site-directed mutagenesis (Fig. 1). Mutations were introduced by SOE PCR (Horton, 1995). Primase alleles were amplified using primer set L001L (Supplementary Table S1) and cloned into the *Pst*I site of pTRK687 in the sense orientation relative to the P6 promoter. In an effort to increase the efficiency of translation, some of the constructs were amplified using primer set L001M (Supplementary Table S1), which mutated the (native) putative leucine (L)

Construct	Start	Mutation	Sfi21-type		7201-type	
			κ3		κ6	
			EOP	RPD (%)	EOP	RPD (%)
pTRK809	TTG		1.0 ± 0.2	100 ± 10	1.0 ± 0.2	
pTRK810	TTG	N151am	0.9 ± 0.2	100 ± 10	0.9 ± 0.2	
pTRK816	TTG	N326A	1.0 ± 0.3	100 ± 10	1.0 ± 0.3	
pTRK812	TTG	E437A	1.1 ± 0.1	100 ± 10	1.1 ± 0.1	
pTRK813	ATG	E437A	1.0 ± 0.2	100 ± 10	1.0 ± 0.2	
pTRK811	ATG	E437D	1.0 ± 0.1	100 ± 10	1.0 ± 0.1	
pTRK815	TTG	K238T	< 10 ⁻⁹	-	< 10 ⁻⁹	
pTRK814	ATG	K238T	< 10 ⁻⁹	-	< 10 ⁻⁹	
pTRK820	ATG	K238A	< 10 ⁻⁹	-	< 10 ⁻⁹	
pTRK823	TTG	N151am K238T	0.9 ± 0.1	100 ± 10	0.9 ± 0.1	
pTRK822	TTG	K238T E437A	0.9 ± 0.2	100 ± 10	0.9 ± 0.2	
pTRK819	ATG	K238T E437A	0.9 ± 0.2	100 ± 10	0.9 ± 0.2	
pTRK817	ATG	R340A R341A	< 10 ⁻⁹	-	< 10 ⁻⁹	
pTRK818	ATG	N151am R340A R341A	1.0 ± 0.1	100 ± 10	1.0 ± 0.1	

Fig. 1. Map of the phage-encoded primase, with graphical depiction of point mutations (○) present in various derivatives of the protein. EOP and relative plaque diameter (RPD) values are expressed as the mean ± SD relative to plaques formed on NCK1125(pTRK687), the control indicator strain. Residues downstream of the N151am point mutation are not translated and are denoted by a dotted line. In an effort to increase the efficiency of translation, some of the constructs were amplified using primer set L001M (Supplementary Table S1), which mutated the (native) putative leucine (L) alternative start codon (5'-TTG-3') to the standard, methionine (M) start codon (5'-ATG-3'). These efforts, however, did not result in increased phage resistance. -, Not applicable.

alternative start codon (5'-TTG-3') to the standard, methionine (M) start codon (5'-ATG-3'). These efforts, however, did not result in increased phage resistance. Phage κ3 was titrated on all 13 of the point mutant constructs during standard plaque assays (Fig. 1). The various primase constructs could easily be differentiated into either phage sensitive or phage resistant based on the relative EOP and plaque diameter. Ten of 14 constructs, including pTRK809, N151am, E437A, E437D, N151am::K238T, K238T::E437A, N326A, and N151am::RR340-341AA, were sensitive to phage κ3, and exhibited parental-type EOP and plaque diameter. The remaining four constructs, including K238T, K238A and RR340-341AA, were completely resistant to phage κ3; no plaques were formed, EOP < 10⁻⁹. No phages resistant to the expression of transdominant K238T, K238A or RR340-341AA have been isolated to date, even after serial enrichment. The expression of these four constructs *in trans* appeared to suppress the native, phage-encoded primase protein in a dominant negative fashion.

Centre of infection assays

NCK1125(pTRK809) and the four constructs resistant to phage κ3 were also tested for their ability to inhibit the formation of phage κ3 infective centres. The strain harbouring pTRK809 exhibited parental-type ECOI and plaque sizes. The four strains harbouring resistance constructs exhibited a 3.5 log reduction in ECOI formation, indicating that most infected cells did not lyse or produce progeny. In addition, the sizes of plaques that did form were reduced by 80 % on all four resistant strains. Phages isolated from plaques generated during COI experiments were titrated on the sensitive indicator host NCK1125(pTRK687) and the four hosts expressing the transdominant primase proteins. These phages plaqued normally on NCK1125(pTRK687), but no plaques were detected on K238T, K238A or RR340-341AA at any dilution, indicating that any progeny phages recovered in the COI assays are not resistant to the expression of mutant primase proteins.

Fig. 2. Consensus motifs among putative phage primases. (a) Schematic of the phage-encoded primase. Amino acid coordinates are numbered relative to the primase translation initiation codon. Where appropriate, the locations of conserved regions and protein domains are demarcated with white and grey bars, respectively. Putative functions attributed to specific protein regions are demarcated by black bars. (b, c) Amino acid sequence comparisons of putative primase orthologues derived from an abbreviated collection of diverse bacteria and phages that, when mutated in *S. thermophilus*, either failed to confer (b) or conferred (c) phage resistance to phage κ 3. Conserved residues and motifs are labelled and highlighted. Asterisks denote the amino acid residues targeted for mutagenesis in the study. The glycine-rich putative phosphate-binding loop is underlined. The numbers on the right indicate the amino acid sequence coordinates.

Transdominant proteins interfere with intracellular phage genome synthesis *in vivo*

NCK1125(pTRK687) and strains expressing K238T, N151am::K238T and RR340-341AA were infected with phage κ 3 at a m.o.i. of approximately 1.0. Genomic DNAs isolated from phage-infected cells over the course of the lytic cycle showed that expression of transdominant primase proteins retarded or abolished the accumulation of phage κ 3-specific DNA bands over time (Fig. 3). In the control NCK1125(pTRK687) parent strain (Fig. 3a, e), phage-specific dsDNA fragments began to accumulate 10 min post-infection, accrued maximally at 30 min post-infection, and decreased at 40 min due to host lysis. Phage-specific DNA bands failed to accumulate over time in hosts expressing primase proteins with the transdominant mutations K238T (Fig. 3b, f) and RR340-341AA

(Fig. 3d). The transdominant phenotype was completely abolished and phage replication restored when the N151am mutation was introduced upstream of the K238T mutation (Fig. 3c). Interestingly, introduction of an E437A mutation downstream of the transdominant K238T mutation completely suppressed phage resistance. These results suggested that E437 might itself be important for protein folding, stability and/or oligomerization. Thus, the E437A mutation is believed to preclude association of the mutant subunits with the phage-encoded (wild-type) primase subunits.

Inhibition of heterologous phages encoding Sfi21-type replication modules

The transdominant primase constructs K238T, K238A and RR340-341AA were challenged with three additional

Fig. 3. Accumulation of *Hind*III-digested phage-specific genomic DNA in sensitive and resistant hosts. (a–d) Agarose gel electrophoresis of phage genomic DNAs isolated from (a) control strain NCK1125(pTRK687), (b) transdominant primase strain K238T, (c) primase truncated strain N151am::K238T and (d) transdominant primase strain RR340-341AA. (e, f) Southern hybridization of *Hind*III-digested phage DNA accumulated on (e) control strain NCK1125(pTRK687) and (f) transdominant primase strain K238T. Abbreviations: ϕ , purified phage κ 3 genomic DNA; M, digoxigenin-labelled molecular mass marker I (Invitrogen). Where appropriate, pTRK687-specific DNA bands are denoted by white triangles, while plasmid-associated primase-specific fragments or hybridization signals are denoted by grey triangles.

phages that encoded variants of the Sfi21-type genome replication module (κ 4, κ 9 and κ 10), and a single phage encoding a 7201-type genome replication module (κ 6) (Fig. 1). The expression of K238T, K238A and RR340-341AA all similarly inhibited the replication of Sfi21-type phages κ 3 (Fig. 1) and κ 4, κ 9, and κ 10 (data not shown), but failed to provide any protection from the 7201-type phage κ 6 (Fig. 1).

DISCUSSION

The genes associated with the Sfi21-type genome replication module, including a putative primase, were found to be among the best candidates for engineered phage defence systems due to their frequency of distribution in industrial phage isolates, striking sequence conservation between independent isolates, and intrinsic strategic importance in early phage development (Sturino & Klaenhammer, 2002). In the present study, multiple alignments of related primase protein sequences were used to identify critical amino acid residues potentially involved in enzyme catalysis and/or protein subunit oligomerization. Directed by this approach, invariant and highly conserved amino acids within a *S. thermophilus* phage primase consensus

sequence were targeted by site-specific mutation(s). Characterizations of these mutant proteins led to the discovery of a novel and highly efficacious subunit poisoning system that was effective against *S. thermophilus* phages encoding variants of the Sfi21-type genome replication module.

The expression *in trans* of the K238(A/T) or RR340-341AA mutant primase proteins suppressed the function of the native, phage-encoded putative primase protein in a dominant negative fashion. The inhibition of phage genome replication and failure to complete the lytic cycle suggested that the mutant primase proteins were structurally intact and formed stable interactions with the native, phage-encoded primase protein. Alternatively, the mutant primase proteins could have formed other non-productive associations, such as substrate binding (e.g. origin of replication) in the absence of catalysis and/or inhibition of DNA replication by titrating away other phage- or host-encoded genome replication factors. Amber mutations (N151am) introduced upstream of the transdominant RR340-341AA and K238(A/T) mutations restored phage genome replication and parental plaquing sensitivity, indicating a complete suppression of primase-encoded resistance. These results indicated that translation of the

transdominant mutant primase proteins was required to confer phage resistance. Given the magnitude of the resistance conferred, it was concluded that the putative primase protein is an essential enzyme required for genome replication in *S. thermophilus* Sfi21-type phages. Further, it was also clear that host-encoded factors were unable to complement the deficiency caused by transdominant primase expression. It is important to note that the predicted phage κ 3-encoded primase has not been biochemically characterized.

Several phage-encoded primase proteins, including gene product 4 (gp4) encoded by coliphage T7 (*Podoviridae*) (Notarnicola & Richardson, 1993) and gp α encoded by coliphage P4 (*Myoviridae*) (Ziegelin *et al.*, 1995), have been characterized at the genetic and biochemical levels. Interestingly, the putative primase from phage κ 3 (family *Siphoviridae*) and its related putative orthologues lack several key functional motifs conserved among these *E. coli* DnaG-like primase proteins (Ilyina *et al.*, 1992). These deficiencies notwithstanding, the phage κ 3-encoded enzyme described in this study has been designated a variant class of putative primase since it possesses significant regions in its carboxy terminus that exhibit weak similarities to other phage-encoded and prokaryotic polymerases, especially primases. For example, the ATPase/helicase domain (COG3387) shows similarities to the putative primase from *Lactobacillus gasserii* phage ϕ adh (Altermann *et al.*, 1999) and the characterized primase from *E. coli* satellite phage P4 (Ziegelin *et al.*, 1993, 1995).

The novel RR340-341 arginine dyad targeted in this study is located within the putative ATPase/helicase domain; however, the role of this dyad in protein function has not yet been determined. In addition, we confirm the essential role of a catalytic lysine residue (K238) in primase function. This residue is positioned within a glycine-rich phosphate-binding loop that is also located within the putative ATPase/helicase domain. This flexible structure is routinely found at the transition point between a β -strand and an α -helix and forms a nucleotide-binding site (NBS) in other ATPase/helicase domains found in a variety of heterologous primase proteins (Notarnicola *et al.*, 1995).

In heterologous phages, amino acid substitutions within the NBS motifs have previously demonstrated that these motifs are essential for phage replication both *in vivo* and *in vitro*. Transcomplementation studies in phage T7 yielded results similar to those observed here (Notarnicola *et al.*, 1995; Notarnicola & Richardson, 1993).

When the E437A mutation was introduced downstream of the transdominant K238T mutation, phage sensitivity was completely restored. These results indicated that the E437A mutation precluded the association of the mutant primase protein with the native, phage-encoded primase. Hence, E437 is likely to be a component of the protein oligomerization domain or important for stabilization of the multimeric protein.

In a previous study, antisense RNAs directed against the phage κ 3-encoded putative primase gene severely retarded phage genome replication and limited the number of progeny phage released in *S. thermophilus* (Sturino & Klaenhammer, 2004). The expression of *pri*-3.10-AS, one of the largest and most effective antisense RNAs, resulted in a 2.7-log reduction in EOP and a 50% reduction in ECOI formation, meaning that only one of every two phage-infected cells released progeny phage, even when only the first lytic cycle was impeded by antisense RNA. In contrast, the expression of transdominant primase proteins was more effective than the most effective primase-specific antisense RNA. The transdominant K238(A/T) and RR340-341AA proteins completely inhibited genome replication and reduced the EOP by more than 9 logs.

Significantly, no phages resistant to transdominant primase proteins have been isolated to date.

In conclusion, this study describes a novel, protein-based defence system effective against *S. thermophilus* phages encoding variants of the Sfi21-type genome replication module. Further, it appears to be the first application of transdominant mutant proteins to inhibit phage replication, via subunit poisoning, in starter culture bacteria that are widely used in industrial fermentations.

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