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

Joseph M. Sturino† and Todd R. Klaenhammer

Genomic Sciences Program, Department of Food Science, Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, NC 27695-7624, USA

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 κ3. PCR products containing the desired mutation(s) within putative ATPase/helicase and/or oligomerization domains of the κ3-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.

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...
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 k3-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

<table>
<thead>
<tr>
<th>Bacterial strain, phage or plasmid</th>
<th>Relevant characteristic(s)*</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><em>Streptococcus thermophilus</em></td>
<td></td>
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<tr>
<td>NCK1125</td>
<td>Industrial isolate; sensitive to phages k3, k4, k6, k9, k10; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Sturino &amp; Klaenhammer (2002)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
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<tr>
<td>MC1061</td>
<td>Transformation host</td>
<td>Huynh <em>et al.</em> (1985)</td>
</tr>
<tr>
<td>Bacteriophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>k3, k4, k6, k9, k10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>k6</td>
<td>Encode Sfi21-type replication module; cos-type encapsidation module</td>
<td>Sturino &amp; Klaenhammer (2002)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
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<tr>
<td>pTRK686</td>
<td>2.4 kb; deletion derivative of pNZ123; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Sturino &amp; Klaenhammer (2002)</td>
</tr>
<tr>
<td>pTRK687</td>
<td>3.0 kb; pTRK686 containing the P6 promoter in z-orientation</td>
<td>Sturino &amp; Klaenhammer (2002)</td>
</tr>
<tr>
<td>pTRK809</td>
<td>4.5 kb; pTRK687 encoding native protein; TTG start; φ&lt;sup&gt;S&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pTRK810</td>
<td>4.5 kb; pTRK809 encoding N151&lt;sup&gt;aa&lt;/sup&gt; truncated wild-type protein; TTG start; φ&lt;sup&gt;S&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pTRK811</td>
<td>4.5 kb; pTRK809 encoding E437D protein; ATG start; φ&lt;sup&gt;S&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pTRK812</td>
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<tr>
<td>pTRK813</td>
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<td>pTRK814</td>
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<td>This study</td>
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<td>pTRK815</td>
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<td>pTRK816</td>
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<td>pTRK817</td>
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<td>pTRK818</td>
<td>4.5 kb; pTRK817 encoding N151&lt;sup&gt;aa&lt;/sup&gt; truncated RR340-341AA protein; ATG start; φ&lt;sup&gt;S&lt;/sup&gt;</td>
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<td>pTRK819</td>
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<td>pTRK822</td>
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<td>This study</td>
</tr>
<tr>
<td>pTRK823</td>
<td>4.5 kb; pTRK815 encoding N151&lt;sup&gt;aa&lt;/sup&gt; truncated K238T protein; TTG start; φ&lt;sup&gt;S&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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*Abbreviations: Cm<sup>S</sup>, sensitive to chloramphenicol; Cm<sup>R</sup>, encodes chloramphenicol resistance; φ<sup>S</sup>, sensitive to phages encoding variants of the Sfi21-type replication module; φ<sup>R</sup>, confers resistance to phages encoding variants of the Sfi21-type replication module.*
**RESULTS**

**Overexpression of the native putative primase protein**

The *S. thermophilus* phage κ3-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 iveron-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 PstI, and cloned into the PstI 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 plating (EOP), the expression of the native protein did not confer resistance to phage κ3 (Fig. 1) or any of the other SfiI1-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 κ3 primase (Fig. 2b, c). Both DNA helicase and ATPase activities associated with the pfam01057 domain, which is required for genome replication in dsDNA paroviruses (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 PstI 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)
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::E347A, 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^(-5). 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.
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 \( \kappa 3 \) 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 \( \kappa 3 \)-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
phages that encoded variants of the Sfi21-type genome replication module (k4, k9 and k10), and a single phage encoding a 7201-type genome replication module (k6) (Fig. 1). The expression of K238T, K238A and RR340-341AA all similarly inhibited the replication of Sfi21-type phages k3 (Fig. 1) and k4, k9, and k10 (data not shown), but failed to provide any protection from the 7201-type phage k6 (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 (N151*am*) 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* Sf121-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 κ3-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 gp2 encoded by coliphage P4 (Myoviridae) (Ziegelin et al., 1995), have been characterized at the genetic and biochemical levels. Interestingly, the putative primase from phage κ3 (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 κ3-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 gasseri* phage φadh (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 κ3-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 K238A(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 Sf121-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.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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