

ABSTRACT

STURINO, JOSEPH MILAND. Bacteriophage Defense Systems and Strategies for *Streptococcus thermophilus*. Under the direction of Dr. Todd Robert Klaenhammer.

The genomes of six *Streptococcus thermophilus* bacteriophages were compared to identify genes that could be targeted by engineered phage defense systems with potentially widespread efficacy. The genes associated with the *S. thermophilus* phage Sfi21-prototype genome replication module, including a putative primase and a putative helicase, were found to be among the best candidates due to their frequency of distribution in industrial phage isolates, striking sequence conservation between independent isolates, and intrinsic strategic importance in early phage development. Fourteen antisense RNA cassettes targeting the phage κ 3-derived helicase (*hel3*) or primase (*pri3*) genes were expressed in *S. thermophilus* NCK1125. These constructs consistently reduced the efficiency of plaquing (EOP) of phage κ 3 to between 5×10^{-1} and 2.0×10^{-3} depending on the (i) gene targeted and (ii) region of the gene that was targeted. The largest antisense RNAs were generally found to confer the largest reductions in EOP, however shorter antisense RNAs designed to the 5' region of the gene retained much of the inhibitory function, especially if they contained sequences complementary to the ribosome binding site. Expression of antisense RNAs correlated with decreased levels of phage encoded primase transcripts, likely due to increased degradation of the dsRNA complex. This, in turn, correlated with diminished phage genome replication and aborted phage development.

In a separate study, invariant and highly conserved amino acids within a primase consensus sequence were targeted by site-specific mutations within the *S. thermophilus* phage κ 3-encoded putative primase. PCR products containing the desired mutation(s) were cloned and expressed in *S. thermophilus* NCK1125. The majority of the examined constructs remained sensitive to phage κ 3, however four constructs conferred strong phage resistance to the bacterial host. The mutated residues resided within a putative ATPase/helicase domain suspected to be critical for primase function *in vivo*. The co-expression *in trans* of the K238(A/T) or RR340-341AA mutant proteins suppressed the function of the native, phage-encoded primase protein in a dominant negative fashion via a proposed subunit poisoning mechanism. According to this model, the plasmid-encoded mutant primase subunits are structurally intact and form stable interactions with the native, phage-encoded primase subunits, thus inhibiting their activity. These constructs completely inhibited phage genome synthesis and reduced the efficiencies of plaquing more than nine log cycles. Given the magnitude of the resistance conferred, it was concluded that the putative primase is essential for genome replication in *S. thermophilus* Sfi21-type phages. Further, it was also clear that host-encoded factors were unable to complement the resultant deficiency. Amber mutations introduced upstream of the transdominant RR340-341AA and K238(A/T) mutations restored phage genome replication and phage sensitivity of the host, indicating that translation was required to confer phage resistance. Residues within a critical oligomerization domain were also identified through genetic analysis. Introduction of an E437A mutation downstream of the transdominant K238T mutation completely suppressed phage resistance, indicating that the E437A mutation precluded the association of the mutant and native subunits. To

our knowledge, this is the first application of a subunit poisoning system to inhibit phage replication in the lactic acid bacteria. It is highly significant that no mutant phages were recovered over the entire course of this study that could circumvent the antisense RNA or subunit poisoning defense systems designed to disrupt the early replication functions of this bacteriophage.

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BACTERIOPHAGE DEFENSE SYSTEMS AND STRATEGIES
FOR *STREPTOCOCCUS THERMOPHILUS*

by

JOSEPH MILAND STURINO

A dissertation submitted to the Graduate Faculty of
North Carolina State University
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Doctor of Philosophy

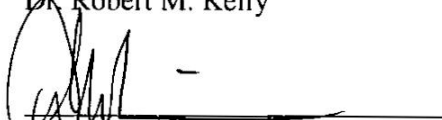
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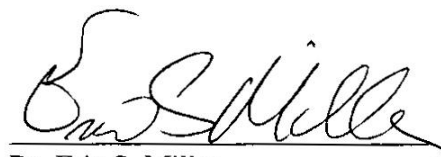
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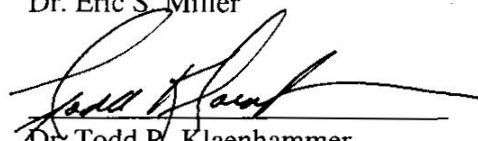
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DEDICATED TO MY WIFE, CHARISSA

NOW WE HAVE TIME

BIOGRAPHY

Joseph Miland Sturino was born on August 4, 1973 in Kenosha, Wisconsin to Frank and Mary Sturino. He attended public schools in Kenosha and graduated from Mary D. Bradford High School in 1991. Following high school, he went on to study music composition and jazz performance for two years at the University of Wisconsin-Parkside and University of Wisconsin-Steven's Point. He transferred to the Bacteriology Department at the University of Wisconsin-Madison in 1993, where he completed his senior honors thesis under the direction of Dr. James Steele and was awarded an Honors Bachelor of Science degree in 1996. He continued to work under the mentorship of Dr. James Steele in the field of starter culture genetics and was awarded a Master of Science degree in Bacteriology in 2000. He then began work on his Doctorate of Philosophy in Genomic Sciences in the Department of Food Sciences at North Carolina State University in 1999 under the mentorship of Dr. Todd Klaenhammer. Together, they went on to develop a variety of novel genetic tools to protect thermophilic starter strains from bacteriophage attack. During his time at North Carolina State University, Joseph was awarded a National Institute of Health Biotechnology Training Program Fellowship and a Research Ethics Fellowship sponsored by the National Science Foundation. Joseph went on to be the first Ph.D. candidate in Functional Genomics to graduate from North Carolina State University in 2003.

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I would like to take this opportunity to extend my sincerest gratitude to those individuals who, through their love, support and/or friendship, made my graduate education possible.

I would like to thank Dr. Todd Robert Klaenhammer, my sponsor and mentor, for allowing me this extraordinary opportunity. I came in his laboratory with foreknowledge of his excellent reputation and academic accomplishments. As I now leave North Carolina, I do so with enormous respect for his scientific insight, work ethic, commitment to science, and dedication to graduate education. Over the years, I have also come to value his sense of humor, generosity, and, perhaps most importantly, his friendship. If my doctorate education was a moving locomotive to which I had tied myself behind, I will always remember Todd as the one who provided me with roller-skates and taught me how to ride the rails.

I would like to thank my wife, Charissa, not only for her commitment to me, but also for her uncompromising commitment to *us*. If it is true that being married to a graduate student is not always easy, then it must be doubly true if that graduate student is me. For instance, it is no secret that I spent far too many hours at home but not *present* (generally in front of the computer). In spite of this, I hope that she understands that I thought of her often during the weekday nights and weekend days that I spent in the laboratory. Balance has never been my fort , however I continue to learn from her example. While those who come to know me now may take note of my new title, it is important to me that she will always be the one who knows best that I can never keep track of my wallet and keys. Thank you for saying *yes*.

I would also like to thank my parents, Frank and Mary, for their support in everything that I have ever attempted. I found much solace in knowing that you would be proud of me even if I had not succeeded in achieving my goals. I am especially indebted to my father, who showed me the importance of education by example. My father attended Cardinal Stritch University part time while working full time on the floor of a factory. I watched him with pride as he was awarded a B. S. in Business Administration. He still works in the same factory, but he now designs the production lines that others use. In many ways, he taught me that the only way to fail is to not try. It may be a little overdue, but thank you both for *not* flipping the bill for my tuition to the Lawrence University Conservatory of Music.

I have been extremely fortunate in having kept such good company while working (and playing) in the laboratory. I would like to thank (in alphabetical order): Eric Altermann, Andrea Azcárate Peril, Rodolphe Barrangou, Logan Buck, Mick Callanan, Tri Duong, Hany Girgis, Olivia McAuliffe, Mike Miller, Mike Russell, Rosemary Sanozky-Dawes, Nicole Souther, and Tracy Tuler for their good humor in times of dropped gels and failed transformations. I am especially thankful for Evelyn Durmaz, a fellow phage-fighter, for her friendship, optimism, and expertise, which she offered freely and free of charge. Evelyn, keep on fighting those phages!

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LIST OF ABBREVIATIONS

Abi	<u>a</u> bortive <u>i</u> nfection
Ads	<u>a</u> dsorption inhibition
BIM	<u>b</u> acteriophage <u>i</u> nsensitive <u>m</u> utant
dsDNA	<u>d</u> ouble <u>s</u> tranded DNA
dsRNA	<u>d</u> ouble <u>s</u> tranded RNA
DVI	<u>d</u> irect <u>y</u> at <u>i</u> noculation
EOP	<u>e</u> fficiency <u>o</u> f <u>p</u> laquing
EPS	<u>e</u> xopolysaccharide
gp	<u>g</u> ene <u>p</u> roduct
HsdS	<u>h</u> ost <u>s</u> pecificity <u>d</u> eterminant
ICE	<u>i</u> ntegrative and <u>c</u> onjugative <u>e</u> lement
IS	<u>i</u> nsertion <u>s</u> equences
kDa	<u>k</u> ilodalton
LAB	<u>l</u> actic <u>a</u> cid <u>b</u> acteria
MTase	<u>m</u> ethyl <u>t</u> ransferase
ORF	<u>o</u> pen <u>r</u> eadin <u>g</u> <u>f</u> rame
Per	origin-conferred <u>p</u> hage- <u>e</u> ncoded <u>r</u> esistance
PFGE	<u>p</u> ulsed- <u>f</u> ield <u>g</u> el <u>e</u> lectrophoresis
pI	<u>i</u> soelectric <u>p</u> oint
PIM	<u>p</u> hage- <u>i</u> nhibitory <u>m</u> edia
Pip	<u>p</u> hage <u>i</u> nfection <u>p</u> rotein
ppm	<u>p</u> arts <u>p</u> er <u>m</u> illion
Prt	<u>p</u> roteinase
RBS	<u>r</u> ibosome <u>b</u> inding <u>s</u> ite
REase	<u>r</u> estriction <u>e</u> ndonuclease
RFLP	<u>r</u> estriction <u>f</u> ragment <u>l</u> ength <u>p</u> olymorphism
R-M	<u>r</u> estriction and <u>m</u> odification
SDS	<u>s</u> odium <u>d</u> odecyl <u>s</u> ulphate
VR	<u>v</u> ariable <u>r</u> egion
w/v	<u>w</u> eight per <u>v</u> olume

The next morning, on opening the incubator, I experienced one of those moments of intense emotion which reward the research worker for all his pains : at the first glance I saw that the culture which the night before had been very turbid, was perfectly clear : all the bacteria had vanished, they had dissolved away like sugar in water. As for the agar spread, it was devoid of all growth and what caused my emotion was that in a flash I had understood: what caused my clear spots was in fact an invisible microbe, a filterable virus, but a virus which is parasitic on bacteria.

Felix d'Herelle, French-Canadian Bacteriologist.

On his discovery of bacteriophages in 1916; Science News **14**: 44-59 (1949).

CHAPTER I

LITERATURE REVIEW:

Bacteriophage Defense Systems and Strategies for
Streptococcus thermophilus

Introduction. Food preservation has been critical for the survival of humankind. Historically, populations depended on a variety of techniques to preserve raw foodstuffs, including fruits, grains, meats, milk, vegetables, and water. Although drying, salting, and smoking are perhaps the most rudimentary forms of food preservation, fermentation is one of the oldest and, arguably, most elegant preservation techniques. Together, these preservation processes enabled humankind to explore the planet in ways not possible before their discovery and allowed commerce to develop between distant populations. These two factors formed an essential platform that allowed increasingly complex civilizations to develop and flourish. Today, with an exploding population and the demand for food growing world wide, the dairy industry is under considerable pressure to produce large-volumes of consistent and high-quality fermented products. The industry has adapted in order to meet these demands. Small-scale production plants have been consolidated into large-scale production facilities that process millions of gallons of milk per day. Modern production processes continue to become increasingly automated and significant efforts have been made to shorten production times while extending production schedules. In fact, many food-processing plants are run at capacity twenty-four hours a day. In addition, industrial scale fermentations no longer rely on spontaneous fermentation and the industry has turned to more consistent inoculation strategies. Today, the industry has turned to the use of concentrated, well-defined, and optimized starter cultures to catalyze their fermentations.

The dairy industry utilizes extensively strains of *Streptococcus thermophilus* and species from the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus* as starter cultures or culture adjuncts for use in the manufacture of a variety of fermented

dairy products. These starter cultures are all members of the lactic acid bacteria (LAB), a heterogeneous family of Gram-positive eubacteria that derive metabolic energy from the fermentation of carbohydrates to lactate via substrate level phosphorylation. These bacteria impart many of the textural, organoleptic, and preservative characteristics that can exclude the growth of many pathogens (for a review, see Ross *et al.*, 2002). Most of these desirable properties are largely a byproduct of cellular metabolism reflecting the activity of enzymes or the production of organic acids, especially lactic acid. From a macroscopic perspective, however, much more than the carbohydrate content changes over the course of food fermentation: the basic characteristics of the foodstuffs, including texture, taste, aroma, and nutritional value are transformed. These changes are primarily due to the gradual hydrolysis of proteins, lipids, polysaccharides, and other compounds associated with the fermentation substrate.

The significance of bacteriophage infections during product manufacture was unknown until Whitehead and Cox (1935) identified the first phages specific for strains of *Lactococcus lactis* (then called *Streptococcus lactis*). Since their discovery, bacteriophages specific for dairy starter cultures, notably lactococci and recently *S. thermophilus*, have been recognized as a significant and persistent problem for the dairy industry. Loss of fermentative capacity associated with starter culture lysis can significantly retard or halt batch fermentations, thereby causing significant losses of time and production capital. 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 infecting it. 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 product manufacture continues to be the leading cause of failed or retarded batch fermentations. The problem endures because the dairy environment is a consistent reservoir for phage contamination, especially the non-sterile fermentation substrate and lysogenic starter cultures (Bruttin *et al.*, 1997a; Moineau *et al.*, 1996). In addition, existing phage populations can evolve resistance to phage defense systems by mutation and recombination (Bouchard and Moineau, 2000; Durmaz and Klaenhammer, 2000). As a result, the phage population as a whole is in a constant state of flux. Together, these selective pressures necessitate the isolation or construction of starter cultures with enhanced phage resistance properties. This chapter will describe the complex relationship that exists between strains of *S. thermophilus* and their bacteriophages. Examples from other genera and species of LAB will be cited only when necessary to fill subtle gaps in current knowledge. In particular, this chapter will highlight the various defense strategies and systems that have been developed in order to curb the propagation and evolution of lytic phages.

***Streptococcus thermophilus*.** The genome of *S. thermophilus* is 1.8 Mb, making it among the smallest genomes of all the dairy LAB. The molar G+C ratio is 40%.

Morphologically, *S. thermophilus* cells are spherical to ovoid, 0.7-0.9 μm in diameter, and grow in pairs to long chains. *S. thermophilus* strains are moderately thermophilic, and can grow in the temperature range of 15 to 45°C (Hardie, 1994). They do not grow at pH

9.6, but growth in 2.0% NaCl is strain dependent. *S. thermophilus* strains are fastidious and require nutrient-rich environments, such as milk, to support growth. In addition, *S. thermophilus* strains have a somewhat limited carbohydrate fermentation profile in comparison to other dairy LAB, making them readily identifiable using API 50 CH fermentation strips (bioMérieux, Marcy l'Etoile, France). Typically, *S. thermophilus* produces L(+)-lactic acid as the principle byproduct of the fermentation of fructose, glucose, lactose, mannose, and sucrose, but not from arabinose, dextrin, glycerol, inulin, mannitol, rhamose, salicin, sorbitol, starch, and xylose (Hardie, 1994). Like most other LAB, *S. thermophilus* strains are chemoorganotrophic, non-sporulating, catalase negative, devoid of cytochromes, facultatively aerobic, and acid-tolerant. They are naturally found in milk and decaying plant material.

Strains of *S. thermophilus* are among the most economically important of the lactic acid bacteria. *S. thermophilus* strains are used during the manufacture of Italian-style cheese varieties, including Asiago, Mozzarella, Parmesan, Provolone, and Romano, and surface ripened cheeses, such as Bel Paese, Limburger, Port du Salut, Tilsit, and Trappist (Olson, 1969; Reinbold, 1963). *S. thermophilus* is also used in combination with the mesophilic *Lactococcus lactis* during in the production of Cheddar cheese. In this case, the thermophilic and mesophilic components are phage unrelated, and one of the two components will continue to produce acid if the other is lysed by bacteriophages. This microorganism is perhaps best known for its use in conjunction with *Lactobacillus* sp., especially *Lactobacillus delbrueckii* spp. *bulgaricus*, during the manufacture of yogurt. These two microorganisms share a remarkable synergistic relationship when mixed together in a 1:1 ratio: they grow faster and produce more lactic acid and

acetaldehyde, the principle volatile flavor compound associated with yogurt, when grown in co-culture (for reviews, see Matalon and Sandine, 1986; Zourari *et al.*, 1992).

Efforts to better understand this relationship are now underway. Milk is very rich in proteins, especially caseins, but contains few free amino acids (Jensen, 1995). Both strains harbor multiple amino acid auxotrophies, however *S. thermophilus* has fewer nutritional requirements in chemically defined media (Letort and Julillard, 2001; Grobber *et al.*, 1998) and in milk (Desmazeaud, 1983). *L. delbrueckii* subsp. *bulgaricus* typically possesses a more robust proteolytic system when compared to *S. thermophilus* (Courtin *et al.*, 2002; Rajagopal and Sandine, 1990). The analysis of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* proteinase (Prt) null strains was recently used to investigate the importance of these two proteinases when the strains are grown in milk in co-culture. These studies revealed that the streptococcal proteinase (PrtS) was essential for *S. thermophilus* grown in milk in pure culture, but its absence had no effect on the final pH when grown in milk in the presence of PrtB⁺ strains of *L. delbrueckii* subsp. *bulgaricus*. The proteinase PrtB, on the other hand, is required for optimal growth of *S. thermophilus* in milk and its absence resulted in a higher final pH and lower streptococcal cell counts when grown in mixed culture (Courtin *et al.*, 2002). These cell wall-associated proteinases cleave caseins into short peptides that are then transported into both organisms via their respective oligopeptide transport permeases and are further degraded intracellularly into free amino acids by a variety of peptidases (for a review, see Kunji *et al.*, 1996). As a result, increased levels of proteolysis stimulate the growth of *S. thermophilus*, which in turn stimulates the growth of *L. delbrueckii* subsp. *bulgaricus* through the production of a variety of compounds, including CO₂ and formate, and by

reducing the redox potential of the growth substrate (for reviews, see Matalon and Sandine, 1986; Zourari *et al.*, 1992). Thus, this synergistic relationship results in a dynamic fermentation where *S. thermophilus* is responsible for driving much of the acid production during the early stages of the fermentation, while acidification is later driven by *L. delbrueckii* subsp. *bulgaricus*.

S. thermophilus also plays an important role as a probiotic, alleviating symptoms of lactose intolerance and other gastrointestinal disorders (Kolars *et al.*, 1984; Martini *et al.*, 1991). This microorganism has shown promise in maintaining remission of ulcerative colitis, and has also been shown to help prevent the post-operative recurrence of Crohn's disease (Venturi *et al.* 1999). *S. thermophilus* also produces high levels of the vitamin folate (vitamin B-9), which plays a variety of important roles in human health (Crittenden *et al.*, 2003). Folate is important for iron metabolism and maintaining cardiovascular function, but is perhaps best known for its role in modulating fetal development in *utero* (Krishnaswamy *et al.*, 2001). Folate deficiency during pregnancy has been linked to the increased incidence of fetal neural tube defects and other congenital anomalies (for a review, see Wilson *et al.*, 2003). The mechanism of action of probiotics may include receptor competition, pathogen antagonism, effects on mucin secretion, vitamin or co-factor production, or probiotic immunomodulation of gut-associated lymphoid tissue (Reid *et al.* 2003).

Discovery of Bacteriophages. Phages were the last of the three major classes of viruses to be discovered, and their discovery was preceded by the discovery of the plant viruses (tobacco mosaic virus) (Ivanovsky, 1892) and the animal viruses (foot-and-mouth

disease virus) (Loeffler and Frosch, 1898). As with many of the great discoveries of our age, phages were discovered by accident. Phages were independently identified early in the twentieth century by British bacteriologist Frederick William Twort (1915) working at the Brown Institution (London, England) and then two years later by the French-Canadian bacteriologist Felix D'Herelle (1917) at the Pasteur Institute (Paris, France). Both researchers observed small, symmetrical zones of clearing, called plaques, on agar plates containing an otherwise confluent lawn of bacteria. When a soft agar overlay containing an abundance of sensitive bacteria and a finite number of phage particles is poured over the surface of a nutrient agar plate and incubated under the appropriate conditions, two distinct topographies will be evident over the surface of the plate. The bacteria will grow to saturation in regions of the plates devoid of phage particles, forming an opaque layer (or lawn) in the nutrient agar overlay. In regions of the plate where phage particles are present, however, the phage particles will come into contact with and lyse the bacteria. Following lysis, the progeny phages are released and diffuse through the semisolid substrate to come into contact with neighboring bacteria, beginning the infection cycle anew. The eventual result of these and subsequent rounds of local infection and lysis is a growing zone of lysis, or plaque, teeming with phage particles and cellular debris. This zone continues to expand while there are actively metabolizing bacteria available. Eventually, the infection becomes visible to the naked eye against the backdrop of an otherwise confluent lawn.

Life cycles. Bacteriophages exist in two alternative states: (i) the extracellular and metabolically inert infectious particle and (ii) the intracellular genetic parasite. In

the extracellular state, the phage particle is defined as a nucleic acid genome encased within a highly symmetric capsid that is normally comprised solely of proteins. The capsid acts as a protective vehicle for the delivery of the phage genome to another suitable host. In order to initiate phage replication, a phage particle must adsorb to an actively growing cell by binding specific receptor(s) associated with the extracellular envelope. Following adsorption, the phage injects its genome into the bacterial cytosol. Once introduced into the cytosol, the genome will respond to its new environment in a manner that is consistent with its genetic makeup.

There are three types of bacteriophage life cycles: lytic, lysogenic, and chronic. Obligate lytic phages must ultimately terminate their infection and lyse their host in order to release progeny phage particles; most of the phages that are problematic to the fermentation industries are obligately lytic in nature. Lysogenic infections do not result in the production of phage particles and do not release progeny into the extracellular environment; this type of life cycle will be described later in this review. A chronically infecting phage can release progeny phages into the extracellular environment without killing its host (Maniloff *et al.*, 1981). In this case, the phage and the host co-exist and bacteriophages are constantly shed over the course of the infection. Bacteriophages capable of chronically infecting LAB have not been described to date.

Intracellularly, bacteriophages exist in a state of flux, and commitment to the lytic life cycle initiates a finely tuned and temporally coordinated cascade of biosynthetic reactions. These reactions rely heavily on the preexisting machinery and energetic capacity of the host system at the time of infection. The degree to which a virus relies on host-encoded factors differs from bacteriophage to bacteriophage, however. During the

lytic life cycle, phage-encoded genes are transcribed and translated. This results in the catalysis of phage genome replication and the synthesis of phage polymers. These polymers are then assembled into mature phage particles. These particles are finally released into the environment when the cellular envelope is breached due to the activity of two phage-encoded proteins, holin and lysin (for reviews, see Wang *et al.*, 2000; Sable and Lortal, 1995).

In addition to the lytic cycle, temperate phages are capable of undergoing a lysogenic life cycle (Figure 1). In this case, the phage does not multiply lytically. Instead, the phage integrates its genome into the host chromosome. As such, the bacterium, which is now referred to as a lysogen, possesses the ability to produce bacteriophages and transmits this ability to its daughter progeny. Hence, the phage is effectively replicated once with every division of the bacterium. The lysogenic state will be maintained until the prophage is induced due to the exposure of the lysogen to exogenous stress(es) (*e.g.* SOS response triggered by DNA damage). Once induced, the integrated phage, called a prophage, will excise from the host genome and enter the lytic cycle. The progeny phages that are subsequently released can go on to lysogenize neighboring cells. As a result, lysogenic starter cultures could be problematic if used industrially since they represent a renewable source of phage within the dairy facility. Lysogenic starter strains appear to be less of a problem for *S. thermophilus* strains than they are for *Lactococcus lactis*, however. Fortunately, very few strains of *S. thermophilus* have been shown to be lysogenic. Le Marrec *et al.* (1997) found one lysogen out of the 51 strains (2%) tested; Brüssow *et al.* (1994a) found two lysogens of the 100 strains (2%) tested; Fayard *et al.* (1993) found 12 lysogens of the 120 strains

(10%) tested; and Charminati and Giraffa (1992) found one lysogen of the 45 strains tested (2%). In addition, candidate starter strains are now routinely testing for the presence of prophages. If the strain exhibits a great deal of economic potential, a prophage-cured derivative of the strain may be constructed.

Interestingly, some prophages appear to be hypersensitive to physiological stresses associated with growth, such that even mild stresses can trigger premature lysis (for a review, see Gasson, 1996). It has been proposed that the prophage-encoded genes (specifically the holin and lysin) are constitutively expressed at a low and nonlethal level within the population and that this expression gives rise to the autolytic phenotype without massive prophage induction (Husson-Kao *et al.*, 2000a; 2000b). While this phenotype is clearly not beneficial for the host, it is of great value to cheese makers. Autolytic strains of LAB are often used as culture adjuncts to deliver intracellular enzymes into the cheese matrix, which can accelerate cheese ripening (for a review, see Gasson, 1996).

Bacteriophage defense strategies and systems defined. In this manuscript, countermeasures designed to minimize the impact of phages will be broken down into strategies and systems. Phage defense *strategies*, which will be discussed only briefly, are specific actions that, when properly administered, reduce the number or kind of phages in the dairy environment. Phage defense *systems*, on the other hand, are defined as genetic constructs that, when expressed in a host bacterium, lead to a bacteriophage resistant phenotype. Phage defense systems will be the primary focus for the remainder of this manuscript.

TRADITIONAL STRATEGIES:

Improved Sanitation and Manufacturing Processes. Bacteriophages are ubiquitously found in milk (Bruttin *et al.*, 1997a; Moineau *et al.*, 1996; for a review, see Brüssow *et al.*, 1998). As a result, the use of higher quality milk substrate(s) and proper pasteurization and sanitation regimes are critical for controlling phage levels within dairy facilities (for a review, see Daly, 1983). In addition, most phages are not completely inactivated by standard pasteurization treatments, and those that do survive are able to infect and lyse starter cultures (Binetti and Reinheimer, 2000). Sodium hypochlorite (100 ppm) and 0.15% (w/v) peracetic acid are very effective at inactivating phages during routine sanitation. Other biocides, such as 75-100% ethanol and isopropanol, exhibit suboptimal biocidal activity and are generally only useful in laboratory settings (Binetti and Reinheimer, 2000).

Inside a dairy facility, phages are spread by improper whey handling and aerosols, and become fixed on fermentation equipment. Special care must be taken to minimize the risk of phage contamination in the starter room. If the bulk starter is infected, then it is possible that the culture will be lysed and yield a cheese with high pH and high lactose content. Other methods, including the use of closed cheese vats and concentrated direct vat inoculation (DVI), which eliminates the need for bulk starter systems, have also helped to minimize the impacts of phage contamination. The use of frozen concentrates has largely eliminated the need for intermediate transfers, which helps to minimize the problems associated with scale up propagations. Prior to the development of DVI

cultures, failures in starter systems usually resulted from phage contamination of the bulk starter.

Phage Inhibitory Media. The use of enriched, phage-inhibitory media (PIM) has been widely adopted since its introduction (Bester and Lombard, 1975; Gulstrom *et al.*, 1979; for a review, see Daly, 1983). In general, calcium ions are required for the efficient adsorption of phages, including those specific for *L. lactis* and *S. thermophilus*. As an example, calcium ions are essential for the adsorption of phage c2 to the extracellular envelope of *L. lactis* (Lowrie and Pearce, 1971; Lubbers *et al.*, 1995). Antibodies specific for the minor capsid protein gp110 (encoded by *110*) specifically bound to the tip of the tail, suggesting that gp110 could be responsible for mediating adsorption of phage c2 to the host receptor (Lubbers *et al.*, 1995). Interestingly, an EF-hand signature (pfam00036) was detected within the deduced gp110 protein sequence, which was not found in any other protein encoded by phage c2. This signature typically facilitates the binding of calcium ions and is comprised of a 12-amino acid residue loop followed by a hydrophobic residue (Nakayama *et al.*, 1992). Based on these requirements, PIM generally contain one or more cation-scavenging compounds (*e.g.* phosphates and citrates) to bind calcium in addition to a variety of standard nutrients, which include milk solids, yeast extract, and peptones that support robust growth of the bacteria (for a review, see Whitehead *et al.*, 1993).

Phage-Unrelated Strains: Rotation. Starter cultures may be of defined or undefined strain composition, and may be used with or without culture or strain rotation

(Cogan *et al.*, 1991). The use of defined strain cultures has provided a significant degree of control over fermentation systems, and has been widely adopted in large-scale production facilities. These starter cultures usually contain two to five well-characterized strains that are phage-unrelated and possess defined fermentation properties (Thunell and Sandine, 1985). In defined systems, rotation is a process whereby sensitive strains are withdrawn as needed upon the emergence of lytic phages and replaced with one or more non-isogenic and phage-unrelated strain(s) with similar fermentative properties. Plasmid intracellular rotation is a novel alternative to traditional strain rotation developed in lactococci, but should be equally useful in *S. thermophilus* (Durmaz and Klaenhammer, 1995; Sing and Klaenhammer, 1993; O’Sullivan *et al.*, 1998). During this process, a number of phage-resistant derivatives of a single strain are constructed by introducing a variety of phage defense plasmids of different natures and specificity (*e.g.* abortive infection and restriction and modification systems). These strains are then rotated as necessary. If the starter strains are to be used for the manufacture of consumer products, the most widely accepted approach for the introduction of heterologous DNA is the use of conjugation (Sanders *et al.*, 1986; for a review, see Klaenhammer and Fitzgerald, 1994). When used properly, these rotation strategies can significantly extend the longevity of strains in the dairy environment—especially when used in conjunction with effective methods to select for bacteriophage insensitive mutants (Heap and Lawrence, 1976; Huggins, 1984; Klaenhammer, 1984). Although phage rotation is a powerful tool, care must be taken to ensure that too many phage unrelated strains are not used at one time. Hull (1985) warned that the concurrent usage of large numbers of phage-unrelated

strains at one time would increase the size of the available gene pool and might stimulate the emergence of new virulent phages by mutation or recombination.

Unfortunately, the degree of phage relatedness must be determined empirically. Since few *S. thermophilus* strains contain plasmids, most strains cannot be differentiated by standard plasmid profiling, which is routinely used for lactococci. Although most *S. thermophilus* strains do not harbor plasmids, they do encode other mobile elements, including insertion sequences (IS), that readily allow for genetic typing (Guedon *et al.*, 1995). Pulsed-field gel electrophoresis (PFGE) in conjunction with ribotyping has proven to be reliable for the differentiation of *S. thermophilus* strains (Roussel *et al.*, 1997). DNA probes have been used to differentiate even very closely related strains by targeting (i) ten single copy genes, (ii) the genes associated with the ribosomal RNA operon (*rrn*), and (iii) three different insertion sequences, IS1191, IS981, and ISS1 (Roussel *et al.*, 1997). Prior to this, Pebay *et al.* (1992) demonstrated that significant variability in hybridization patterns were observed among strains of *S. salivarius* subsp. *thermophilus* based on (i) restriction fragment length polymorphism (RFLP) and (ii) variability in the number of *rrn* operons present.

MOLECULAR STRATEGIES:

Elimination or Alteration of Host-Encoded Factors. Following phage adsorption, the phage genome is injected into the bacterial cytoplasm in order to establish a lytic infection. The receptors and other factors required for phage replication, such as membrane-associated genome carrier proteins, are encoded by the host, but have been poorly characterized in *S. thermophilus*. Quiberoni *et al.* (2000) have recently made

some progress in identifying the character of these receptors in *S. thermophilus*. In this study, purified cell walls from two strains of *S. thermophilus*, YSD10 and BJ15, were treated with various macromolecule antagonists. Treatment of these cell walls with sodium dodecyl sulphate (SDS) and proteinase K failed to reduce phage adsorption, whereas mutanolysin and trichloroacetic acid reduced phage adsorption. These results suggested that a component of the phage receptor is either the peptidoglycan itself or moieties associated with the peptidoglycan. In separate experiments, the authors tested several carbohydrates for their ability to inhibit phage adsorption (Quiberoni *et al.*, 2000). These experiments indicated that phage CYM adsorbed to the glucosamine and rhamnose moieties associated with the YSD10 cell wall, while phage 0BJ adsorbed to glucosamine and ribose moieties associated with the BJ15 cell wall. In *L. lactis*, the adsorption of c2-type phages is a two-step process (Monteville *et al.*, 1994). In the first step, the phage tail reversibly adsorbs to a carbohydrate component (rhamnose) of the cell wall. In the second step, the phage particle becomes irreversibly anchored to a membrane associated phage infection protein (Pip) (Geller *et al.*, 1993; Monteville *et al.*, 1994). The involvement of a previously identified unnamed 32-kDa protein in phage genome infection is yet unknown (Valyasevi *et al.*, 1991).

The generation of bacteriophage insensitive mutants (BIM) by spontaneous mutation or chemical mutagenesis has a rich history in starter culture development (for reviews, see Forde and Fitzgerald, 1999; Coffey and Ross, 2002). The random introduction of one or more specific mutation(s) may confer partial or complete insensitivity to phages, which enables the straightforward selection of BIMs. Although BIMs are generally easy to isolate, they often exhibit a variety of negative qualities that

may exclude them from use during product manufacture. Among the least desirable qualities that often accompany phage insensitivity are slow growth, diminished capacity to produce lactic acid and/or flavor compounds, and altered agglutination properties. Other problems commonly associated with the use of with BIMs are frequent reversion to the phage sensitive phenotype and insensitivity to only closely related phage. The study of BIMs has facilitated the study of the phage receptors involved in adsorption, however it is often difficult to localize the gene(s) that have been mutated, since they may be located anywhere in the bacterial genome.

In order to address these difficulties, Lucchini *et al.* (2000) described the use of pG⁺host9::ISSI-based insertional mutagenesis to identify genes involved in bacteriophage sensitivity. The plasmid pG⁺host9::ISSI encodes an antibiotic resistance marker, a temperature sensitive replicon, and a single copy of ISSI, which has previously been shown to integrate randomly in *S. thermophilus* (Maguin *et al.*, 1996). One of the principle advantages of plasmid-based mutagenesis systems over the use of spontaneous or chemically induced mutagenesis, is that the genes interrupted by the integrated plasmid are readily cloned. Further, the vector sequences can be removed from the chromosome by recombination while leaving a single integrated copy of ISSI in the chromosome. Using this approach, four distinct host-encoded loci involved in bacteriophage sensitivity have been identified (Lucchini *et al.*, 2000). Among the most effective loci identified was an open reading frame (*orf394*) that encoded a putative transmembrane protein, gene product (gp394). When mutated, gp394 conferred complete resistance to all *S. thermophilus* phages tested. As a result, the authors proposed that gp394 is functionally

analogous to the lactococcal Pip, which is essential for infection of *L. lactis* by c2-type bacteriophages (Garbutt *et al.*, 1997).

NATIVE DEFENSE SYSTEMS:

The dairy has proven to be a dynamic venue for the study of the interactions between LAB and their bacteriophages. Through prolonged exposure over time, the persistence of phages within this environment has enriched for robust bacterial strains that have acquired a variety of bacteriophage defense systems. The characterization of these defenses allows researchers to (i) better understand the dynamics that exist between phage and host and (ii) exploit these findings through the construction of strains with greater phage resistance that exhibit enhanced fitness in the industrial setting.

In lactococci, these defenses are typically, but not exclusively, plasmid-encoded. Industrial strains of lactococci typically carry a multiple plasmids in a wide range of sizes and multiple phage defense systems are often stacked within the same strain (Klaenhammer, 1989). Strikingly, this contrasts the situation found in *S. thermophilus*, where strains encode fewer plasmids. Herman and McKay (1985) examined 23 strains of *S. thermophilus* examined and found that five (22%) contained a single small cryptic plasmid. In a more recent study, Turgeon and Moineau (2001) assayed the plasmid content of 22 strains of *S. thermophilus*. Thirteen of them (59%) were found to contain one or two plasmids. Fifteen *S. thermophilus* plasmids were divided into four DNA homology groups (*i.e.* groups A-D). Two thirds of these plasmids belonged to group A. Three of the plasmids belonged to group B, while groups C and D each contained a single plasmid. The authors went on to determine that groups A, C, and D replicated via

a rolling-circle mechanism, but were unable to determine the replication mechanism for those plasmids in group B.

The scarcity of plasmid-associated defense mechanisms has made the study of native bacteriophage defense systems more challenging in *S. thermophilus*. Although studies on native bacteriophage defense systems in *S. thermophilus* remain limited, three distinct classifications of native bacteriophage defense strategies will be discussed. They are listed below in order of their interference with the lytic life cycle. Whenever possible, each of these functional classes will be discussed, and representative examples of each will be highlighted as appropriate.

Adsorption Blocking. The first line of extracellular defense against bacteriophage infection is to prevent the adsorption of the phage particle to the extracellular envelope (for reviews, see Coffey and Ross, 2002; Forde and Fitzgerald, 1999; Klaenhammer and Fitzgerald, 1994). As seen in other bacterial systems, electron microscopic examinations of lactococcal phage-host complexes indicated that phage attachment to the host is mediated by the tail structure (Budde-Niekiel and Teuber, 1987). Further, phage attachment to host-encoded receptors may be uniformly distributed over the surface of a bacterium, or may occur in localized hot spots randomly distributed over the surface of the extracellular envelope (Budde-Niekiel and Teuber, 1987). Adsorption inhibition (Ads) has been proposed to occur by two distinct mechanisms (for a review, see Klaenhammer and Fitzgerald, 1994). The first mechanism involves the expression of extracellular factors, including exopolysaccharide (EPS), that either bind to- or sterically mask the phage receptor(s). The second mechanism results in the reduction or complete

elimination of the phage receptors expressed on the extracellular surface. These mechanisms have been extensively characterized in lactococci, however they have not yet been identified in strains of *S. thermophilus*, although it is very likely to occur since strains of *S. thermophilus* produce a variety of chemically distinct EPSs (for a review, see Broadbent *et al.*, 2002).

Restriction and Modification. Following particle adsorption and genome injection, replication of the phage genome can be terminated through the activity of restriction and modification (R-M) systems, which act as the first line of intracellular defense against bacteriophages (Klaenhammer, 1989; for reviews, see Coffey and Ross, 2002; Forde and Fitzgerald, 1999; Allison and Klaenhammer, 1998; Klaenhammer *et al.*, 1991). One of the greatest benefits of R-M systems is that they terminate the infection prior to the initiation of phage-directed cell death. As a result, many of the R-M⁺ infected cells will continue to be viable following the degradation of the phage genome. R-M systems are comprised of two complementary enzymatic functions that work in concert to differentiate endogenous from exogenous DNA. The first functional component is a restriction endonuclease (REase), which cleaves double stranded DNA (dsDNA). The second component is a modification enzyme, typically a methyltransferase (MTase) that covalently modifies DNA at sequence specific loci located throughout the genome. Currently, four functional classifications of R-M systems have been described based on the nature and complexity of their (i) target recognition sequences, (ii) cleavage site(s), and (iii) enzyme structure (for a review, see Roberts *et al.*, 2003a). In Type I, II, and III R-M systems, the MTase is responsible for protecting endogenous DNA from REase-

mediated cleavage. The converse is true for Type IV R-M systems, where the MTase targets modified DNA for REase-mediated cleavage.

To date, only members of the Type I, II, and III R-M systems have been identified in the dairy LAB. In *S. thermophilus*, two Type I and eight Type II R-M systems have been described and partially characterized in strains of *S. thermophilus* (Table 1). The vast majority of these R-M systems have never been cloned, sequenced, or used to augment the levels of phage resistance, however. Recently, Burrus *et al.* (2001) cloned, sequenced, and functionally characterized Sth368I, a Type II R-M system associated with the 34,734-bp integrative and conjugative element ICE*StI*, which was isolated from *S. thermophilus* CNRZ368 (Burrus *et al.*, 2002). Sth368I is comprised of two different genes, *sth368IR* and *sth368IM*. The *sth368IR*-encoded protein exhibited significant sequence similarity to a variety of Type II endonucleases, including R.LlaKR2I and R.Sau3AI, that recognize and cleave (\downarrow) the sequence 5'- \downarrow GATC-3'. In addition, the *sth368IM*-encoded protein exhibited similarity to a variety of Type II 5-methylcytosine methyltransferases, including M.LlaKR2I and M.Sau3AI. Cloning and integration of the *sth368IRM* genes into the chromosome of *S. thermophilus* A054 resulted in significant resistance to phage ϕ ST84 and reduced the efficiency of plaquing (EOP) to approximately 10^{-4} . This was the first report of using a native R-M system to enhance the phage resistance of a closely related strain of *S. thermophilus*. Prior to that, the only other report that described the use of an R-M system to enhance the level of phage resistance in *S. thermophilus* was due to the expression of a heterologous R-M system. Moineau *et al.* (1995) found that the plasmid-borne expression of the lactococcal LlaDCHI (formerly LlaII) conferred broad-range bacteriophage resistance in various strains of *S.*

thermophilus. When expressed from pNZ123, a high-copy-number vector, the expression of LlaDCHI reduced the EOP to between 10^{-5} and 10^{-8} , depending on the phage and host background tested (Moineau *et al.*, 1995).

As seen in Table 1, two complete Type I R-M systems have been identified in *S. thermophilus*. Type I systems differ from Type II, III, and IV R-M systems, in that sequence specificity is not determined by their respective REase or MTase subunits (Roberts *et al.* 2003a). Rather, specificity is directed by a third subunit called a host specificity determinant (HsdS). Schouler *et al.* (1998) first described the existence of plasmid-borne *hsdS* genes in lactococci and demonstrated that the encoded specificity subunits interacted with chromosomally encoded HsdR and HsdM proteins. Since then, specificity domain stacking has been suggested to be a means of augmenting the phage resistance of *S. thermophilus* strains (O'Sullivan *et al.*, 1999). For example, the 6.5 kb plasmid pCI65st isolated from the *S. thermophilus* strain NDI-6 encodes a putative HsdS protein (O'Sullivan *et al.*, 1999). Interestingly, the plasmid-bearing parent was resistant to phage ϕ bas19, while a pCI65st-cured derivative was found to be sensitive to phage ϕ bas19, suggesting that the HsdS played a role in modulating phage resistance. It had been reported previously that another *S. thermophilus* plasmid, pCRB33, was similarly able to confer bacteriophage resistance to a variety of *S. thermophilus* strains (Cocconcelli *et al.*, 1995). This is believed to be a natural process in both organisms since several HsdS domains (*i.e.* without the cognate REase and or MTase) have been found on the same or different native plasmids (Seegers *et al.*, 2000; Forde *et al.*, 1999; O'Sullivan *et al.*, 1999; Schouler *et al.*, 1998), but are also found in on the host chromosome (Seegers *et al.*, 2000; Schouler *et al.*, 1998). In addition, variable specificity

domains can be shuffled between different *hsdS* genes via recombination and can result in novel HsdS subunits with altered specificities (Fuller-Pace and Murray, 1986).

R-M systems confer an added benefit at the population level since they actually remove lytic phages from the environment that might have otherwise gone on to infect other R-M⁻ hosts that might be grown in co-culture. R-M systems are powerful defenses, however failure of either component can have dire consequences not only for the infected bacterium, but also for the population as a whole (for reviews, see Bickle and Kruger, 1993; Kruger and Bickle, 1983). At some frequency, a finite number of phage genomes will escape restriction and become modified by the bacterial MTase during replication. Once modified, the phage genome becomes impervious to the cognate REase and can then initiate its developmental program unimpeded. As a result, the progeny phages that result from this infection will be modified by the host MTase by default. Once released, the phages will be able to circumvent the R-M systems of neighboring bacteria in subsequent infections, thereby rendering this particular R-M system ineffective at the population level. The initial modification event is a random epigenetic event that leads to heritable resistance to the cognate REases and REases that share similar core recognition and/or cleavage sequences. The genetic capacity of the bacteriophage is unchanged, however. As such, the conferred resistance is host dependent. Invasion of a homologous, R-M⁺ host by the modified phage will lead to the generation of R-M resistant phages, whereas invasion of an R-M⁻ host will lead to the generation of R-M sensitive phages. The chances of evading restriction via modification of the genome are dependent on the R-M system and the number of restriction sites encoded on the phage genome. Since it has been found that the EOP of the phage decreases logarithmically as the number of sites

in the phage genome increases the elimination of even a single REase recognition site by deletion or point mutation can dramatically increase a phage's chances of evading the REase via this method (Moineau *et al.*, 1993; Powell and Davidson, 1986; for a review, see Wilson and Murray, 1991).

Industrial strains of *S. thermophilus* typically encode one or more R-M systems. As a result of these extreme selective pressures, phages have evolved a variety of genetic countermeasures that allow mutant derivatives to circumvent these defenses (for reviews, see Bickle and Kruger, 1993; Kruger and Bickle, 1983). Among these countermeasures are (i) the acquisition of methyltransferase genes from the host genome via recombination (Hill *et al.*, 1991b), (ii) the incorporation of modified nucleotides into bacteriophage DNA, (iii) the production of antagonistic proteins that inhibit the activity of bacterially-encoded restriction endonucleases, and (iv) the elimination of restriction endonuclease recognition sites throughout the genome (Moineau *et al.*, 1993). To date, none of these escape countermeasures have been observed in *S. thermophilus*.

In lactococci, the identification, characterization and, importantly, the functional exploitation of these and other defense systems has extended the utility of many industrially important strains, especially when used in rotation (Durmaz and Klaenhammer, 1995; Sing and Klaenhammer, 1993). This has been greatly facilitated by the fact that R-M systems are typically, although not exclusively, plasmid-encoded in lactococci. Of the ten R-M systems that have been identified in lactococci, eight have been plasmid-encoded (80%). In addition, several of these plasmids, such as pTR2030, are self-transmissible, which greatly facilitates the introduction of the phage resistance plasmids into lactococcal starter cultures (Klaenhammer and Sanozky, 1985). Increased

use of *S. thermophilus* starter cultures worldwide has resulted in an increased incidence of phage attacks, which has prompted the study of *S. thermophilus* R-M systems. The vast majority of R-M systems identified in *S. thermophilus* have been chromosomally encoded (70%), which has made them more difficult to clone and characterize (Table 1).

Abortive Infection. If a phage circumvents restriction, abortive infection (Abi) defense systems act as the second line of intracellular defense against bacteriophages (for reviews, see Klaenhammer, 1987; Klaenhammer *et al.*, 1991). In lactococci, abortive defense systems that interfere with many steps in the lytic life cycle have been identified, including genome replication, transcription, translation, encapsidation, and particle morphogenesis. Twenty-four genes have been shown to abort the replication of lactococcal bacteriophages (for reviews, see Coffey and Ross, 2002; Forde and Fitzgerald, 1999; Allison and Klaenhammer, 1998). The vast majority of these genes show no homology to one another or to any other entry in the GenBank database. Of the twelve phage species that have been identified, only three are responsible for the majority of industrial phage attacks (*i.e.* P335, c2, and 936) (Jarvis *et al.*, 1991). Based on this, the spectrum of phage protection conferred by any one of the Abi defenses may be rated as narrow or broad. Defenses with narrow specificity are confined to affect a single phage species (*e.g.* AbiB, AbiE, AbiH, and AbiJ); whereas defenses with broad specificity are able to affect constituents of two (*e.g.* AbiC, AbiD, AbiD1, AbiF, AbiG, AbiI, and AbiL) or three (AbiA, AbiK and AbiU) phage types. Not all of the defenses have been tested against all three phage species, however. It is important to note that these defenses may not be effective against all phages within a given species, but may only be effective

against a handful of phages within the species in question. Abi systems are typically characterized as acting either prior to- or at the level of genome replication (*i.e.* early) or after genome replication (*i.e.* late) (Garvey *et al.*, 1995). Native abortive defense mechanisms typically result in the death of the host cell, however it is not known if these systems kill the host by design (*i.e.* a form of altruism) or if this is simply a function of arresting phage development.

A single reference to native abortive infection in *S. salivarius* subsp. *thermophilus* NST5 has been described in the literature, however the putative defense system(s) have never been cloned or characterized (Larbi *et al.*, 1992). The activity was found to be temperature dependent, active at 42°C but not at 30°C, as measured by a significant increase of both plaque size and EOP at the non-permissive temperature. This temperature dependant activity of Abi systems had previously been shown in lactococci (Klaenhammer and Sanozky, 1985). Recently, the AbiA and AbiG abortive defense systems isolated from *L. lactis* subsp. *cremoris* AC8147 and LOC735, respectively, were tested for their ability to confer resistance to phages in *S. thermophilus* 4035 (Tangney and Fitzgerald, 2002). In this study, the AbiA system was shown to be effective at 30°C, but not at 37 or 42°C. It is important to note that 30°C is optimal for the growth of many lactococci. Unfortunately, the permissive temperature for AbiA function in *S. thermophilus* is well below its optimum for propagation and, importantly, the temperatures at which thermophilic processes are generally conducted during product manufacture (*i.e.* between 40 and 45°C). As seen against lactococcal phages, AbiA interfered with phage genome replication (Hill *et al.*, 1991a). BlastP analysis detected a putative reverse transcriptase domain (pfam00078) within the AbiA primary amino acid

structure, however the function of AbiA is not yet known. In contrast, the AbiG system failed to interfere with *S. thermophilus* phage replication at any temperature tested.

These two systems were specifically chosen because they are effective against lactococcal P335 phages, which share up to 60% sequence similarity at the nucleotide level with regions of *S. thermophilus* phages Sfi19 and Sfi21 genomic DNA (Chopin *et al.*, 2001).

RECENT ADVANCEMENTS IN GENOMICS:

Comparative Genomics. Our understanding of *S. thermophilus* bacteriophages has progressed rapidly since the release of six bacteriophage whole-genome sequences: DT1 (Tremblay and Moineau, 1999), ϕ O1205 (Stanley *et al.*, 1997), Sfi11 (Lucchini *et al.*, 1998), Sfi19 (Lucchini *et al.*, 1999a), Sfi21 (Lucchini *et al.*, 1999a), and ϕ 7201 (Stanley *et al.*, 2000). With regard to the genetic content and genomic organization of these phages, they exhibit significant similarities to other members of the λ super family of phages (Brüssow and Desiere, 2001). Bioinformatic analyses have provided significant insight into the evolution of *S. thermophilus* phages, revealing that their genomes are molecular mosaics assembled upon a relatively simple scaffold consisting of four independently evolving modules such that each module directs distinct developmental processes (*e.g.* DNA replication) (Lucchini *et al.*, 1999b). In addition, functionally coupled genes were found to be arranged into conserved clusters within these four modules, such that gene order was predicated by developmental order, or *vice versa*. These clusters were generally, but not exclusively, found to be arranged into putative operons such that they would be co-transcribed and, likely, co-regulated. Interestingly,

one or more distinct pathways encoded on alternative and interchangeable modules can carry out each developmental process (Lucchini *et al.*, 1999b). For example, two DNA replication modules have been identified among the various *S. thermophilus* phages: Sfi21-type and 7201-type. The Sfi21-type replication module is comprised of a single origin of DNA replication and several open reading frames that encode a putative helicase, a putative primase, and a number of other proteins of undetermined function (Lucchini *et al.*, 1999b). The phage 7201-type replication module, on the other hand, includes two distinct *oris* and encodes a probable single stranded DNA binding protein, a putative replication protein, a putative DnaC homologue, and a number of other proteins of undetermined function (Stanley *et al.*, 2000). The variants of the Sfi21-type genome replication module are found among the majority of industrial phage isolates (Brüssow *et al.*, 1994b).

These modules are subject to both horizontal and vertical evolution. The horizontal evolutionary component is primarily the result of recombination between interbreeding phage populations, including resident prophages and remnants thereof, but may also occur between an invading phage and the host genome (Desiere *et al.*, 1998). As a result of horizontal evolution, on the other hand, two parent phages produce a chimeric third. The vertical evolutionary component is the gradual accumulation of spontaneous mutations, predominantly point mutations, but also short insertions and deletions, passed from parent to progeny (Desiere *et al.*, 1998).

The availability of a growing number of bacteriophage and bacterial genomes has become an invaluable resource for the understanding these interactions. Bacteriophage genomes may be analyzed to identify conserved gene targets. Developmental pathways

and can be scrutinized to anticipate evolutionary escape routes. Analysis of bacterial sequences may be used to identify native defense systems or facilitate the removal of host-encoded factors involved in phage replication or evolution. Given this, special focus will be given to the impact of genomic data on the development of novel, genetically engineered solutions to the phage problem. These information-based strategies include the use of origin-conferred phage-encoded resistance, phage-derived antisense RNAs, and the overexpression of phage-encoded proteins, or mutant derivatives thereof.

Anti-Receptor Identification. Until recently, phage-host interactions remain poorly understood in most Gram-positive bacteria, including the LAB. Significant advances have been made in this area, largely due to insights garnered from comparative genomic analyses. Based on these analyses, several clues suggested that the *S. thermophilus* phage DT1-encoded *orf18* gene the phage anti-receptor (Tremblay and Moineau, 1999). First, *orf18* is located within the same region as the coliphage λ anti-receptor gene, *J* (Werts *et al.*, 1994). Second, the deduced proteins, gp18 from DT1 and gpJ from coliphage λ , have similar molecular weights and isoelectric points (pI). Finally, gp18 exhibits a modular organization of conserved and hyper-variable regions shared with the anti-receptor genes of T-even phages (Tétart *et al.*, 1996; 1998). This last point was explored further in a later study by Duplessis and Moineau (2001). The authors aligned the *orf18* gene from *S. thermophilus* phage DT1 with alleles from six other phages. The deduced gp18 proteins were divided into three distinct domains. The first, N-terminal domain (491 residues) was highly conserved among the seven phages, exhibiting 83-100% identity at the amino acid level between variants. The second domain

(approximately 400 residues) was found in only two of the seven phages (MD2 and DT2). Domain 2 also contained a short (133 residue) internal variable region (VR) called VR1. The second (central) domain was demarcated by two collagen-like repeats. The third, C-terminal domain was present in all seven phages, and contained another variable region (VR2) (145 residues). VR2 was also found in phages Sfi11, Sfi19, Sfi21, ϕ O1205, and ϕ 7201, the five other phages that have been completely sequenced.

The authors went on to use recombination-mediated domain swapping to generate chimeric variants of phage DT1 that had acquired the host range of MD4 to provide convincing biological evidence in support of their claim that *orf18* encoded the phage host specificity determinant (Duplessis and Moineau, 2001). Prior to this, a spontaneous deletion mutant of phage Sfi21, designated D3, had previously been isolated through routine serial propagation. In this case, the second domain between the collagen-like repeats III and I was deleted in the mutant, which indicated that these recombination hotspots contribute to the allelic diversity within the population (Bruttin and Brüssow, 1996). The nucleotide sequences encoding the collagen-like repeats had previously been suggested to be hotspots for recombination-mediated gene shuffling (Desiere *et al.*, 1998). These motifs consist of repeated amino acid triplets where glycine is the first residue in each triplet (GX₂)_n (Beck and Brodsky, 1998).

ENGINEERED DEFENSE SYSTEMS:

In addition to those native bacteriophage defenses described above, a wide variety of engineered phage resistance strategies have been constructed. With regard to the practical efficacy of antisense cassettes in the dairy environment, identification of target

genes that are effective against a variety of industrially relevant bacteriophages is of utmost importance. In many cases, these defense systems have been tested in the laboratory but have not been utilized during product manufacture due to consumer reluctance and/or government mandate (Scannerini, 2003).

Superinfection Exclusion and Immunity. Approximately one half of the sequenced bacterial genomes contain prophage-associated sequences (Lawrence *et al.*, 2001). Examination of these lysogens reveals that prophage or prophage-remnant sequences comprise between 3-10% of the total genomic content of lysogens (Brüssow and Hendrix, 2002). This extra genomic content significantly increases the metabolic burden of the host, which should significantly decrease the fitness of the lysogen relative to prophage-free strains. Interestingly, prophage-containing strains abound in nature. This is apparently due to the fact that prophages often provide benefits to the lysogen by encoding factors that may increase the fitness of the bacterium, such as the lysogenic conversion genes (Desiere *et al.*, 2002). The genes encoding lysogenic conversion functions are located between the lysin gene and the phage attachment site (Desiere *et al.*, 2002). In the pathogenic streptococci, prophages often encode virulence factors. For instance, the streptococcal erythrogenic toxin A gene is encoded by a variety of *S. pyogenes* prophages, including phage T12 (Yu and Ferretti, 1991). While prophages associated with LAB certainly do not encode virulence factors, they do encode genes that may provide some benefit to the lysogen. In the *S. thermophilus*, the lysogenic conversion genes are among the small handful of phage-encoded genes that are actively and consistently transcribed by the prophage (Ventura *et al.*, 2002).

From a phage resistance point of view, superinfection exclusion and immunity genes are well-characterized examples of beneficial genes associated with the prophages of Gram-positive bacteria, including lactococci (Bruttin *et al.*, 1997b; McGrath *et al.*, 2002). These genes protect lysogens from becoming infected with additional phages. In the *S. thermophilus* temperate phage Sfi21, RNAs specific for *orf203* were found to be the predominant phage-specific transcripts detected in Sfi21 lysogens (Ventura *et al.*, 2002). This gene, which is located upstream of the phage integrase, was shown to mediate superinfection exclusion when it was expressed from a high-copy-number plasmid, and resulted in significant protection from a diverse collection of lytic bacteriophages (Bruttin *et al.*, 1997b). In a related form of resistance, Durmaz *et al.* (2002) recently cloned two derivatives of the *cI* repressor from the lactococcal P335-type phage ϕ 31 into the high-copy-number plasmid, pTRKH2. The first construct (pTRKH2::*CI*-per1) harbored an ochre mutation in the *cI* gene after the first 128 amino acids of the predicted 180-amino-acid protein, whereas the second construct (pTRKH2::*CI*-per2) was completely devoid of the sequences downstream of the ochre mutation. The EOP of ϕ 31 was reduced to 10^{-6} in the presence of pTRKH2::*CI*-per1 construct, whereas EOP was further reduced to less than 10^{-7} in the presence of pTRKH2::*CI*-per2 construct. The authors went on to show that 12 of 16 heterologous lytic P335-type phages were completely inhibited by pTRKH2::*CI*-per2, while four phages were completely resistant to the defense system.

Antisense RNA. Antisense RNAs may be used to interfere with phage development by inhibiting the translation of phage-encoded genes necessary for normal

development. Mechanistically, antisense RNA hybridizes to the sense RNA strand and creates a translationally inactive double stranded RNA (dsRNA) molecule (for a review, see Inouye, 1988) (Figure 2). Formation of the dsRNA molecule silences gene expression through the cooperative action of one or more intermolecular mechanisms. If the antisense RNA includes sequences complementary to the ribosome-binding site (RBS), then the formation of dsRNA may mask the RBS, thereby preventing efficient ribosome loading and reducing translation of the gene of interest. Formation of dsRNA downstream of the RBS may also interfere with translation by sterically impeding, to some degree, the procession of the mRNA through the ribosome. In addition, the formation of dsRNA may destabilize the sense mRNA by promoting the action of dsRNA-specific ribonucleases. Lastly, if the gene of interest is transcribed on a polycistronic mRNA, then antisense targeting may also negatively impact the expression of translationally-coupled genes located downstream, causing pleotropic effects that might further inhibit bacteriophage proliferation.

Watson-Crick base pairings between the antisense RNA and its complementary, phage-encoded target mRNA(s) are initiated through a limited number of intermolecular nucleation events occurring within complementary unstructured regions, including 5' or 3' single-stranded tails and internal loops and/or bulges (Hjalt and Wagner, 1995; Kolb *et al.*, 2001). These associations are subsequently stabilized through progressive Watson-Crick base-pairings proximal to the initial nucleation site(s). During this process, intermolecular base pairing occurs at the expense of intramolecular interactions individually associated with both the antisense and target RNA molecules. Previous studies *in vitro* demonstrated that two strands of even short (less than 200-nt)

complementary RNAs become fixed in thermodynamic troughs and may not form double stranded RNA over the entire length of both molecules (Kolb *et al.*, 2001). The remaining single stranded regions were found to either remain unpaired or undergo extensive intramolecular associations. If this is also true *in vivo*, it is possible that a single antisense RNA may interact with and negatively impact more than one target RNA at a time, especially when the antisense RNA is exceptionally long, as is the case of the engineered varieties that have been routinely expressed in the dairy LAB for phage defense.

See Table 2 for a list of antisense RNA targets that were effective against lactococcal phages. In lactococci, six genes putatively involved in lactococcal P335-type phage genome replication were targeted with antisense RNA (McGrath *et al.*, 2001). The targeted genes were: *orf14* (encoding a putative topoisomerase), *orf15* (putative single-stranded DNA binding protein), *orf16* (putative replisome organizer), *orf18* (putative methylase), and two open reading frames 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 phages Q30, Q33, or ul36. In contrast, the expression of *orf16* and *orf17* conferred significant and highly variable resistance to all four phages, as measured by 0.5- to a 10^{-6} log reductions in EOP. Antisense RNA specific for *orf19* failed to inhibit any of the four phages. Kim *et al.* (1992) 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.* (1992) 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 (1991) 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.

Throughout these and other studies, the effectiveness of antisense RNA-based phage defense strategies has been highly variable, exhibiting both target- and phage-specific differences. See Table 3 for a list of antisense RNA targets that were ineffective against lactococcal phages. These observations raise questions about the key characteristics of an ideal 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, however, the target RNA must be essential for phage development, or at least critical for efficient synthesis or maturation of virulent progeny phages and/or their release in order to be effective. In general, antisense RNAs targeting early-expressed genes involved in genome replication (McGrath *et al.*, 2001) have been more effective targets than genes expressed later in the lytic cycle (McGrath *et al.*, 2001). It is important to note, however, that not all genes involved in genome replication are effective targets. Polzin *et al.* (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) were all ineffective in their ability to inhibit the replication of the lactococcal prolate-headed phage c2, regardless of the gene dosage tested. In this context, the failure of these strategies to inhibit phage replication may have been due to functional complementation by host-encoded factors or a more general recalcitrance to antisense RNA, which might be mediated by differences in phage c2-directed RNA metabolism.

Origin-Derived Phage-Encoded Resistance. Origin-derived phage-encoded resistance (PER) was first reported to be effective in *L. lactis* (Hill *et al.*, 1990). When a bacteriophage origin of genome replication (*ori*) is provided in *trans* on a recombinant plasmid, the origin acts as a molecular decoy that competes for and titrates away both bacteriophage- and host-derived replication factors that catalyze phage genome replication. As a result, the number of bacteriophage genomes replicated over the course of the lytic infection is reduced. In addition, the plasmid-associated replication factors catalyze a dramatic increase in plasmid copy-number. Origin-derived PER has been found to be highly dependent on gene dosage, and can be dependent on plasmid copy number (O'Sullivan *et al.*, 1993) or the number of copies of the *ori* that are cloned within the same plasmid (McGrath *et al.*, 2001).

Origin-derived PER has recently been exported to *S. thermophilus* strains. Foley *et al.* (1998) were the first to use origin-derived PER as a means of increasing phage resistance in *S. thermophilus*. They found that the phage Sfi21-derived *ori* conferred strong resistance to related phages, as measured by at least seven log cycle reductions in

the number of PFU/ml obtained when grown in broth. The authors pointed out that the phage Sfi21-derived *ori* shows 80% sequence similarity to the putative single-strand origin of the cryptic *S. thermophilus* plasmid, pST1. These results suggested that the phage- and plasmid-encoded *oris* might share a common ancestor.

More recently, Stanley *et al.* (2000) identified four loci from two different *S. thermophilus* phage genomes that were able provide origin-derived PER. These authors divided 11 phages into two replication groups: group I and group II. At least one of these phages in replication group I (*i.e.* phage O1205) encoded an Sfi21-type DNA replication module, while at least one member of the replication group II phages (*i.e.* phage 7201) encoded a different (non-Sfi21-type) genome replication module. It is not known, however if all of the group I or group II phages encoded variants of the Sfi21-type or 7201-type replication modules, respectively.

As seen in lactococci (O'Sullivan *et al.*, 1993), the presentation of the phage O1205-derived origin (*ori1205*) on a low-copy-number vector failed to provide protection from the homologous phage, but provided protection from group I type phages when cloned onto the high-copy-number plasmid pNZ8048 (Stanley *et al.*, 2000). The range of protection provided by this plasmid was phage-specific and the EOP for these phages ranged between 10^{-3} to less than 10^{-7} . Interestingly, the pORI1205 construct was also able to reduce the plaque diameter of three out of the five group II phages that were tested, but did not have any impact on EOP of any of these phages. These results suggested that a common host encoded protein is required for the replication of the two phage groups, albeit to varying degrees. In addition, one of the phage O1205-derived PER constructs (p1205-*orf9*) provided significant protection to bacteriophages from both

replication groups in one host (*i.e.* strain CNRZ1205-3), however it failed to provide detectable levels of phage protection in another background (*i.e.* strain 4035). These results clearly implicated the importance of certain host-encoded factors in phage replication and suggested that the available levels of these factors were also critical to confer phage resistance (Stanley *et al.*, 2000).

Hurdle Techniques: Explosive Delivery of Antisense RNA. The food industry routinely uses a series of intrinsic barriers, such as high salt content, low pH, and heat processing in order to control the outgrowth of and toxin production by food-borne pathogens. These barriers have come to be referred to as hurdles (Leistner and Rodel, 1976). It has been found that these hurdles have synergistic (*i.e.* non-additive) effects when they are used together in combination (*e.g.* pasteurization *and* high salt content) such that the level of protection provided is significantly greater than if the hurdles were used individually (*e.g.* pasteurization *or* high salt content). In fermented dairy products, especially cheeses, some of the most important hurdles for pathogen inhibition are high salt content, which is added to the fermentation substrate, low pH, and low carbohydrate availability. The low pH and carbohydrate availability are a direct result of the growth of the microflora, especially the starter culture, and are perhaps the most important characteristics in a safe fermented product. As such, phage infections during food fermentations are important not only from quality control and stability perspectives, but also one of safety as well.

It has recently been demonstrated that the hurdle concept is also applicable to phage defense strategies. Walker and Klaenhammer (2000) 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 $\phi 31$ when expressed from the high-copy-number vector pTRKH2. The presentation of the putative origin of replication from phage $\phi 31$ (*ori31*) on the low-copy-number vector pSA3, however, reduced the EOP to 0.36 ± 0.92 . 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 the *ori31*-containing plasmid. Following $\phi 31$ invasion, the expression of phage-derived DNA replication factors triggered the explosive replication of the plasmid from *ori31*, and produced inhibiting levels of antisense RNA (Walker and Klaenhammer, 2000). The EOP of these constructs depended on the gene targeted by antisense RNA and ranged from 0.11 ± 0.03 to 0.33 ± 0.06 . From these results, the authors proposed that antisense RNAs must be delivered in sufficiently high dosage and at the appropriate time during the lytic cycle in order to be an effective phage defense strategy.

Justification. As the demand for fermented food products made with strains of *S. thermophilus* has increased, so has the incidence and severity of bacteriophage attacks against these strains. In addition, strains of LAB are being further exploited for the manufacture of industrial chemicals (*e.g.* L-lactate) and employed as vehicles for the delivery of biologics (*e.g.* vaccines, enzymes). With the expansion of fermentation and bioprocessing systems reliant on LAB, disruption by bacteriophages remains a growing concern. These persistent pressures necessitate the continued development of starter cultures with enhanced phage resistance properties. This dissertation describes the

construction and characterization of a variety of information-based systems to defend against bacteriophage attack. Use of these phage defense systems will prove invaluable to protecting bioprocessing strains of LAB that are expected to perform consistently and over extended time frames within industrial applications.

REFERENCES

Allison, G. E., and T. R. Klaenhammer. 1998. Phage resistance mechanisms in lactic acid bacteria. *Int. Dairy J.* **8**:207-226.

Beck, K., and B. Brodsky. 1998. Supercoiled protein motifs: the collagen triple-helix and the α -helical coiled coil. *J. Struct. Biol.* **122**:17-29.

Bester, B. H., and S. H. Lombard. 1975. Protection of starter cultures against bacteriophages by propagation in a phage-resistant medium. *South African J. Dairy Technol.* **7**:235-450.

Benbadis, L., J. R. Garel, and D. L. Hartley. 1991. Purification, properties, and sequence specificity of SsII, a new type II restriction endonuclease from *Streptococcus salivarius* subsp. *thermophilus*. *Appl. Environ. Microbiol.* **57**:3677-3678.

Bickle, T.A., and D. H. Kruger. 1993. Biology of DNA restriction. *Microbiol. Rev.* **57**:434-450.

Binetti, A. G., and J. A. Reinheimer. 2000. Thermal and chemical inactivation of indigenous *Streptococcus thermophilus* bacteriophages isolated from Argentinean dairy plants. *J. Food Prot.* **63**:509-515.

Bouchard, J. D., and S. Moineau. 2000. Homologous recombination between a lactococcal bacteriophage and the chromosome of its host strain. *Virology*. **270**:65-75.

Broadbent, J. R., D. J. McMahon, D. L. Welker, C. J. Oberg, and S. Moineau. 2003. Biochemistry, genetics, and applications of exopolysaccharide production in *Streptococcus thermophilus*: a review. *J. Dairy Sci.* **86**:407-423.

Burrus, V., C. Bontemps, B. Decaris, and G. Guédon. 2001. Characterization of a novel type II restriction-modification system, Sth368I, encoded by the integrative element ICESt1 of *Streptococcus thermophilus* CNRZ368. *Appl. Environ. Microbiol.* **67**:1522-1528.

Burrus, V., G. Pavlovic, B. Decaris, and G. Guedon. 2002. The ICESt1 element of *Streptococcus thermophilus* belongs to a large family of integrative and conjugative elements that exchange modules and change their specificity of integration. *Plasmid*. **48**:77-97.

Brüssow, H., M. Fremont, A. Bruttin, J. Sidoti, A. Constable, and V. Fryder V. 1994a. Detection and classification of *Streptococcus thermophilus* bacteriophages isolated from industrial milk fermentation. *Appl. Environ. Microbiol.* **60**:4537-4543.

Brüssow, H., A. Probst, M. Fremont, and J. Sidoti. 1994b. Distinct *Streptococcus thermophilus* bacteriophages share an extremely conserved DNA fragment. *Virology*. **200**:854-857.

Brüssow, H., A. Bruttin, F. Desiere, S. Lucchini, and S. Foley. 1998. Molecular ecology and evolution of *Streptococcus thermophilus* bacteriophage—a review. *Virus Genes*. **16**:95-109.

Brüssow, H., and F. Desiere. 2001. Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. *Mol. Microbiol.* **39**:213-222.

Brüssow, H., and R. W. Hendrix. 2002. Phage genomics: Small is beautiful. *Cell*. **108**:13-16.

Bruttin, A., and H. Brüssow. 1996. Site-specific spontaneous deletions in three genome regions of a temperate *Streptococcus thermophilus* phage. *Virology*. **219**:96-104.

Bruttin, A., F. Desiere, N. d'Amico, J. P. Guerin, J. Sidoti, B. Huni, S. Lucchini, and H. Brüssow. 1997a. Molecular ecology of *Streptococcus thermophilus* bacteriophage infections in a cheese factory. *Appl. Environ. Microbiol.* **63**:3144-3150.

Bruttin, A., F. Desiere, S. Lucchini, S. Foley, and H. Brüssow. 1997b.

Characterization of the lysogeny DNA module from the temperate *Streptococcus thermophilus* bacteriophage Sfi21. *Virology*. **233**:136-148.

Budde-Niekiet, A., and M. Teuber. 1987. Electron microscopy of the adsorption of bacteriophages to lactic acid streptococci. *Milchwissenschaft*. **42**:551-553.

Carminati, D., and G. Giraffa. 1992. Evidence and characterization of temperate bacteriophages in *Streptococcus salivarius* subsp. *thermophilus* St18. *J. Dairy Res.* **59**:71-79.

Chopin, A., A. Bolotin, A. Sorokin, S. D. Ehrlich, and M. Chopin. 2001. Analysis of six prophages in *Lactococcus lactis* IL1403: different genetic structure of temperate and virulent phage populations. *Nucleic Acids Res.* **29**:644-651.

Chung, D. K., S. K. Chung, and C. A. Batt. 1992. Antisense RNA directed against the major capsid protein of *Lactococcus lactis* subsp. *cremoris* bacteriophage F4-1 confers partial resistance to the host. *Appl. Microbiol. Biotechnol.* **37**:79-83.

Cocconcelli, P. S., M. L. Callegari, D. Porro, and L. Morelli. Plasmid recombination mediated by IS-like structures in *Streptococcus thermophilus* T003. *In* Abstracts of Posters, European Commission Biotechnology and FAIR Programmes Conference on

Lactic Acid Bacteria: From Fundamental Research to Innovative Applications, Abstract F-8. Cork, Ireland, 22-26 October, 1995.

Coffey, A., and R. P. Ross. 2002. Bacteriophage-resistance systems in dairy starter strains: molecular analysis to application. *Antonie van Leeuwenhoek*. **82**:303-321.

Cogan, T. M., N. Peitersen, and R. L. Sellars. 1991. Starter systems, p.16-23. *In* Bulletin of the International Dairy Federation, no. 263/1991. Practical phage control. International Dairy Federation, Brussels.

Courtin, P., V. Monnet, and F. Rul. 2002. Cell-wall proteinases PrtS and PrtB have a different role in *Streptococcus thermophilus/Lactobacillus bulgaricus* mixed cultures in milk. *Microbiology*. **148**:3413-3421.

Crittenden, R. G., N. R. Martinez, and M. J. Playne. 2003. Synthesis and utilization of folate by yogurt starter cultures and probiotic bacteria. *Int. J. Food Microbiol.* **80**:217-222.

Daly, C. 1983. The use of mesophilic cultures in the dairy industry. *Antonie Van Leeuwenhoek*. **49**:297-312.

D'Herrelle, F. 1917. Sur un microbe invisible antagoniste des bacilles dysenteriques. *Comp. Rend. Acad. Sci.* **165**:373-375. *In* T. D. Brock (ed.), *Milestones in Microbiology*:

1556 to 1940. American Society for Microbiology Press, Washington, D. C., 1998, pp. 157.

Desiere, F., S. Lucchini, A. Bruttin, M. C. Zwahlen, and H. Brüssow. 1997. A highly conserved DNA replication module from *Streptococcus thermophilus* phages is similar in sequence and topology to a module from *Lactococcus lactis* phages. *Virology*. **234**:372-382.

Desiere, F., S. Lucchini, and H. Brüssow. 1998. Evolution of *Streptococcus thermophilus* bacteriophage genomes by modular exchanges followed by point mutations and small deletions and insertions. *Virology*. **241**:345-356.

Desiere, F., S. Lucchini, C. Canchaya, M. Ventura, and H. Brüssow. 2002. Comparative genomics of phages and prophages in lactic acid bacteria. *Antonie Van Leeuwenhoek*. **82**:73-91.

Desmazeaud, M. 1983. L'état des connaissances en matière de nutrition des bactéries lactiques. *Lait*. **63**:267-316.

Duplessis, M., and S. Moineau. 2001. Identification of a genetic determinant responsible for host specificity in *Streptococcus thermophilus* bacteriophages. *Mol. Microbiol.* **41**:325-336.

- Durmaz, E., and T. R. Klaenhammer.** 1995. A starter culture rotation strategy incorporating paired restriction/modification and abortive infection bacteriophage defenses in a single *Lactococcus lactis* strain. Appl. Environ. Microbiol. **61**:1266-1273.
- Durmaz, E., and T. R. Klaenhammer.** 2000. Genetic analysis of chromosomal regions of *Lactococcus lactis* acquired by recombinant lytic phages. Appl. Environ. Microbiol. **66**:895-903.
- Durmaz, E., S. M. Madsen, H. Israelsen, and T. R. Klaenhammer.** 2002. *Lactococcus lactis* lytic bacteriophages of the P335 group are inhibited by overexpression of a truncated CI repressor. J. Bacteriol. **184**:6532-6544.
- Fayard, B., M. Haefliger, and J. P. Accolas.** 1993. Interactions of temperate bacteriophages of *Streptococcus salivarius* subsp. *thermophilus* with lysogenic indicators affect phage DNA restriction patterns and host ranges. J. Dairy Res. **60**:385-399.
- Foley, S., S. Lucchini, M. C. Zwahlen, and H. Brüssow.** 1998. A short noncoding viral DNA element showing characteristics of a replication origin confers bacteriophage resistance to *Streptococcus thermophilus*. Virology. **250**:377-387.
- Forde, A., and G. F. Fitzgerald.** 1999. Bacteriophage defense systems in lactic acid bacteria. Antonie van Leeuwenhoek. **76**:89-113.

Forde, A., C. Daly, and G. F. Fitzgerald. 1999. Identification of four phage resistance plasmids from *Lactococcus lactis* subsp. *cremoris* HO2. Appl. Environ. Microbiol. **65**:1540-1547.

Fuller-Pace, F. V., and N. E. Murray. 1986. Two DNA recognition domains of the specificity polypeptides of a family of type I restriction enzymes. Proc. Natl. Acad. Sci. USA. **83**:9368-9372.

Garbutt, K. C., J. Kraus, and B. L. Geller. 1997. Bacteriophage resistance in *Lactococcus lactis* engineered by replacement of a gene for a bacteriophage receptor. J. Dairy Sci. **80**:1512-1519.

Garvey, P., D. van Sinderen, D. P. Twomey, C. Hill, and G. F. Fitzgerald. 1995. Molecular genetics of bacteriophage and natural phage defense systems in the genus *Lactococcus*. In. Dairy J. **5**:905-947.

Gasson, M. J. 1996. Lytic systems in lactic acid bacteria and their bacteriophages. Antonie Van Leeuwenhoek. **70**:147-159.

Geis, A., H. A. Demerdash, and K. J. Heller. 2003. Sequence analysis and characterization of plasmids from *Streptococcus thermophilus*. Plasmid. **50**: 53-69.

Geller, B. L., R. G. Ivey, J. E. Trempey, and B. Hettinger-Smith. 1993. Cloning of a chromosomal gene required for phage infection in *Lactococcus lactis* subsp. *lactis* C2. J. Bacteriol. **175**:5510-5519.

Grobben, G. J., I. Chin-Joe, V. A. Kitzen, I. C. Boels, F. Boer, J. Sikkema, M. R. Smith, and J. A. M. Bont. 1998. Enhancement of exopolysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 with a simplified defined medium. Appl. Environ. Microbiol. **64**:1333-1337.

Guedon, G., F. Bourgoïn, M. Pebay, Y. Roussel, C. Colmin, J. M. Simonet, and B. Decaris. 1995. Characterization and distribution of two insertion sequences, IS1191 and iso-IS981, in *Streptococcus thermophilus*: does intergeneric transfer of insertion sequences occur in lactic acid bacteria co-cultures? Mol. Microbiol. **16**:69-78.

Guimont, C., P. Henry, and G. Linden. 1993. Restriction/modification in *Streptococcus thermophilus*: isolation and characterization of a type II restriction endonuclease Sth455I. Appl. Microbiol. Biotechnol. **39**:216-220.

Gulstrom, T. J., L. E. Pearce, W. E. Sandine and P. R. Elliker. 1979. Evaluation of commercial phage inhibitory media. J. Dairy Sci. **62**:208-221.

Hardie, J. M. 1994. *Streptococcus*. In J. G. Holt (ed.), Bergy's manual of determinative bacteriology, 9th edition. Williams and Wilkins. Baltimore, MD.

- Heap, H. A., and R. C. Lawrence.** 1976. The selection of starter strains for cheese making. N. Z. J. Dairy Sci. Technol. **11**:16-53.
- Herman, R. E., and L. L. McKay.** 1985. Isolation and partial characterization of plasmid DNA from *Streptococcus thermophilus*. Appl. Environ. Microbiol. **50**:1103-1106.
- Hill, C., L. A. Miller, and T. R. Klaenhammer.** 1990. Cloning, expression, and sequence determination of a bacteriophage fragment encoding bacteriophage resistance in *Lactococcus lactis*. J. Bacteriol. **172**:6419-6426.
- Hill, C., I. J. Massey, and T. R. Klaenhammer.** 1991a. A rapid method to characterize lactococcal bacteriophage genomes. Appl. Environ. Microbiol. **57**:283-288.
- Hill, C., L. A. Miller, and T. R. Klaenhammer.** 1991b. In *vivo* genetic exchange of a functional domain from a type IIA methylase between lactococcal plasmid pTR2030 and a virulent bacteriophage. J. Bacteriol. **173**:4363-4370.
- Hjalt, T. A., and E. G. Wagner.** 1995. Bulged-out nucleotides in an antisense RNA are required for rapid target RNA binding in *vitro* and inhibition in *vivo*. Nucleic Acids Res. **23**:580-587.

Huggins, A. R. 1984. Progress in dairy starter culture technology. Food Technol. **38**:41.

Hull, R. R. 1985. Recent developments in the genetics of lactic acid bacteria. CSIRO Food Res. Q. **45**:40-46.

Husson-Kao, C., J. Mengaud, J. C. Gripon, L. Benbadis, and M. P. Chapot-Chartier. 2000a. Characterization of *Streptococcus thermophilus* strains that undergo lysis under unfavorable environmental conditions. Int. J. Food Microbiol. **55**:209-213.

Husson-Kao, C., J. Mengaud, B. Cesselin, D. van Sinderen, L. Benbadis, and M. P. Chapot-Chartier. 2000b. The *Streptococcus thermophilus* autolytic phenotype results from a leaky prophage. Appl. Environ. Microbiol. **66**:558-565.

Inouye, M. 1988. Antisense RNA: its functions and applications in gene regulation—a review. Gene. **72**:25-34.

Ivanowski, D. I. 1892. On two diseases of tobacco. Sel' Khoz. Lesov. **169**:108-121; an English translation appears as an appendix to S. S. Hughes, 1972. In The origins and development of the concept of the virus in the late nineteenth century. Ph.D. thesis, London University.

- Jarvis, A. W., G. F. Fitzgerald, M. Mata, A. Mercenier, H. Neve, I. B. Powell, C. Ronda, M. Saxelin, and M. Teuber.** 1991. Species and type phages of lactococcal bacteriophages. *Intervirology*. **32**:2-9.
- Jensen, R. G.** (ed.). 1995. Handbook of milk composition. Academic Press, New York, NY.
- Kim, J. H., and C. A. Batt.** 1991. Antisense RNA mediated bacteriophage resistance in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **57**:1039-1045.
- Kim, S. G., Y. C. Bor, and C. A. Batt.** 1992. Bacteriophage resistance in *Lactococcus lactis* spp. *lactis* using antisense ribonucleic acid. *J. Dairy Sci.* **75**:1761-1767.
- Klaenhammer, T. R.** 1984. Interactions of bacteriophages with lactic streptococci, pp. 1-29. *In* A. I. Laskin (ed.), *Advances in Applied Microbiology*, Volume 30. Academic Press, New York, NY.
- Klaenhammer, T. R., and R. Sanozky.** 1985. Conjugal transfer from *Streptococcus lactis* ME2 of plasmids encoding phage resistance, nisin resistance and lactose-fermenting ability: evidence for a high-frequency conjugative plasmid responsible for abortive infection of virulent bacteriophage. *J. Gen. Microbiol.* **131**:1531-1541.
- Klaenhammer, T.R.** 1987. Plasmid-directed mechanisms for bacteriophage defense in lactic streptococci. *FEMS Microbiol. Rev.* **46**:313-325.

Klaenhammer, T. R. 1989. Genetic characterization of multiple mechanisms of phage defense from a prototype phage-insensitive strain *Lactococcus lactis* ME2. *J. Dairy Sci.* **72**:3429-3443.

Klaenhammer, T.R., D. Romero, W. Sing, and C. Hill. 1991. Molecular analysis of pTR2030 gene systems that confer phage resistance to lactococci, pp. 124-130. *In* Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci. G. Dunny, P. Cleary, and L. McKay (ed). American Society for Microbiology Press, Washington, D. C.

Klaenhammer, T. R., and G. F. Fitzgerald. 1994. Bacteriophage and bacteriophage resistance, pp. 106-168. *In* M. J. Gasson and W. M. de Vos (ed.), Genetics and biotechnology of lactic acid bacteria. Chapman and Hall, London, England.

Kolars, J. C., M. D. Levitt, M. Aouji, and D. A. Savaiano. 1984. Yogurt—an autodigesting source of lactose. *N. Engl. J. Med.* **310**:1-3.

Kolb, F. A., E. Westhof, C. Ehresmann, B. Ehresmann, E. Gerhart, H. Wagner, and P. Romby. 2001. Bulged residues promote the progression of a loop–loop interaction to a stable and inhibitory antisense–target RNA complex. *Nucleic Acids Res.* **29**:3145-3153.

Krishnaswamy, K., and N. K. Madhavan. 2001. Importance of folate in human nutrition. *Br. J. Nutr. Suppl.* **2**:S115-124.

Kruger, D. H., and T. A. Bickle. 1983. Bacteriophage survival: multiple mechanisms for avoiding the deoxyribonucleic acid restriction systems of their hosts. *Microbiol. Rev.* **47**:345-360.

Kunji, E. R., I. Mierau, A. Hagting, B. Poolman, and W. N. Konings. 1996. The proteolytic systems of lactic acid bacteria. *Antonie Van Leeuwenhoek.* **70**:187-221.

Larbi, D., B. Decaris, and J. M. Simonet. 1992. Different bacteriophage resistance mechanisms in *Streptococcus salivarius* subsp. *thermophilus*. *J. Dairy Res.* **59**: 349-357.

Lawrence, J. G., R. Hendrix, and S. Casjens. 2001. Where are the pseudogenes in bacterial genomes? *Trends Microbiol.* **9**:535-540.

Leistner, L., and W. Rodel. 1976. The stability of intermediate moisture foods with respect to microorganisms, pp. 120-137. *In* R. Davies, G. G. Birch, and K. J. Parker (ed.), *Intermediate moisture foods*. Applied Science Publication, London, England.

Le Marrec, C., D. van Sinderen, L. Walsh, E. Stanley, E. Vlegels, S. Moineau, P. Heinze, G. Fitzgerald, and B. Fayard. 1997. Two groups of bacteriophages infecting *Streptococcus thermophilus* can be distinguished on the basis of mode of packaging and

genetic determinants for major structural proteins. Appl. Environ. Microbiol. **63**:3246-3253.

Letort, C., and V. Juillard. 2001. Development of a minimal chemically defined medium for the exponential growth of *Streptococcus thermophilus*. J. Appl. Microbiol. **91**:1023-1029.

Loeffler, F., and P. Frosch. 1898. Berichte der Kommission zur Erforschung der Maul- und Klauenseuche bei dem Institut für Infektionskrankheiten. **23**:371-391. In T. D. Brock (ed.), Milestones in Microbiology: 1556 to 1940, American Society for Microbiology Press, Washington, D. C., 1998, pp. 149.

Lowrie, R. J., and L. E. Pearce. 1971. The plating efficiency of bacteriophages of lactic streptococci. N. Z. J. Dairy Sci. Technol. **6**:166-171.

Lubbers, M. W., N. R. Waterfield, T. P. Beresford, R. W. Le Page, and Jarvis AW. 1995. Sequencing and analysis of the prolate-headed lactococcal bacteriophage c2 genome and identification of the structural genes. Appl. Environ. Microbiol. **61**:4348-4356.

Lucchini, S., F. Desiere, and H. Brüssow. 1998. The structural gene module in *Streptococcus thermophilus* bacteriophage ϕ Sfil1 shows a hierarchy of relatedness to *Siphoviridae* from a wide range of bacterial hosts. Virology. **246**:63-73.

Lucchini, S., F. Desiere, and H. Brüssow. 1999a. The genetic relationship between virulent and temperate *Streptococcus thermophilus* bacteriophages: whole genome comparison of *cos*-site phages Sfi19 and Sfi21. *Virology*. **260**:232-243.

Lucchini, S., F. Desiere, and H. Brüssow. 1999b. Comparative genomics of *Streptococcus thermophilus* phage species supports a modular evolution theory. *J. Virology*. **73**:8647-8656.

Lucchini, S., J. Sidoti, and H. Brüssow. 2000. Broad-range bacteriophage resistance in *Streptococcus thermophilus* by insertional mutagenesis. *Virology*. **275**:267-277.

Maguin, E., H. Prevost, S. D. Ehrlich, and A. Gruss. 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *J. Bacteriol.* **178**:931-5.

Maniloff, J., S. P. Cadden, and R. M. Putzrath. 1981. Maturation of an enveloped budding phage: mycoplasmavirus L2. *Prog. Clin. Biol. Res.* **64**:503-513.

Martini, M. C., E. C. Lerebours, W. J. Lin, S. K. Harlander, N. M. Barrada, J. M. Antoine, and D. A. Savaiano. 1991. Strains and species of lactic acid bacteria in fermented milks (yogurts): effect on *in vivo* lactose digestion. *Am. J. Clin. Nutr.* **54**:1041-1046.

Matalon, M. E., and W. E. Sandine. 1986. *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and yogurt: a review. Cult. Dairy Prod. J. **21**:6-23.

McGrath, S., G. F. Fitzgerald, and D. van Sinderen. 2001. Improvement and optimization of two engineered phage resistance mechanisms in *Lactococcus lactis*. Appl. Environ. Microbiol. **67**:608-616.

McGrath, S., G. F. Fitzgerald, D. van Sinderen. 2002. Identification and characterization of phage-resistance genes in temperate lactococcal bacteriophages. Mol. Microbiol. **43**:509-520.

Moineau, S., S. Pandian, and T.R. Klaenhammer. 1993. Restriction/modification systems and restriction endonucleases are more effective on lactococcal bacteriophages that have emerged recently in the dairy industry. Appl. Environ. Microbiol. **59**:197-202.

Moineau, S., S. A. Walker, B. J. Holler, E. R. Vedamuthu, and P. A. Vandenberg. 1995. Expression of a *Lactococcus lactis* phage resistance mechanism by *Streptococcus thermophilus*. Appl. Environ. Microbiol. **61**:2461-2466.

Moineau, S., M. Borkaev, B. J. Holler, S. A. Walker, J. K. Kondo, E. R. Vedamuthu, and P. A. Vandenberg. 1996. Isolation and characterization of lactococcal bacteriophages from cultured buttermilk plants in the United States. J. Dairy Sci. **79**:2104-2111.

Monteville, M. R., B. Ardestani, and B. L. Geller. 1994. Lactococcal bacteriophages require a host cell wall carbohydrate and a plasma membrane protein for adsorption and ejection of DNA. *Appl. Environ. Microbiol.* **60**:3204-3211.

Nakayama, S., N. D. Moncrief, and R. H. Kretsinger. 1992. Evolution of EF-hand calcium-modulated proteins. II. Domains of several subfamilies have diverse evolutionary histories. *J. Mol. Evol.* **34**:416-448.

Olson, N. F. 1969. Ripened semisoft cheeses. Pfizer Cheese Monographs, vol . 4. Pfizer, Inc., New York, NY.

O'Sullivan, D. J., C. Hill, and T. R. Klaenhammer. 1993. Effect of increasing the copy number of bacteriophage origins of replication in *trans*, on incoming-phage proliferation. *Appl. Environ. Microbiol.* **59**:2449-2456.

O'Sullivan, D., A. Coffey, G. F. Fitzgerald, C. Hill, and R. P. Ross. 1998. Design of a phage-insensitive lactococcal dairy starter via sequential transfer of naturally occurring conjugative plasmids. *Appl. Environ. Microbiol.* **64**:4618-4622.

O'Sullivan, T., D. van Sinderen, and G. Fitzgerald. 1999. Structural and functional analysis of pCI65st, a 6.5 kb plasmid from *Streptococcus thermophilus* NDI-6. *Microbiology.* **145**:127-34.

Pebay, M., C. Colmin, G. Guedon, C. DeGasperi, B. Decaris, and J. M. Simonet.

1992. Detection of intraspecific DNA polymorphism in *Streptococcus salivarius* subsp. *thermophilus* by a homologous rDNA probe. Res. Microbiol. **143**:37-46.

Poch, M. T., G. A. Somkuti, and D. K. Y. Solaiman. 1997. Sth132I, a novel class-IIS restriction endonuclease of *Streptococcus thermophilus* ST132. Gene. **195**:201-206.

Polzin, K. M. , L. J. Collins, M. W. Lubbers, and A. W. Jarvis. Effect of various antisense mRNAs on bacteriophage c2 replication. In Abstracts of Posters at the Fifth Symposium on Lactic Acid Bacteria: Genetics, Metabolism, and Applications, Abstract F2. Veldhoven, The Netherlands, 8-12 October, 1996.

Powell, I. A., and B. E. Davidson. 1986. Resistance to *in vitro* restriction of DNA from lactic streptococcal bacteriophage c6A. Appl. Environ. Microbiol. **51**:1358-1360.

Quiberoni, A., J. I. Stiefel, and J. A. Reinheimer. 2000. Characterization of phage receptors in *Streptococcus thermophilus* using purified cell walls obtained by a simple protocol. J. Appl. Microbiol. **86**:1059-1065.

Rajagopal, S. N., and W. E. Sandine. 1990. Associative growth and proteolysis of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in skim milk. J. Dairy Sci. **73**:894-899.

Reid, G., M. E. Sanders, H. R. Gaskins, G. R. Gibson, A. Mercenier, R. Rastall, M. Roberfroid, I. Rowland, C. Cherbut, and T. R. Klaenhammer. 2003. New scientific paradigms for probiotics and prebiotics. *J. Clin. Gastroenterol.* **37**:105-108.

Reinbold, G. W. 1963. Italian cheese varieties. Pfizer Cheese Monographs, vol 1. Pfizer, Inc., New York, NY.

Roberts, R. J., M. Belfort, T. Bestor, A. S. Bhagwat, T. A. Bickle, J. Bitinaite, R. M. Blumenthal, S. K. Degtyarev, D. T. Dryden, K. Dybvig, K. Firman, E. S. Gromova, R. I. Gumpert, S. E. Halford, S. Hattman, J. Heitman, D. P. Hornby, A. Janulaitis, A. Jeltsch, J. Josephsen, A. Kiss, T. R. Klaenhammer, I. Kobayashi, H. Kong, D. H. Kruger, S. Lacks, M. G. Marinus, M. Miyahara, R. D. Morgan, N. E. Murray, V. Nagaraja, A. Piekarowicz, A. Pingoud, E. Raleigh, D. N. Rao, N. Reich, V. E. Repin, E. U. Selker, P. C. Shaw, D. C. Stein, B. L. Stoddard, W. Szybalski, T. A. Trautner, J. L. Van Etten, J. M. Vitor, G. G. Wilson, and S. Y. Xu. 2003a. A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Res.* **31**:1805-1812.

Roberts, R. J., T. Vincze, J. Posfai, and D. Macelis. 2003b. REBASE: restriction enzymes and methyltransferases. *Nucleic Acids Res.* **31**:418-420.

Ross, R. P., S. Morgan, and C. Hill. 2002. Preservation and fermentation: past, present and future. *Int. J. Food Microbiol.* **79**:3-16.

Roussel, Y., F. Bourgoïn, G. Guedon, M. Pebay, and B. Decaris. 1997. Analysis of the genetic polymorphism between three *Streptococcus thermophilus* strains by comparing their physical and genetic organization. *Microbiology.* **143**:1335-1343.

Sable, S., and S. Lortal. 1995. The lysins of bacteriophages infecting lactic acid bacteria. *Appl. Microbiol. Biotechnol.* **43**:1-6.

Sanders, M. E., P. J. Leonhard, W. D. Sing, and T. R. Klaenhammer. 1986. Conjugal strategy for construction of fast-acid producing, bacteriophage resistant lactic streptococci for use in dairy fermentations. *Appl. Environ. Microbiol.* **52**:1001-1007.

Scannerini, S. 2003. The GMO war. *Riv. Biol.* **96**:181-187.

Schouler, C., M. Gautier, D. Ehrlich, and M. C. Chopin. 1998. Combinational variation of restriction modification in *Lactococcus lactis*. *J. Mol. Microbiol.* **28**:169-178.

Seegers, J. F., D. van Sinderen, and G. F. Fitzgerald. 2000. Molecular characterization of the lactococcal plasmid pCIS3: natural stacking of specificity subunits of a type I restriction/modification system in a single lactococcal strain. *Microbiology.* **146**:435-443.

Sing, W. D., and T. R. Klaenhammer. 1993. A strategy for rotation of different bacteriophage defenses in a lactococcal single-strain starter culture system. Appl. Environ. Microbiol. **59**:365-372.

Solow, B. T., and G. A. Somkuti. 2001. Molecular properties of *Streptococcus thermophilus* plasmid pER35 encoding a restriction modification system. Curr. Microbiol. **42**:122-128.

Solaiman, D. K. Y., and G. A. Somkuti. 1990. Isolation and characterization of a type II restriction endonuclease from *Streptococcus thermophilus*. FEMS Microbiol. Lett. **67**:261-266.

Solaiman, D. K. Y., and G. A. Somkuti. 1991. A type II restriction endonuclease of *Streptococcus thermophilus* ST117. FEMS Microbiol. Lett. **80**:75-80.

Stanley, E., G. F. Fitzgerald, M. C. Le Marrec, B. Fayard, and D. van Sinderen. 1997. Sequence analysis and characterization of ϕ O1205, a temperate bacteriophage infecting *Streptococcus thermophilus* CNRZ1205. Microbiology. **143**:3417-3429.

Stanley, E., L. Walsh, A. van der Zwet, G. F. Fitzgerald, and D. van Sinderen. 2000. Identification of four loci isolated from two *Streptococcus thermophilus* phage genomes responsible for mediating bacteriophage resistance. FEMS Microbiol. Lett. **182**:271-277.

Tangney, M., and G. F. Fitzgerald. 2002. AbiA, a lactococcal abortive infection mechanism functioning in *Streptococcus thermophilus*. Appl. Environ. Microbiol. **68**:6388-6391.

Tétart, F., R. Repolla, C. Monod, and H. M. Krisch. 1996. Bacteriophage T4 host range is expanded by duplication of a small domain of the tail fiber adhesin. J. Mol. Biol. **258**:726-731.

Tétart, F., C. Desplats, and H. M. Krisch. 1998. Genome plasticity in the distal tail fiber locus of the T-even bacteriophage recombination between conserved motifs swaps adhesin specificity. J. Mol. Biol. **282**:543-556.

Thunell, R. K., and W. E. Sandine. 1985. Types of starter cultures, pp.127-144. In S. E. Gilland (ed.), Bacterial starter cultures for foods. CRC Press, BocaRaton, FL.

Tremblay, D. M., and S. Moineau. 1999. Complete genomic sequence of the lytic bacteriophage DT1 of *Streptococcus thermophilus*. Virology. **255**:63-76.

Turgeon, N., and S. Moineau. 2001. Isolation and characterization of a *Streptococcus thermophilus* plasmid closely related to the pMV158 family. Plasmid. **45**:171-183.

Twort, F. 1915. An investigation on the nature of ultra-microscopic viruses. *Lancet*. **2**:1241-1243.

Valyasevi, R., W. E. Sandine, and B. L. Geller. 1991. A membrane protein is required for bacteriophage c2 infection of *Lactococcus lactis* subsp. *lactis* C2. *J. Bacteriol.* **173**:6095-6100.

Ventura, M., A. Bruttin, C. Canchaya, and H. Brüssow. 2002. Transcription analysis of *Streptococcus thermophilus* phages in the lysogenic state. *Virology*. **302**:21-32.

Ventura, M., S. Foley, A. Bruttin, S. Chennoufi, C. Canchaya, and H. Brüssow. 2002. Transcription mapping as a tool in phage genomics: the case of the temperate *Streptococcus thermophilus* phage Sfi21.

Venturi, A., P. Gionchetti, F. Rizzello, R. Johansson, E. Zucconi, P. Brigidi, D. Matteuzzi, and M. Campieri. 1999. Impact on the composition of the fecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. *Aliment Pharmacol. Ther.* **13**:1103-1108.

Wang, I. N., D. L. Smith, and R. Young. 2000. Holins: the protein clocks of bacteriophage infections. *Annu. Rev. Microbiol.* **54**:799-825.

- Walker, S. A., and T. R. Klaenhammer.** 2000. An explosive antisense RNA strategy for inhibition of a lactococcal bacteriophage. *Appl. Environ. Microbiol.* **66**:310-319.
- Werts, C., V. Michel, M. Hofnung, and C. Charbit.** 1994. Adsorption of bacteriophage lambda on the LamB protein of *Escherichia coli* K-12: point mutations in gene *J* of lambda responsible for extended host range. *J. Bacteriol.* **176**:941-947.
- Whitehead, H. R., and G. A. Cox.** 1935. The occurrence of bacteriophage in cultures of lactic streptococci, a preliminary note. *N. Z. J. Sci. Technol.* **16**:319.
- Whitehead, W. E., J. W. Ayres, and W. E. Sandine.** 1993. A review of starter media for cheese making. *J. Dairy Sci.* **76**:2344-2353.
- Wilson, G. G., and N. E. Murray.** 1991. Restriction and modification systems. *Annu. Rev. Genet.* **25**:585-627.
- Wilson, R. D., G. Davies, V. Desilets, G. J. Reid, A. Summers, P. Wyatt, and D. Young.** 2003. The use of folic acid for the prevention of neural tube defects and other congenital anomalies. *J. Obstet. Gynaecol. Can.* **25**:959-973.
- Yu, C. E., and J. J. Ferretti.** 1991. Molecular characterization of new group A streptococcal bacteriophages containing the gene for streptococcal erythrogenic toxin A (*speA*). *Mol. Gen. Genet.* **231**:161-168.

Zourari, A., J. P. Accolas, and M. J. Desmazeaud. 1992. Metabolism and biochemical characteristics of yogurt bacteria: a review. *Lait*. **72**:1-34.

TABLE 1 ^a
 Characterized R-M systems in *Streptococcus thermophilus*

Name ^b	Type	Specificity ^{c,d}	Host ^e	Location	Accession Number	Reference
S.SthCI65IP	I.hsdS	– ^f	ST NDI-6	pCI65st	AF02167	O’Sullivan <i>et al.</i> (1999)
SthSFil	I	–	ST Sfi1	chromosome	-	Lucchini <i>et al.</i> (2000)
SthER35IP	I	–	ST 135	pER35	AF177167	Solow & Somkuti (2001)
Sth134I	II	5′ –C↓CGG–3′	ST 134	chromosome	-	Solaiman & Somkuti (1990)
Sth117I	II	5′ –CC↓WGG–3′	ST 117	chromosome	-	Solaiman & Somkuti (1991)
SsII	II	5′ –CCWGG–3′	ST T	chromosome	-	Benbadis <i>et al.</i> (1991)
Sth455I	II	5′ –CCWGG–3′	ST CNRZ455	chromosome	-	Guimont <i>et al.</i> (1993)
Sth132I	II	5′ –CCC GNNNN ↓ NNNN –3′ 3′ –GGG CNNNN NNNN ↑ –5′	ST 132	chromosome	-	Poch <i>et al.</i> (1997)
Sth368I	II	5′ –GATC–3′	ST CNRZ368	chromosome	AJ271594	Burrus <i>et al.</i> (2001)
Sth0I	II	–	ST 0	pSt0	AJ242480	Geis <i>et al.</i> (2003)
Sth8I	II	–	ST 8	pSt08	AJ239049	Geis <i>et al.</i> (2003)

^a Adapted from table provided by Aidan Coffey (personal communication)

^b Nomenclature guidelines according to REBASE (<http://rebase.neb.com>; Roberts *et al.* 2003b)

^c When known, the cleavage point is indicated (↓)

^d W = A or T; R = A or G; Y = C or T; N = ACG or T

^e ST = *Streptococcus thermophilus*

^f –, unknown specificity or no accession number

TABLE 2Antisense RNA-based phage defense strategies effective (EOP < 1.0) against P335-type phages in *Lactococcus lactis*

Antisense Construct	Base Vector	Promoter	Phage	Predicted Target	Function	Length ^a	ORF ^b	anti-RBS ^c	EOP	Reference
pNZ44::topo-rev	pNZ123	P44	Tuc2009	topoisomerase	early	640	C	yes	1×10^{-1}	McGrath <i>et al.</i> (2001)
pNZ44::ssb-rev	pNZ123	P44	Tuc2009	ssDNA binding protein	early	478	C	yes	1×10^{-1}	McGrath <i>et al.</i> (2001)
pNZ44::rep2009-rev	pNZ123	P44	Tuc2009	replisome organizer	early	802	C	yes	1×10^{-6}	McGrath <i>et al.</i> (2001)
pNZ44::orf17-rev	pNZ123	P44	Tuc2009	topoisomerase	early	747	C	yes	1×10^{-6}	McGrath <i>et al.</i> (2001)
pNZ44::meth-rev	pNZ123	P44	Tuc2009	methylase	early	773	C	yes	5×10^{-1}	McGrath <i>et al.</i> (2001)
pSGK1.0R::gp18C::gp24C	pGKV210	P59	Φmi7-9	glycoprotein::(unknown)	early	1.0 kb	P::C	no:: yes	6.9×10^{-1}	Kim <i>et al.</i> (1992)
pSGK1.5R::gp18C::gp24C	pGKV210	P59	Φmi7-9	glycoprotein::(unknown)	early	1.5 kb	C::C	yes::yes	4.5×10^{-1}	Kim <i>et al.</i> (1992)
pDC100::mcp	pGKV210	P59	ΦF4-1	major capsid protein	late	926	C	yes	5.8×10^{-1}	Chung <i>et al.</i> (1992)
pSC1::mcp-222	pGKV210	P59	ΦF4-1	major capsid protein	late	301	P	yes	5.0×10^{-1}	Chung <i>et al.</i> (1992)
pDC101::mcp-246	pGKV210	P59	ΦF4-1	major capsid protein	late	227	P	yes	7.7×10^{-1}	Chung <i>et al.</i> (1992)
pSGK1.6R::gp51C	pGKV210	P59	Φmi7-9	translation factor?	?	1,654	C	yes	4×10^{-3}	Kim and Batt (1991)

^a Length in nucleotides unless otherwise indicated^b C, complete open reading frame (ORF) from start to stop codon; P, partial open reading frame^c Presence or absence of sequences complementary for the predicted ribosome binding site (RBS)

TABLE 3Antisense RNA-based phage defense strategies ineffective (EOP = 1) against various phages in *Lactococcus lactis*

Antisense Construct	Base Vector	Promoter	Phage	Predicted Target	Function	Length ^a	ORF ^b	anti-RBS ^c	Reference
pNZ44::orf19-rev	pNZ123	P44	Tuc2009	resolvase	early	410	C	yes	McGrath <i>et al.</i> (2001)
pGKV259::e5	pGKV210	P59	c2	DNA pol. subunit	early	?	?	?	Polzin <i>et al.</i> (personal 1996)
pGKV259::e12	pGKV210	P59	c2	transcription factor	early	?	?	?	Polzin <i>et al.</i> (personal 1996)
pGKV259::e15	pGKV210	P59	c2	recombinase	early	?	?	?	Polzin <i>et al.</i> (personal 1996)
pTRK594::anti-orf1	pTRKH2	P6	φ31	?	early or middle	?	C	?	Walker and Klaenhammer (2000)
pTRK595::anti-(orf1::tac31A)	pTRKH2	P6	φ31	? / transcriptional activ.	early or middle	?	C::C	?	Walker and Klaenhammer (2000)
pNZ44::msp1-rev	pNZ123	P44	Tuc2009	major structural prot.	late	551	C	yes	McGrath <i>et al.</i> (2001)
pNZ44::msp2-rev	pNZ123	P44	Tuc2009	major structural prot.	late	546	C	yes	McGrath <i>et al.</i> (2001)
pTRK596::anti-orf3	pTRKH2	P6	φ31	?	late	358	C	?	Walker and Klaenhammer (2000)
pTRK597::anti-orf4H	pTRKH2	P6	φ31	?	late	361	P	yes	Walker and Klaenhammer (2000)
pTRK598::anti-orf5H	pTRKH2	P6	φ31	?	late	493	P	yes	Walker and Klaenhammer (2000)
pTRK599::anti-orf6H	pTRKH2	P6	φ31	?	late	467	P	yes	Walker and Klaenhammer (2000)
pTRK600::anti-orf6	pTRKH2	P6	φ31	?	late	?	C	yes	Walker and Klaenhammer (2000)
pGKV259::l7	pGKV210	P59	c2	major tail protein	late	?	?	?	Polzin <i>et al.</i> (personal 1996)
pGKV259::l12	pGKV210	P59	c2	terminase	late	?	?	?	Polzin <i>et al.</i> (personal 1996)
pSGK1.0R::gp51C	pGKV210	P59	φmi7-9	translation factor	?	695	P	no	Kim and Batt (1991)
pSGK0.8R::gp51C	pGKV210	P59	φmi7-9	translation factor	?	422	P	no	Kim and Batt (1991)

^a Length in nucleotides unless otherwise indicated^b C, complete open reading frame (start to stop codon); P, partial open reading frame^c Presence or absence of sequences complementary for the predicted ribosome binding site (RBS)

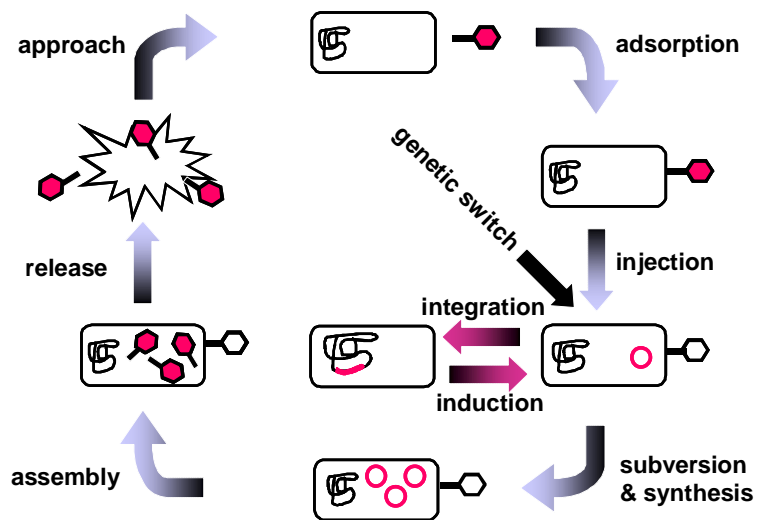


Figure 1. Simplification of the life cycles of a temperate bacteriophage.

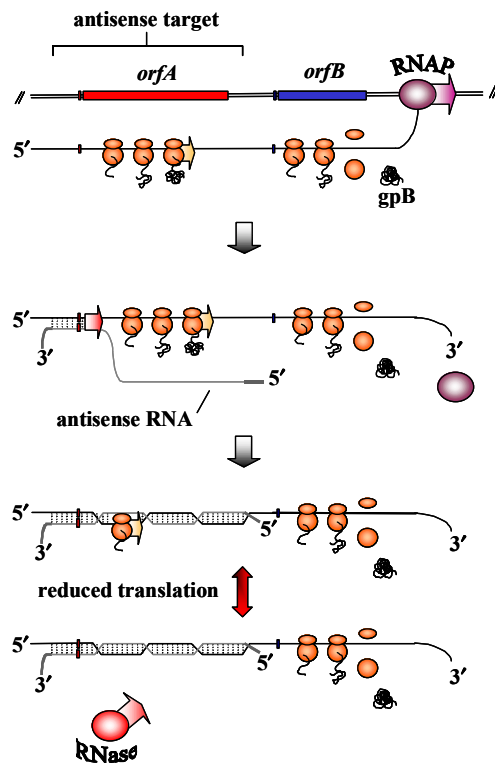


Figure 2. Illustration depicting the proposed mechanism of antisense RNA-mediated gene silencing. Antisense RNAs interfere with phage development by inhibiting the translation of phage-encoded genes necessary for normal development. Mechanistically, antisense RNA hybridizes to the sense RNA strand and creates a translationally inactive double stranded RNA (dsRNA) molecule and the action of dsRNA specific RNases.

CHAPTER II

Expression of Antisense RNA Targeted Against
Streptococcus thermophilus Bacteriophages

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ABSTRACT

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

INTRODUCTION

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

In recent years, an increased incidence of bacteriophage-related problems have been observed for *S. thermophilus* strains, which are an essential component of starter systems for yogurt and Italian cheese varieties. Our understanding of *S. thermophilus* bacteriophages has progressed rapidly since the release of six bacteriophage whole-genome sequences: DT1 (42), ϕ O1205 (40), Sfi11 (24), Sfi19 (25), Sfi21 (25), and ϕ 7201 (41). In contrast to bacteriophages that infect *Lactococcus* species, *S. thermophilus* bacteriophages are closely related, both at the genetic and morphological levels, making differentiation difficult. Electron microscopy studies revealed that *S. thermophilus* bacteriophages, both temperate and lytic, are nearly identical, and that all belong to the

Siphoviridae family (morphotype B1), having small isometric heads, long, non-contractile tails, and genomes comprised of double-stranded DNA (31). It has recently been shown that *S. thermophilus* strains are attacked by two groups of highly related bacteriophages that differ in their mechanism of genome encapsidation: *cos*-type and *pac*-type (23). These bacteriophages can be identified and differentiated by examination of their distinct capsid protein profiles or through the detection of the genes that encode those structural proteins (23).

Studies on native bacteriophage defense systems remain limited. *S. thermophilus* strains have been found to possess both chromosomal and plasmid-borne restriction and modification (R/M) systems (2, 15, 37, 38, 39). At this juncture, however, these native R/M systems have not yet been exploited as means of augmenting the intrinsic level of resistance of industrial starter strains. The plasmid-borne expression of *LlaDCHI* (formerly *LlaII*), a heterologous R/M system derived from *Lactococcus lactis*, does confer broad-range bacteriophage resistance in various strains of *S. thermophilus* (32). In addition, efforts to construct strains of *S. thermophilus* with passive resistance properties are also underway. Lucchini et al. (27) recently described the use of pG⁺host9::ISSI-mediated insertional mutagenesis to identify four distinct host-encoded loci involved in bacteriophage sensitivity. A putative transmembrane protein (*orf394*) was discovered and proposed to be functionally analogous to the lactococcal Pip protein, which is essential for infection of *L. lactis* by c2-type bacteriophages (13).

The application of molecular biology and modern functional genomics is accelerating the development of novel bacteriophage resistance mechanisms through genetic engineering. Sequence analysis can be used to interpret the encoded genetic

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

Origin-conferred phage-encoded resistance (PER) was first reported to be effective in *L. lactis* (16, 28), and has since been demonstrated to be effective in *S. thermophilus* (12, 41). When a bacteriophage *ori* is provided *in trans* on a recombinant plasmid, the origin acts as a molecular decoy that competes for and titrates away

bacteriophage-specific replication factors. The result is a reduction in the number of bacteriophage genomes replicated and a dramatic increase in plasmid copy-number. The efficacy of PER is bacteriophage-specific, and the conferred level of resistance correlates with the copy-number of the false origin presented *in trans* (29, 34). Further, when an antisense RNA expression cassette was linked, *in cis*, to a PER vector, the result was the expression of an explosive dose of antisense RNA that effectively inhibited a lactococcal bacteriophage (43).

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

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All bacterial stocks were maintained at –80°C in fresh culture medium supplemented with 10% glycerol. All bacteriological media and components were purchased from Difco Laboratories (Detroit, MI).

Escherichia coli strains were grown at 37°C with constant aeration in Luria-Bertani broth. Unless otherwise indicated, *S. thermophilus* derivatives were propagated aerobically at 42

°C in Elliker broth supplemented with 1% (w/v) beef extract (Elliker-B).

Chloramphenicol (Cm) was added at 2.5 µg/ml for both *E. coli* and *S. thermophilus*, when appropriate. For solid media, Bacto Agar was added at a final concentration of 1.5% (w/v) for base agar and 0.75% (w/v) for soft agar.

Plasmids, bacteriophages and propagation assays. Plasmids and bacteriophages used in this study are listed in Table 1. Bacteriophages described in this study were isolated from mozzarella whey. Bacteriophages were propagated in Elliker-B broth supplemented with 10 mM CaCl₂ (Elliker-BC) at 42°C and diluted in 0.1X Elliker-B broth supplemented with 10 mM CaCl₂. For plaque assays, 20-ml ± 1-ml of M17-G base agar supplemented with 10 mM CaCl₂ (M17-GC) was dispensed using a Bellco Biotechnology automatic medium dispenser (Vineland, NJ) to limit volume-dependant variations in plaque size, and incubated aerobically at 37°C for 18 hours prior to analysis. The efficiency of plaquing (EOP) was calculated by dividing the bacteriophage titer, in plaque forming units per milliliter (PFU/ml), of the test strain by the bacteriophage titer of the parental strain. Bacteriophages were characterized as *cos*- or *pac*-type bacteriophages as described by Le Marrec et al. (23). Lysis-in-broth assays were performed in Elliker-BC medium as described previously, except that samples were taken every 15 minutes for a period of 4 hours (41).

Enzymes and reagents. Restriction enzymes, *Taq* DNA polymerase and deoxynucleoside triphosphates were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). T4 DNA ligase, SuperScript reverse transcription kit, and DNA molecular weight markers were obtained from Gibco-BRL Life Technologies, Inc. (Gaithersburg, MD). *Pwo* DNA polymerase was obtained from Roche (Indianapolis, IN),

and all enzymes were used according to the manufacturer's specifications. All other chemicals were of analytical grade and obtained from Sigma Chemical Company (St. Louis, MO).

Bacterial transformation. All electroporations were performed using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA) apparatus configured to 25 μ F, 2.5 kV, and 200 Ω . Preparation of electrocompetent *E. coli* strain MC1061 (18) was conducted as described by Sambrook et al. (36), and electrocompetent *S. thermophilus* strains were prepared utilizing a method modified from Holo and Nes (17). A stationary phase culture of *S. thermophilus* was diluted 100-fold into 42°C Elliker-B broth, incubated at 42°C, and allowed to reach an optical density at 600 nm (OD₆₀₀) of 0.2 prior to the addition of one tenth volume of 42°C 15% glycine (w/v) and one tenth volume of 42°C 2X Elliker-B. Cells were harvested by centrifugation at an OD₆₀₀ between 0.6-0.8, washed three times with two volumes of sterile, deionized water, washed once with two volumes of SG buffer (0.5 M sucrose and 10% glycerol), resuspended in 0.003 volumes of SG buffer, and incubated on ice prior to use. Plasmid DNA (1- μ g) was mixed with 40- μ l of cells in a chilled Gene Pulser 0.2 cm cuvette. Following electroporation, cells were immediately resuspended in 960- μ l of recovery medium (Elliker-B broth supplemented with 20 mM MgCl₂ and 2 mM CaCl₂) and incubated for 2 h at 42°C, before being spread onto Elliker-B base agar supplemented with chloramphenicol (5.0 μ g/ml).

Plasmid and genomic DNA preparation. Small-scale preparations of plasmid DNA were isolated from *E. coli* (36) and *S. thermophilus* (35) as described previously. Large-scale preparations of plasmid DNA were isolated using the Qiagen Midi Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. PCR products

were purified by using the Qiagen PCR Purification Kit prior to further manipulation. When required, DNA was extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

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

PCR and DNA sequencing. PCR was performed in a Hybaid Limited (Middlesex, UK) PCR Express thermal cycler using either *Taq* or *Pwo* DNA polymerase. DNA primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). When appropriate, restriction endonuclease recognition sites were incorporated into the 5' end of DNA primers to facilitate the cloning of PCR products. A list of primers utilized in this study can be found in Table 2. Cycle sequencing reactions and DNA sequence determination were performed by the University of California-Davis Automated DNA Sequencing Facility (Davis, CA) using an ABI Prism 377 DNA sequencer with a 96-lane upgrade (Applied Biosystems, Foster City, CA). DNA sequences were analyzed using

the GCG sequence analysis package v10.0 (Genetics Computer Group, Inc., Madison, WI) and Clone Manager v6.0 (Scientific and Educational Software, Durham, NC). The Basic Local Alignment Search Tool (1) for nucleic acid (BlastN) and protein (BlastX) homology searches were performed using National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>).

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

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

RESULTS

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

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

Sequence and phylogenetic analyses of the κ 3-derived *hel3.1* cassette. The 1,431-bp PCR fragment amplified from bacteriophage κ 3 was sequenced and revealed a single open reading frame of 1,331-bp (GenBank accession number AF442520). This open reading frame, designated *hel3.1*, begins with a 5'-ATG-3' translation initiation codon, ends with a 5'-TAA-3' stop codon, and is preceded by a putative RBS (5'-AAATTTGGTGA-3'). The putative Hel3.1 protein is 443 amino acids long, and has a predicted molecular mass of 50.5 kDa. Conserved structural motifs that are characteristic of ATP-dependent helicases were found in the deduced primary sequence, including the NTP-binding (SPPRSGKT), NTP-hydrolysis (DEAH), and variant zinc-finger (CDECYATFWSAERICPLC) motifs (14).

The coding region of *hel3.1* was compared to the putative helicase genes from *S. thermophilus* bacteriophages DT1, ϕ O1205, Sfi11, Sfi18, Sfi19, and Sfi21. BlastN sequence analysis revealed the coding region of the bacteriophage κ 3 putative helicase gene to have 99% sequence similarity to bacteriophage DT1 and 90% similarity to bacteriophages Sfi11, Sfi18, Sfi19, Sfi21, and ϕ O1205. Therefore, *hel3.1* was more closely related to the helicase gene from bacteriophage DT1 than to the helicase alleles

from the other bacteriophages. Over the entire length of the proteins, BlastX analysis indicated the following amino acid similarities to: DT1 (99%), ϕ O1205 (98%), Sfi11 (97%), Sfi18 (97%), Sfi19 (97%), and Sfi21 (97%).

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

the κ 3-derived *hel3.1* allele. These results indicated that a majority of the bacteriophages listed in Table 1 possessed the Sfi21-type DNA replication module.

Construction of a basal antisense RNA expression vector (pTRK687). Of the vector systems tested in our laboratory to date, only those that replicate via a rolling circle mechanism are transformable into strains of *S. thermophilus* (unpublished observations). As a result, antisense RNA expression systems were assembled on a stable derivative of the high-copy number plasmid pNZ123 (9). This vector, which was recovered from *E. coli* as a deletion derivative and designated pTRK686, was completely sequenced (2,410-bp). This plasmid transforms *S. thermophilus* NCK1124, which is plasmidless, and NCK1125, which contains two native plasmids, at frequencies between 10^4 - 10^5 transformants per μ g of supercoiled plasmid DNA. The 0.6-kb *Bgl*II expression cassette from pTRK593 (43), containing the *Lactobacillus acidophilus* ATCC 4356 P6 promoter (11) and a mini-MCS with a downstream coliphage T7 transcriptional terminator, was cloned into the *Sau*3AI site of pTRK686. The resulting (3,018-bp) plasmid, designated pTRK687, was used as a basal RNA expression vector.

Construction of a DNA helicase-based antisense RNA expression system. The 1.4-kb *hel3.1*-containing fragment amplified from *cos*-type bacteriophage κ 3 was digested with PstI and cloned, in either orientation (*i.e.* sense vs. antisense) relative to the P6 promoter, into the PstI site of pTRK687. The resulting sense (*hel3.1*-S) and antisense (*hel3.1*-AS) constructs, designated pTRK688::*hel3.1*-S and pTRK689::*hel3.1*-AS, respectively (Figure 2), were electroporated into *S. thermophilus* NCK1124, NCK1125, and NCK1434 to determine their impact on bacteriophage infection during standard plaque assays. The plasmid pTRK688::*hel3.1*-S was included in this study as a sense

RNA control to exclude the possibility that any observed drop in EOP or plaque size might be attributed to the increased metabolic burden associated with RNA expression from these high copy-number expression vectors.

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

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

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

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

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

Strand-specific RT-PCR. Non-quantitative, strand-specific RT-PCR was performed to confirm that the *hel3.1* antisense RNA was expressed in the appropriate strains. Primer JMSp4 was used during first strand (RT) synthesis of cDNA and primers JMSp4 and JMSp5 were used during the subsequent PCR amplification (Figure 4). The expected 0.7-kb fragment was amplified only from total RNA isolated from NCK1125

(pTRK689::*hel3.1*-AS), indicating that (i) antisense *hel3.1* RNA was being expressed from pTRK689::*hel3.1*-AS and (ii) antisense *hel3.1* RNA was not expressed in strains that did not harbor the plasmid.

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

Construction of PER and explosive antisense RNA vectors. *Eco*RI-tagged primer JMSp6 was designed from the DT1-, ϕ O1205-, Sfi11-, Sfi19-, and Sfi21-derived consensus region upstream of the putative origin of DNA replication (*ori*) consensus sequence while *Eco*RI-tagged primer JMSp7 was designed exclusively from the DT1 genomic sequence. Regions downstream of the putative *ori* diverged among the above bacteriophages so preference was given to DT1 since it was derived from North American cheese fermentations. Primers JMSp6 and JMSp7 were used to amplify the putative *ori* from the *S. thermophilus* bacteriophages listed in Table 1, except κ 1 was not tested. The expected 0.7-kb amplicon was generated only from bacteriophages DT1, κ 3

and $\kappa 5$. The 677-bp $\kappa 3$ -derived fragment, designated *ori3.1*, was sequenced (GenBank accession number AF442521), digested with *EcoRI*, and cloned into the *EcoRI* site of pTRK687, which is located upstream of the P6 promoter. The resulting PER plasmid, designated pTRK690::*ori3.1*, served as a base vector for the construction of an explosive antisense RNA expression system. The *hel3.1* fragment described above was subsequently cloned into the *PstI* site of pTRK690::*ori3.1* to yield pTRK691::*ori3.1::hel3.1*-AS. The plasmids pTRK690::*ori3.1* and pTRK691::*ori3.1::hel3.1*-AS were then electroporated into *S. thermophilus* NCK1125 to determine their impact on the infection of bacteriophages $\kappa 3$, $\kappa 4$, $\kappa 6$, $\kappa 9$, $\kappa 10$, and $\kappa 12$ during standard plaque assays (Table 3). The presence of the $\kappa 3$ -derived origin alone on the PER plasmid pTRK690::*ori3.1* had a significant impact on the proliferation of bacteriophages $\kappa 3$, $\kappa 4$, $\kappa 9$, and $\kappa 10$, but did not effect the replication of $\kappa 6$ or $\kappa 12$. The pTRK690::*ori3.1* construct reduced the EOP of sensitive bacteriophages to less than 10^{-6} relative to the NCK1125 parental strain and gave rise to irregularly shaped pinpoint plaques. The addition of the antisense *hel3.1* cassette to the PER plasmid further impeded bacteriophage $\kappa 3$ replication significantly beyond the level of the PER parent plasmid alone (Table 3). NCK1125 (pTRK691::*ori3.1::hel3.1*-AS) lowered the EOP of sensitive bacteriophages to less than 10^{-7} and 10^{-8} and gave rise to irregularly shaped pinpoint plaques, often with faint halos, that were difficult to enumerate. Bacteriophages picked from these plaques failed to propagate to detectable levels, even after multiple propagations on NCK1125. No antisense *hel3.1* RNA resistant bacteriophages have been isolated to date.

DISCUSSION

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

The magnitude of bacteriophage inhibition via antisense RNA expression is similar to results reported previously in *L. lactis*. Kim et al. (21) found that antisense expression of two polycistronic open reading frames, designated *gp18C* and *gp24C*, inhibited the P335-type bacteriophage $\phi 7-9$, as measured by a 55% reduction in EOP. Interestingly, the reduction in EOP dropped to 30% if the RBS and coding region for the first 15 amino-terminal residues of Gp18C were omitted from the antisense construct. In both cases, the plaque size was also reduced by approximately 10-fold. Chung et al. (7) obtained variable reductions in EOP, which ranged between 0.5 and 0.8, as they expressed different lengths of the $\phi F4-1$ major coat protein (*mcp*) gene. Kim and Batt

(20) found that antisense expression of the full-length, bacteriophage ϕ 7-9-derived *gp15C* mediated a reduction in EOP of ϕ 7-9 (and other *gp15C*-containing bacteriophages) to 10^{-2} . More recently, McGrath et al. (29) targeted DNA replication functions, and found a 50% to a 10^{-6} log reduction in EOP, depending on the targeted gene and bacteriophage tested.

The results from these and other studies clearly indicate that certain genes are better targets than others for silencing by antisense RNA. Polzin et al. (K. M. Polzin, L. J. Collins, M. W. Lubbers, and A. W. Jarvis, Abstr. 5th Symp. Lactic Acid Bacteria, abstr. F2, 1996) found that the antisense expression of four early open reading frames, including *e5*, a putative subunit of DNA polymerase; *e12*, a putative transcription regulator; and *e15*, a putative recombinase and four late ORFs, including *l7*, a major tail protein and *l12*, a putative terminase were all ineffective in their ability to inhibit the replication of the *L. lactis* prolate-headed bacteriophage c2, regardless of the gene dosage tested. In addition, Walker and Klaenhammer (43) found that two middle-expressed open reading frames, including *orf1* and *orf2*, and four late-expressed open reading frames, *orf3* through *orf6*, were also ineffective at inhibiting the *L. lactis* P335-type bacteriophage ϕ 31 when expressed from the *L. acidophilus* P6 promoter on the high-copy-number vector pTRKH2. In order to increase the ratio of antisense RNA to sense RNA, the authors cloned the aforementioned antisense expression cassettes into pTRK360, a low-copy-number vector containing the bacteriophage ϕ 31 putative origin of DNA replication (*ori31*). Following bacteriophage ϕ 31 invasion, the expression of bacteriophage-derived DNA replication factors triggered the explosive replication of

pTRK360 from *ori31*, and produced inhibiting levels of antisense RNA during the later stages of the lytic cycle.

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

The variation in efficacy of antisense RNA-mediated gene silencing raises questions about the key characteristics of an ideal target gene or locus. Essentiality of the target RNA(s) for the replication, maturation, or release of progeny bacteriophages is perhaps the most obvious criterion. Unfortunately, essentiality must be derived empirically, cannot be garnered from the analysis of genomic data and, often, should not be extrapolated from prior observations in heterologous systems. Analysis of a bacteriophage's transcriptome could provide additional insights into the choice of targets. In theory, optimal candidates will be genes that are transiently expressed, expressed at a

very low level, and/or coded for by unstable, inefficiently-translated mRNA species. In addition, the secondary structure(s) of potential mRNA species may also be examined and should be able to form structures that are conducive to recognition of the expressed antisense RNA molecule(s).

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

When using nucleotide sequence similarity as an indication of evolutionary descent, it was clear that the putative helicase genes of *S. thermophilus* bacteriophages can be divided into two groups based on either (i) the fermented product (*i.e.* yogurt vs. cheese) or (ii) geographic location (*i.e.* North America vs. Europe). Phylogenetically, one group contained bacteriophages isolated from North American cheese plants (DT1

and κ3), while the other included bacteriophages that were isolated from European yogurt plants (Sfi11, Sfi19, Sfi21, and φO1205).

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

In conclusion, we have exploited the conservation of the Sfi21-type DNA replication module and constructed effective antisense RNA expression strategies that are effective against *S. thermophilus* bacteriophages. Both *cos*- and *pac*-type *S. thermophilus* bacteriophages that attack two different strains of *S. thermophilus* are sensitive to these technologies. The combination of a bacteriophage origin of replication with a high expression antisense RNA cassette provided significant protection from specific

bacteriophages at levels higher than for each mechanism alone. Work is continuing to target other components of the putative DNA replication module.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403-410.
2. **Benbadis, L., J. R. Garel, and D. L. Hartley.** 1991. Purification, properties, and sequence specificity of SsII, a new type II restriction endonuclease from *Streptococcus salivarius* subsp. *thermophilus*. *Appl. Environ. Microbiol.* **57**:3677-3678.
3. **Brüssow, H., A. Probst, M. Fremont, and J. Sidoti.** 1994. Distinct *Streptococcus thermophilus* bacteriophages share an extremely conserved DNA fragment. *Virology* **200**:854-857.
4. **Brüssow, H., A. Bruttin, F. Desiere, S. Lucchini, and S. Foley.** 1998. Molecular ecology and evolution of *Streptococcus thermophilus* bacteriophage—a review. *Virus Genes* **16**:95-109.
5. **Brüssow, H., and F. Desiere.** 2001. Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. *Mol. Microbiol.* **39**:213-222.
6. **Bruttin, A., F. Desiere, N. d'Amico, J. P. Guerin, J. Sidoti, B. Huni, S. Lucchini, and H. Brüssow.** 1997. Molecular ecology of *Streptococcus*

- thermophilus* bacteriophage infections in a cheese factory. Appl. Environ. Microbiol. **63**:3144-3150.
7. **Chung, D. K., S. K. Chung, and C. A. Batt.** 1992. Antisense RNA directed against the major capsid protein of *Lactococcus lactis* subsp. *cremoris* bacteriophage F4-1 confers partial resistance to the host. App. Microbiol. Biotechnol. **37**:79-83.
 8. **Desiere, F., S. Lucchini, A. Bruttin, M. C. Zwahlen, and H. Brüssow.** 1997. A highly conserved DNA replication module from *Streptococcus thermophilus* phages is similar in sequence and topology to a module from *Lactococcus lactis* phages. Virology **234**:372-382.
 9. **De Vos, W.** 1997. Gene cloning and expression in lactic streptococci. FEMS Microbiol. Rev. **46**:281-295.
 10. **Dinsmore, P. K., and T. R. Klaenhammer.** 1997. Molecular characterization of a genomic region in a *Lactococcus* bacteriophage that is involved in its sensitivity to the phage defense system AbiA. J. Bacteriol. **179**:2949-2957.
 11. **Djordjevic, G., B. Bojovic, N. Miladinov, and L. Topisirovic.** 1997. Cloning and molecular analysis of promoter-like sequences isolated from the chromosomal DNA of *Lactobacillus acidophilus* ATCC 4356. Can. J. of Microbiol. **43**:61-9.

12. **Foley, S., S. Lucchini, M. C. Zwahlen, and H. Brüssow.** 1998. A short noncoding viral DNA element showing characteristics of a replication origin confers bacteriophage resistance to *Streptococcus thermophilus*. *Virology*. **250**:377-387.
13. **Garbutt, K. C., J. Kraus, and B. L. Geller.** 1997. Bacteriophage resistance in *Lactococcus lactis* engineered by replacement of a gene for a bacteriophage receptor. *J. Dairy Sci.* **80**:1512-1519.
14. **Gorbalenya, A. E., and E. V. Koonin.** 1993. Helicases: amino acid sequence comparisons and structure-function relationships. *Curr. Opin. Struct. Biol.* **3**:419-429.
15. **Guimont, C., P. Henry, and G. Linden.** 1993. Restriction/modification in *Streptococcus thermophilus*: isolation and characterization of a type II restriction endonuclease *Sth455I*. *Appl. Microbiol. Biotechnol.* **39**:216-220.
16. **Hill, C., L. A. Miller, and T. R. Klaenhammer.** 1990. Cloning, expression, and sequence determination of a bacteriophage fragment encoding bacteriophage resistance in *Lactococcus lactis*. *J. Bacteriol.* **172**:6419-6426.

17. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl. Environ. Microbiol.* **55**:3119-3123.
18. **Huynh, T. V., R. A. Young, and R. W. Davis.** 1985. Construction and screening cDNA libraries in λ gt10 and λ gt11, p. 49-78. *In* D. M. Glover (ed.), *DNA cloning*, vol. I. IRL Press Ltd., Oxford, United Kingdom.
19. **Inouye, M.** 1988. Antisense RNA: its functions and applications in gene regulation—a review. *Gene* **72**:25-34.
20. **Kim, J. H., and C. A. Batt.** 1991. Antisense RNA mediated bacteriophage resistance in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **57**:1039-1045.
21. **Kim, S. G., Y. C. Bor, and C. A. Batt.** 1992. Bacteriophage resistance in *Lactococcus lactis* ssp. *lactis* using antisense ribonucleic acid. *J. Dairy Sci.* **75**:1761-1767.
22. **Labrie, S., and S. Moineau.** 2000. Multiplex PCR for detection and identification of lactococcal bacteriophages. *Appl. Environ. Microbiol.* **66**:987-994.

23. **Le Marrec, C., D. van Sinderen, L. Walsh, E. Stanley, E. Vlegels, S. Moineau, P. Heinze, G. Fitzgerald, and B. Fayard.** 1997. Two groups of bacteriophages infecting *Streptococcus thermophilus* can be distinguished on the basis of mode of packaging and genetic determinants for major structural proteins. Appl. Environ. Microbiol. **63**:3246-3253.
24. **Lucchini, S., F. Desiere, and H. Brüssow.** 1998. The structural gene module in *Streptococcus thermophilus* bacteriophage ϕ Sfi11 shows a hierarchy of relatedness to *Siphoviridae* from a wide range of bacterial hosts. Virology **246**: 63-73.
25. **Lucchini, S., F. Desiere, and H. Brüssow.** 1999. The genetic relationship between virulent and temperate *Streptococcus thermophilus* bacteriophages: whole genome comparison of *cos*-site phages Sfi19 and Sfi21. Virology **260**: 232-243.
26. **Lucchini, S., F. Desiere, and H. Brüssow.** 1999. Comparative genomics of *Streptococcus thermophilus* phage species supports a modular evolution theory. J. of Virology **73**: 8647-8656.
27. **Lucchini, S., J. Sidoti, and H. Brüssow.** 2000. Broad-range bacteriophage resistance in *Streptococcus thermophilus* by insertional mutagenesis. Virology **275**:267-277.

28. **McGrath, S., J. F. Seegers, G. F. Fitzgerald, and D. van Sinderen.** 1999. Molecular characterization of a phage-encoded resistance system in *Lactococcus lactis*. Appl. Environ. Microbiol. **65**:1891-1899.
29. **McGrath, S., G. F. Fitzgerald, and D. van Sinderen.** 2001. Improvement and optimization of two engineered phage resistance mechanisms in *Lactococcus lactis*. Appl. Environ. Microbiol. **67**:608-616.
30. **McKillip, J. L., and M. Drake.** 2000. Molecular beacon polymerase chain reaction detection of *Escherichia coli* O157:H7 in milk. J. Food. Prot. **63**:855-859.
31. **Mercenier, A., P. H. Pouwels, and B. M. Chasy.** 1994. Genetic engineering of lactobacilli, leuconostocs, and *Streptococcus thermophilus*, p. 253-293. In M. J. Gasson and W. M. DeVos (ed.), Genetics and biotechnology of lactic acid bacteria. Blackie Academic and Professional, Glasgow, United Kingdom.
32. **Moineau, S., S. A. Walker, B. J. Holler, E. R. Vedamuthu, and P. A. Vandenberg.** 1995. Expression of a *Lactococcus lactis* phage resistance mechanism by *Streptococcus thermophilus*. Appl. Environ. Microbiol. **61**:2461-2466.

33. **Moineau, S., M. Borkaev, B. J. Holler, S. A. Walker, J. K. Kondo, E. R. Vedamuthu, and P. A. Vandenberg.** 1996. Isolation and characterization of lactococcal bacteriophages from cultured buttermilk plants in the United States. *J. Dairy Sci.* **79**:2104-2111.
34. **O'Sullivan, D. J., C. Hill, and T. R. Klaenhammer.** 1993. Effect of increasing the copy number of bacteriophage origins of replication *in trans*, on incoming-phage proliferation. *Appl. Environ. Microbiol.* **59**:2449-2456.
35. **O'Sullivan, D. J., and T. R. Klaenhammer.** 1993. Rapid mini-prep isolation of high-quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. *Appl. Environ. Microbiol.* **59**:2730-2733.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1982. Molecular cloning: a laboratory manual, 2nd ed. Spring Harbor Laboratory, Cold Spring Harbor. N.Y.
37. **Solaiman, D. K. Y., and G. A. Somkuti.** 1990. Isolation and characterization of a type II restriction endonuclease from *Streptococcus thermophilus*. *FEMS Microbiol. Lett.* **67**:261-266.
38. **Solaiman, D. K. Y., and G. A. Somkuti.** 1992. A type II restriction endonuclease of *Streptococcus thermophilus* ST117. *FEMS Microbiol. Lett.* **80**:75-80.

39. **Solow, B. T., and G. A. Somkuti.** 2001. Molecular properties of *Streptococcus thermophilus* plasmid pER35 encoding a restriction modification system. *Curr. Microbiol.* **42**:122-128.
40. **Stanley, E., G. F. Fitzgerald, M. C. Le Marrec, B. Fayard, and D. van Sinderen.** 1997. Sequence analysis and characterization of ϕ O1205, a temperate bacteriophage infecting *Streptococcus thermophilus* CNRZ1205. *Microbiology* **143**:3417-3429.
41. **Stanley, E., L. Walsh, A. van der Zwet, G. F. Fitzgerald, and D. van Sinderen.** 2000. Identification of four loci isolated from two *Streptococcus thermophilus* phage genomes responsible for mediating bacteriophage resistance. *FEMS Microbiol. Lett.* **182**:271-277.
42. **Tremblay, D. M., and S. Moineau.** 1999. Complete genomic sequence of the lytic bacteriophage DT1 of *Streptococcus thermophilus*. *Virology* **255**: 63-76.
43. **Walker, S. A. and T. R. Klaenhammer.** 2000. An explosive antisense RNA strategy for inhibition of a lactococcal bacteriophage. *Appl. Environ. Microbiol.* **66**:310-319.

TABLE 1
Bacterial strains, bacteriophages and plasmids

Bacterial strain, bacteriophage or plasmid	Relevant characteristic(s) ^a	Source or Reference
<i>Streptococcus thermophilus</i>		
NCK1434	Industrial isolate; sensitive to κ1; Cm ^S	This study
NCK1125	Industrial isolate; sensitive to κ3; κ4, κ5, κ6, κ9, κ10, κ12; Cm ^S	This study
NCK1435	Industrial isolate; sensitive to κ2; Cm ^S	This study
NCK1124	Industrial isolate; sensitive to κ13; Cm ^S	This study
SMQ495	Industrial isolate; sensitive to DT1; Cm ^S	42
<i>Escherichia coli</i>		
MC1061	Transformation host	18
Bacteriophages		
κ1	<i>pac</i> -type bacteriophage	This study
κ2	No information available	This study
κ3	<i>cos</i> -type bacteriophage	This study
κ4	<i>cos</i> -type bacteriophage	This study
κ5	<i>cos</i> -type bacteriophage	This study
κ6	<i>pac</i> -type bacteriophage	This study
κ9	<i>cos</i> -type bacteriophage	This study
κ10	<i>cos</i> -type bacteriophage	This study
κ12	<i>pac</i> -type bacteriophage	This study
κ13	No information available	This study
DT1	<i>cos</i> -type bacteriophage	42
Plasmids		
pNZ123	2.8-kb; high-copy-number shuttle vector; Cm ^R	9
pTRK686	2.4-kb; deletion derivative of pNZ123; Cm ^R	This study
pTRK687	3.0-kb; pTRK686 containing the high-expression P6 promoter	This study
pTRK688:: <i>hel3.1</i> -S	4.5-kb; pTRK687 containing 1.4-kb sense <i>hel3.1</i> cassette	This study
pTRK689:: <i>hel3.1</i> -AS	4.5-kb; pTRK687 containing 1.4-kb antisense <i>hel3.1</i> cassette	This study
pTRK690:: <i>ori3.1</i>	3.7-kb; pTRK687 containing 0.7-kb <i>ori3.1</i> cassette; Per ⁺	This study
pTRK691:: <i>ori3.1</i> :: <i>hel3.1</i> -AS	5.1-kb; pTRK690 containing 1.4-kb antisense <i>hel3.1</i> cassette	This study

^a Abbreviations: Cm^R, resistant to chloramphenicol; Cm^S, sensitive to chloramphenicol; Per⁺, origin-conferred phage encoded resistance

TABLE 2
Primers used in this study

Primer Name	Nucleic Acid Sequence ^a	Position ^b
JMSp1:	5'-AA ACTGCAGG CCTTGCAAGATTGAAGACC-3'	25,911
JMSp2:	5'-AA ACTGCAG CCGTCTTTGATAGATCCG-3'	27,341
JMSp3:	5'-GGAGCGTGATTTTATGG-3'	-
JMSp4:	5'- G TTAAAGCTAAGACCTACC-3'	26,275
JMSp5:	5'-CCCTTTAGTGACCATTCACGG-3'	26,963
JMSp6:	5'- GGAATTC AGTTAGGTTCTTGTGG-3'	29,810
JMSp7:	5'- GGAATTC CCCATAATCTTCGTCGGTCC-3'	30,486

^a PstI (5'-CTGCAG-3') and EcoRI (5'-GAATTC-3') restriction sites are underlined

^b The position of the 5' nucleotide (bold) relative to the DT1 genomic sequence is denoted, when appropriate

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

ϕ^a	Strain (construct)	EOP ^b	Δ PS ^c	ϕ^a	Strain (construct)	EOP ^b	Δ PS ^c
κ1	NCK1434	1.0	–	κ9	NCK1125	1.0	–
	NCK1434 (pTRK687)	0.8 ± 0.2	SE		NCK1125 (pTRK687)	0.9 ± 0.1	SE
	NCK1434 (<i>hel3.1</i> -S)	0.8 ± 0.2	SE		NCK1125 (<i>hel3.1</i> -S)	1.0 ± 0.1	SE
	NCK1434 (<i>hel3.1</i> -AS)	0.3 ± 0.2	PP		NCK1125 (<i>hel3.1</i> -AS)	0.5 ± 0.2	PP
	NCK1434 (<i>ori3.1</i>)	NT	NT		NCK1125 (<i>ori3.1</i>)	< 10 ⁻⁶	IP
	NCK1434 (<i>ori3.1::hel3.1</i> -AS)	NT	NT		NCK1125 (<i>ori3.1::hel3.1</i> -AS)	< 10 ⁻⁸	IP
κ3	NCK1125	1.0	–	κ10	NCK1125	1.0	–
	NCK1125 (pTRK687)	0.9 ± 0.2	SE		NCK1125 (pTRK687)	0.9 ± 0.2	SE
	NCK1125 (<i>hel3.1</i> -S)	0.9 ± 0.2	SE		NCK1125 (<i>hel3.1</i> -S)	1.1 ± 0.2	SE
	NCK1125 (<i>hel3.1</i> -AS)	0.5 ± 0.1	PP		NCK1125 (<i>hel3.1</i> -AS)	0.4 ± 0.1	PP
	NCK1125 (<i>ori3.1</i>)	< 10 ⁻⁶	IP		NCK1125 (<i>ori3.1</i>)	< 10 ⁻⁶	IP
	NCK1125 (<i>ori3.1::hel3.1</i> -AS)	< 10 ⁻⁸	IP		NCK1125 (<i>ori3.1::hel3.1</i> -AS)	< 10 ⁻⁸	IP
κ4	NCK1125	1.0	–	κ12	NCK1125	1.0	–
	NCK1125 (pTRK687)	1.1 ± 0.1	SE		NCK1125 (pTRK687)	0.8 ± 0.1	SE
	NCK1125 (<i>hel3.1</i> -S)	1.2 ± 0.1	SE		NCK1125 (<i>hel3.1</i> -S)	0.8 ± 0.2	SE
	NCK1125 (<i>hel3.1</i> -AS)	0.6 ± 0.2	PP		NCK1125 (<i>hel3.1</i> -AS)	0.9 ± 0.2	SE
	NCK1125 (<i>ori3.1</i>)	< 10 ⁻⁶	IP		NCK1125 (<i>ori3.1</i>)	0.8 ± 0.2	SE
	NCK1125 (<i>ori3.1::hel3.1</i> -AS)	< 10 ⁻⁷	IP		NCK1125 (<i>ori3.1::hel3.1</i> -AS)	0.8 ± 0.2	SE
κ6	NCK1125	1.0	–	κ13	NCK1124	1.0	–
	NCK1125 (pTRK687)	0.8 ± 0.1	SE		NCK1124 (pTRK687)	0.8 ± 0.2	SE
	NCK1125 (<i>hel3.1</i> -S)	0.9 ± 0.1	SE		NCK1124 (<i>hel3.1</i> -S)	0.8 ± 0.2	SE
	NCK1125 (<i>hel3.1</i> -AS)	0.8 ± 0.1	SE		NCK1124 (<i>hel3.1</i> -AS)	0.9 ± 0.2	SE
	NCK1125 (<i>ori3.1</i>)	0.9 ± 0.2	SE		NCK1124 (<i>ori3.1</i>)	NT	SE
	NCK1125 (<i>ori3.1::hel3.1</i> -AS)	1.0 ± 0.1	SE		NCK1124 (<i>ori3.1::hel3.1</i> -AS)	NT	SE

^a ϕ , bacteriophage isolate^b EOP, efficiency of plaquing expressed as mean ± standard deviation^c Δ PS, change in plaque size; –, reference plaque size; SE, slightly enlarged; PP, pinpoint; IP, irregularly-shaped pinpoint plaques; NT, not tested

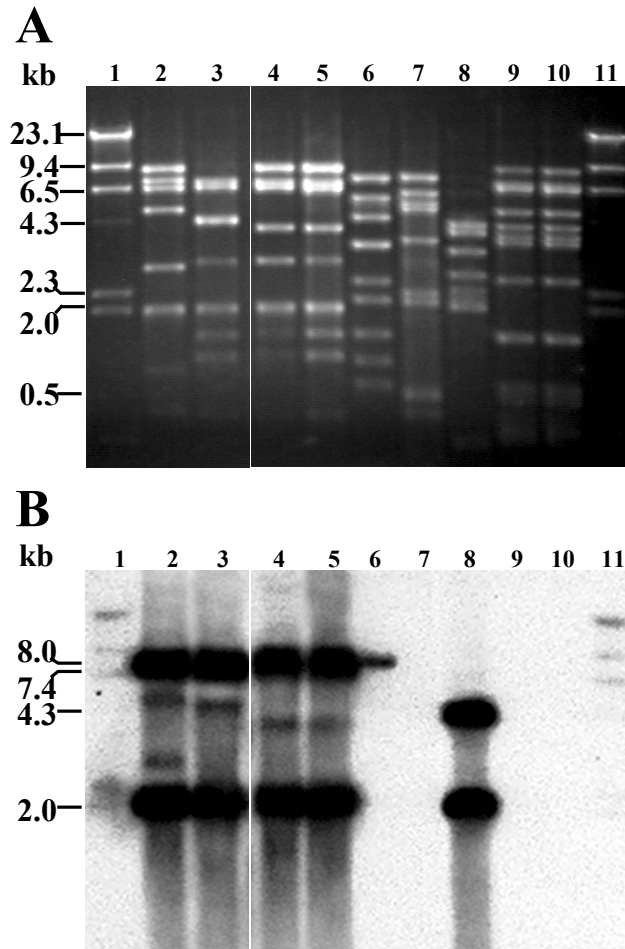
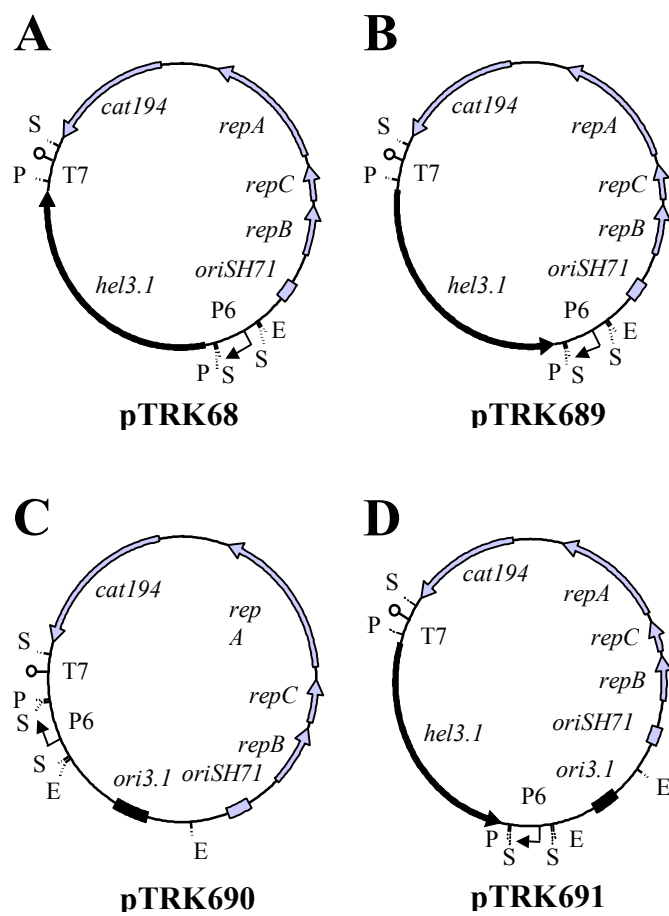


Figure 1. (A), Restriction profiles of *Hind*III-restricted bacteriophage genomic DNA. (B), Southern hybridization using an internal *hel3.1* probe. Lanes 1 and 11, DIG-labeled λ DNA molecular weight marker; Lane 2, DT1; Lane 3, κ 3; Lane 4, κ 9; Lane 5, κ 10; Lane 6, κ 13; Lane 7, κ 2; Lane 8, κ 1;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

Figure 2. (A), Sense RNA control plasmid pTRK688::*hel3.1*-S; (B), antisense RNA plasmid pTRK689::*hel3.1*-AS.; (C), PER plasmid pTRK690::*ori3.1*; (D), explosively replicated antisense RNA expression plasmid pTRK691::*ori3.1*::*hel3.1*-AS.

Abbreviations: T7, coliphage T7 transcription terminator; P6, *L. acidophilus* P6 promoter; *repBCA*, genes encoding plasmid replication factors; *cat194*, chloramphenicol resistance gene; *oriSH71*, plasmid origin of DNA replication; *ori3.1*, bacteriophage κ -derived origin of DNA replication; *hel3.1*, bacteriophage κ 3-derived putative helicase. Restriction endonuclease recognition sites: E, EcoRI; S, Sau3AI; P, PstI.



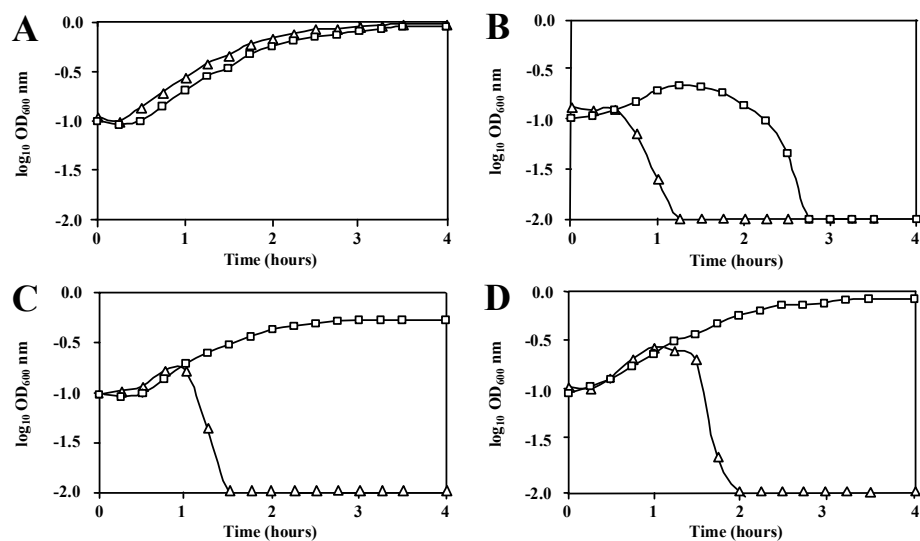


Figure 3. Effect of bacteriophage $\kappa 3$ on the growth of Δ , NCK1125 and , NCK1125 (pTRK688::*hel3.1*-AS) at varying multiplicities of infection (MOI). (A), Growth in the absence of bacteriophage $\kappa 3$ (*i.e.* MOI = 0). Growth in the presence of bacteriophage $\kappa 3$ at (B) MOI ≈ 5 ; (C), MOI ≈ 1 ; (D), and MOI ≈ 0.1 . Abbreviation: OD₆₀₀, optical density at 600 nm.

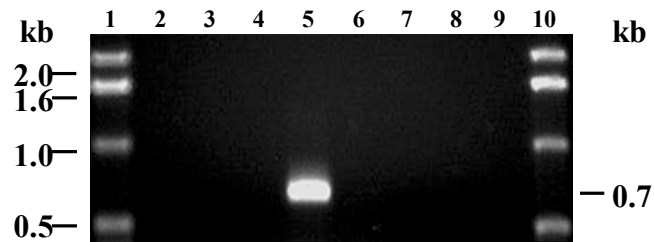


Figure 4. DNase-treated total RNA preparations were subjected to RT-PCR (Lanes 2-5) and control PCR reactions (without cDNA synthesis; Lanes 6-9) in order to detect *hel3.1* antisense RNA expression in: Lanes 2 and 6, NCK1125; Lanes 3 and 7, NCK1125 (pTRK687); Lanes 4 and 8, NCK1125 (pTRK688::*hel3.1*-S);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

CHAPTER III

Antisense RNA Targeting Primase Interferes with Bacteriophage Replication in
Streptococcus thermophilus

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ABSTRACT

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 $\kappa 3$ provided significant protection from $\kappa 3$ 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 $\kappa 3$ 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*.

INTRODUCTION

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 ribonucleic acid (RNA) targeted against phage-encoded transcripts. These antisense RNAs have been constitutively expressed by starter strains (reviewed in 2, 34, 24, 32) 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, six *Streptococcus thermophilus*-, eleven *Lactococcus*-, and three *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 non-orthologous 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 un-sequenced 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 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 $\kappa 3$ (*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. Phages used in this study are listed in Table 1. Bacteriophages were propagated as described previously (32). The EOP was calculated by dividing 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 diameter of phage plaques were measured using

a caliper, and values represent the average 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 $0.1 \pm 20\%$, as described previously (32). Burst size determinations were performed at 42°C in Elliker-BC medium, but otherwise as described previously (21). *L. lactis* phages were propagated and enumerated as described previously (13).

Polymerase chain reaction (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 using the primer P6 with either of the two primers used to amplify the PCR fragment (Table 2). Electrocompetent *E. 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 using the Lambda Kit (Qiagen) from cells infected with phage $\kappa 3$ at a MOI of $1 \pm 20\%$ or from uninfected control cultures as described previously (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, TX), 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 $\kappa 3$ using primers S4 and A3 (Fig. 2; Table 2). Double stranded DNA templates used during downstream *in vitro* transcription reactions were generated by PCR 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 - 32 P]UTP (NEN, Boston, MA) using the high yield MEGAscript transcription kit (Ambion). Radiolabeled RNA probes were purified using NucTrap Probe Purification Columns (Stratagene, La Jolla, CA). Total RNAs were isolated (i) from cultures 10, 20, 30, and 60 minutes after infection with phage $\kappa 3$ at a MOI of $1 \pm 20\%$ and (ii) from uninfected control cultures grown in parallel. RNAs were isolated using the TRIzol reagent (Gibco-BRL) as described previously (11). RNA-RNA slot hybridizations were performed using the Bio-Dot-SF apparatus and Zeta-probe membranes (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions.

Nucleotide sequence accession numbers. The DNA and deduced protein sequences for phage DT1 (33) and the $\kappa 3$ -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. 4A). 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 $\kappa 3$, $\kappa 4$, and $\kappa 9$ (data not shown). The 1.7-kb fragment was not generated from the negative control, phage $\kappa 6$, which encodes a variant of the 7201-type genome replication module.

The 1.7-kb PCR fragment amplified from phage $\kappa 3$ 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'-AGGAGG-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 $\kappa 3$ using primers S4 and A1, digested with PstI, and cloned in the antisense (-AS) orientation into the PstI site of pTRK687, which is downstream from the *Lactobacillus acidophilus* 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. 3). The control strain NCK1125 (pTRK687) lysed within 120 minutes 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 were cloned in an antisense orientation into the PstI site of pTRK687. The antisense constructs were then electroporated into NCK1125 and the resulting transformants 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 $\kappa 3$ is illustrated in Fig. 4B. 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 were systemically 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, non-overlapping 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, 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 $\phi 31$.

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 lead 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 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-minutes 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 the NCK1125 (pTRK687) control. Of these, the phage-infected strain expressing *pri3.19-AS* yielded the strongest sense mRNA signal, while 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. 4), 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 primase antisense RNA-specific signal was significantly weaker at every time point tested relative to 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 $1 \pm 20\%$. Total genomic DNAs were isolated from infected cells over the course of the lytic cycle and digested with *HindIII*. The *HindIII*-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 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 thirteen different antisense RNA constructs targeting specific regions of the *S. thermophilus* phage κ 3-encoded putative primase gene were 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 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 $\kappa 3$ 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 twelve additional subclones that spanned various structural or putative regulatory regions of the primase gene. The expression of all thirteen 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 at which phages might overcome antisense inhibition via point mutation(s).

As the length of the antisense fragments were 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, non-overlapping regions of the entire gene, exhibited significant differences in their ability to reduce the EOP of phage $\kappa 3$. 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. 4). Further, expression of antisense RNAs that strongly inhibited 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). This may not only effect 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 40-70% 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* (putative single-stranded DNA binding protein), *orf16* (putative replisome organizer), *orf18* (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 phages 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 a 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). It is important to 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) were all ineffective in their ability to inhibit the replication of the lactococcal prolate-headed phage c2, 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 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 engineering phage-encoded resistance systems, there are potential benefits of 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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REFERENCES

1. **Alatossava, T., and T. R. Klaenhammer.** 1991. Molecular characterization of three small isometric-headed bacteriophages which vary in their sensitivity to the lactococcal phage resistance plasmid pTR2030. *Appl. Environ. Microbiol.* **57**: 1346-1353.
2. **Allison, G. E., and T. R. Klaenhammer.** 1998. Phage resistance mechanisms in lactic acid bacteria. *Int. Dairy J.* **8**: 207-226.
3. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST, a new generation of protein database search programs. *Nucleic Acids Res.* **25**: 3389-3402.
4. **Bouchard, J.D., and S. Moineau.** 2000. Homologous recombination between a lactococcal bacteriophage and the chromosome of its host strain. *Virology.* **270**: 65-75.
5. **Brüssow, H., and F. Desiere.** 2001. Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. *Mol. Microbiol.* **39**: 213-222.

6. **Brüssow, H., A. Probst, M. Fremont, and J. Sidoti.** 1994. Distinct *Streptococcus thermophilus* bacteriophages share an extremely conserved DNA fragment. *Virology*. **200**: 854-857.
7. **Bruttin, A., F. Desiere, N. d'Amico, J. P. Guerin, J. Sidoti, B. Huni, et al.** 1997. Molecular ecology of *Streptococcus thermophilus* bacteriophage infections in a cheese factory. *Appl. Environ. Microbiol.* **63**: 3144-3150.
8. **Bull, J. J., A. Jacobson, M. R. Badgett, and J. Molineux.** 1998. Viral escape from antisense RNA. *Mol. Microbiol.* **28**:853-846.
9. **Chung, D. K., S. K. Chung, and C. A. Batt.** 1992. Antisense RNA directed against the major capsid protein of *Lactococcus lactis* subsp. *cremoris* bacteriophage F4-1 confers partial resistance to the host. *App. Microbiol. Biotechnol.* **37**: 79-83.
10. **Desiere, F., S. Lucchini, C. Canchaya, M. Ventura, and H. Brüssow.** 2002. Comparative genomics of phages and prophages in lactic acid bacteria. *Antonie Van Leeuwenhoek.* **82**: 73-91.
11. **Dinsmore, P. K., and T. R. Klaenhammer.** 1997. Molecular characterization of a genomic region in a *Lactococcus* bacteriophage that is involved in its sensitivity to the phage defense system AbiA. *J. Bacteriol.* **179**: 2949-2957.

12. **Djordjevic, G., B. Bojovic, N. Miladinov, and L. Topisirovic.** 1997. Cloning and molecular analysis of promoter-like sequences isolated from the chromosomal DNA of *Lactobacillus acidophilus* ATCC 4356. *Can. J. Microbiol.* **43**: 61-69.
13. **Durmaz, E., and T. R. Klaenhammer.** 2000. Genetic analysis of chromosomal regions of *Lactococcus lactis* acquired by recombinant lytic phages. *Appl. Environ. Microbiol.* **66**: 895-903.
14. **Hill, C., Massey, L.J., and T. R. Klaenhammer.** 1991. Rapid method to characterize lactococcal bacteriophage genomes. *Appl. Environ. Microbiol.* **57**: 283-288.
15. **Hjalt, T. A, and E. G. Wagner.** 1995. Bulged-out nucleotides in an antisense RNA are required for rapid target RNA binding in vitro and inhibition in vivo. *Nucleic Acids Res.* **23**: 580-587.
16. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl. Environ. Microbiol.* **55**: 3119-3123.

17. **Huynh, T. V., R. A. Young, and R. W. Davis.** 1985. Construction and screening cDNA libraries in λ gt10 and λ gt11. In *DNA cloning, vol. I*. D. M. Glover (ed.). Oxford: IRL Press Ltd., pp. 49-78.
18. **Inouye, M.** 1988. Antisense RNA: its functions and applications in gene regulation—a review. *Gene*. **72**: 25-34.
19. **Kim, J. H., and C. A. Batt.** 1991. Antisense RNA mediated bacteriophage resistance in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **57**: 1039-1045.
20. **Kim, S. G., Y. C. Bor, and C. A. Batt.** 1992. Bacteriophage resistance in *Lactococcus lactis* ssp. *lactis* using antisense ribonucleic acid. *J Dairy Sci.* **75**: 1761-1767.
21. **Klaenhammer, T. R., and R. B. Sanozky.** 1985. Conjugal transfer from *Streptococcus lactis* ME2 of plasmid encoding phage resistance, nisin resistance and lactose-fermenting ability: evidence for a high-frequency conjugative plasmid responsible for abortive infection of virulent bacteriophage. *J. Gen. Microbiol.* **131**: 1531-1541.
22. **Kolb, F. A., E. Westhof, C. Ehresmann, B. Ehresmann, E. Gerhart, H. Wagner, and P. Romby.** 2001. Bulged residues promote the progression of a

- loop–loop interaction to a stable and inhibitory antisense–target RNA complex. *Nucleic Acids Res.* **29**: 3145-3153.
23. **Lucchini, S., F. Desiere, and H. Brüssow.** 1999. Comparative genomics of *Streptococcus thermophilus* phage species supports a modular evolution theory. *J. Virol.* **73**: 8647-8656.
24. **McGrath, S., G. F. Fitzgerald, and D. van Sinderen.** 2001. Improvement and optimization of two engineered phage resistance mechanisms in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **67**: 608-616.
25. **Moineau, S., M. Borkaev, B. J. Holler, S. A. Walker, J. K. Kondo, E. R. Vedamuthu, and P. A. Vandenberg.** 1996. Isolation and characterization of lactococcal bacteriophages from cultured buttermilk plants in the United States. *J. Dairy Sci.* **79**: 2104-2111.
26. **Nuesch J. P., and P. Tattersall.** 1993. Nuclear targeting of the parvoviral replicator molecule NS1: evidence for self-association prior to nuclear transport. *Virology.* **196**: 637-651.
27. **O'Sullivan, D. J., and T. R. Klaenhammer.** 1993. Rapid mini-prep isolation of high-quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. *Appl. Environ. Microbiol.* **59**: 2730-2733.

28. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.
29. **Sanders, M. E., and T. R. Klaenhammer.** 1980. Restriction and modification in group N streptococci: effect of heat on development of modified lytic bacteriophage. *Appl. Environ. Microbiol.* **40**: 500-506.
30. **Sanders, M. E., P. J. Leonhard, W. D. Sing, and T. R. Klaenhammer.** 1986. Conjugal strategy for construction of fast acid-producing, bacteriophage-resistant lactic streptococci for use in dairy fermentations. *Appl. Environ. Microbiol.* **52**: 1001-1007.
31. **Sing, W. D., and T.R. Klaenhammer.** 1990. Characteristics of phage abortion conferred in lactococci by the conjugal plasmid pTR2030. *J. Gen. Microbiol.* **136**: 1807-1815.
32. **Sturino, J. M., and T. R. Klaenhammer.** 2002. Expression of antisense RNA targeted against *Streptococcus thermophilus* bacteriophages. *Appl. Environ. Microbiol.* **68**: 588-596.

33. **Tremblay, D. M., and S. Moineau.** 1999. Complete genomic sequence of the lytic bacteriophage DT1 of *Streptococcus thermophilus*. *Virology*. **255**: 63-76.
34. **Walker, S. A. and T. R. Klaenhammer.** 2000. An explosive antisense RNA strategy for inhibition of a lactococcal bacteriophage. *Appl. Environ. Microbiol.* **66**: 310-319.
35. **Wang, D., W. Yuan, I. Davis, and C. R. Parrish.** 1998. Nonstructural protein-2 and the replication of canine parvovirus. *Virology*. **240**: 273-281.

TABLE 1
Bacterial strains, bacteriophages and plasmids

Bacterial 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
Bacteriophages		
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 7201-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
Plasmids		
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 Abbreviations: Cm^R, encodes chloramphenicol resistance; Cm^S, sensitive to chloramphenicol

TABLE 2
Primers used in this study

Name ^a	Nucleic Acid Sequence ^b	Position ^c
S1	5'-AAACTGCAGCAGAGAACAATTGCAAGC-3'	28,575
S2	5'-AAACTGCAGCAACACCCAAGAGCC-3'	28,466
S3	5'-AAACTGCAGTAAGGAGGATTGGACTTGAC-3'	28,534
S4	5'-AAACTGCAGTTGACAACGATTGATTTCG-3'	28,549
S5	5'-AAACTGCAGATTAAATTTTAGTACCATTG-3'	29,053
S6	5'-AAACTGCAGGGTACATATCGACGTATCG-3'	29,557
S7	5'-AAACTGCAGTAGCTATATATGATCCAG-3'	28,787
S8	5'-CCCAAGAGCCTTTGGGCAATAAGG-3'	28,471
A1	5'-AAACTGCAGGTTGCAATAACCTGCGG-3'	30,080
A2	5'-AAACTGCAGCTGAGTAACCATAACCAAC-3'	30,060
A3	5'-AAACTGCAGAAACTTATGGTCAAACGATAG-3'	29,052
A4	5'-AAACTGCAGGTTTGACTTATTCTTAAACAC-3'	29,556
A5	5'-AAACTGCAGCTTTCCCATTTTCGAGGG-3'	28,786
A6	5'-CTAAGTAACTAAAGCAACCGAACCC-3'	30,135
P6	5'-GGAGCGTGATTTTATGG-3'	-
T7	5'-GCTCCGGCTCGTATGTTGTGTGG-3'	-

^a S- and A- primers are derived from the sense- and antisense strands, respectively
^b PstI (5'-CTGCAG-3') restriction sites are underlined, when appropriate
^c The 5' nucleotide position relative to the DT1 sequence is bolded, when appropriate

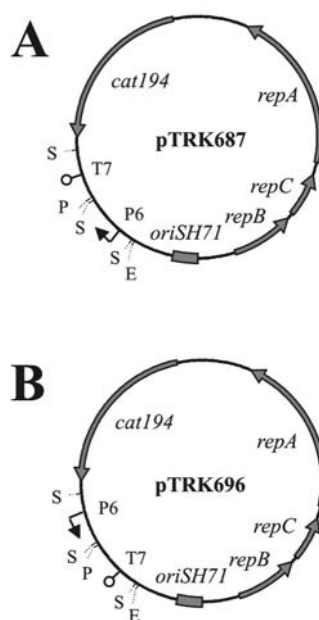


Figure 1. The antisense RNA expression vectors used in this study that contain the P6 promoter cassette cloned in opposite orientations. (A), pTRK687 and (B) pTRK696. Abbreviations: T7, coliphage T7 transcription terminator; P6, *L. acidophilus* P6 promoter; *repBCA*, genes encoding plasmid replication factors; *cat194*, chloramphenicol resistance gene; *oriSH71*, origin of DNA replication; *pri3.1*, phage κ 3-derived putative primase. Restriction endonuclease recognition sites: E, EcoRI; S, Sau3AI; P, PstI.

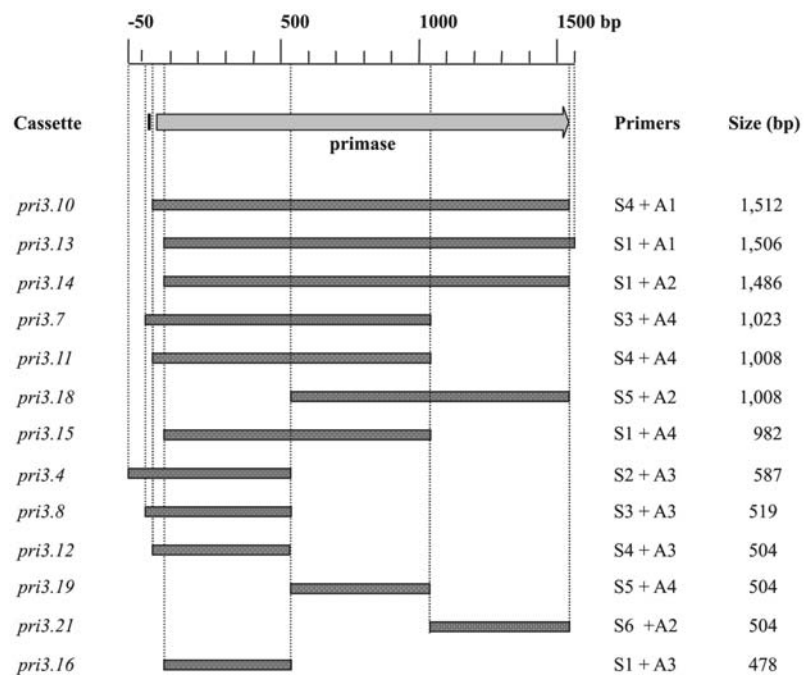
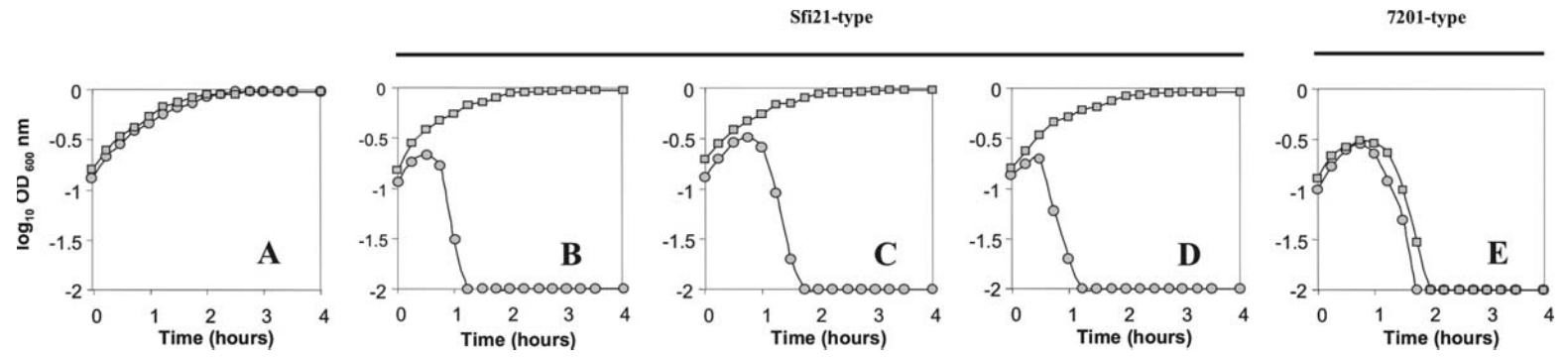


Figure 2. Schematic of the *pri3.1* gene (arrow) and upstream putative ribosome binding site (■). The boundaries of the subcloned fragments are shown as bars demarcated with dotted lines. Base pair (bp) coordinates are numbered relative to the 5' position of the primase translation initiation codon.

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



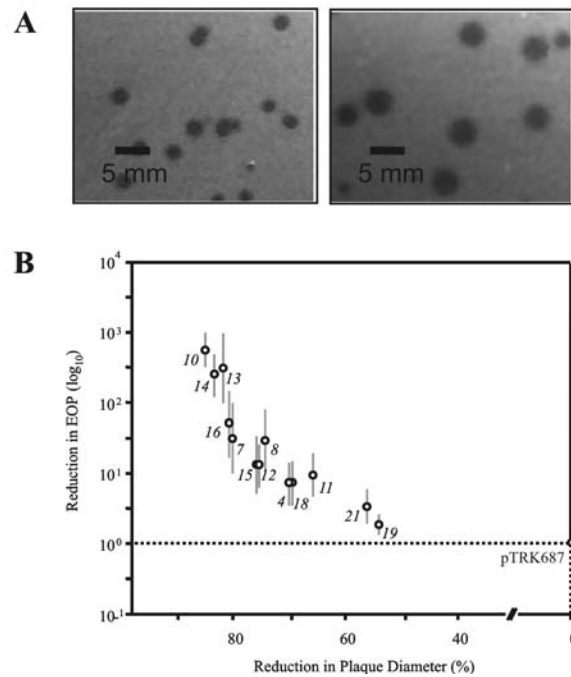


Figure 4. (A) Plaque size of phage $\kappa 3$ when titrated on NCK1125 (left) and NCK1125 (pTRK687) (right). (B) Comparison of primase-derived antisense RNA constructs on the EOP and plaque size of phage $\kappa 3$ relative to plasmid control, pTRK687. Horizontal and vertical dotted lines represent an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

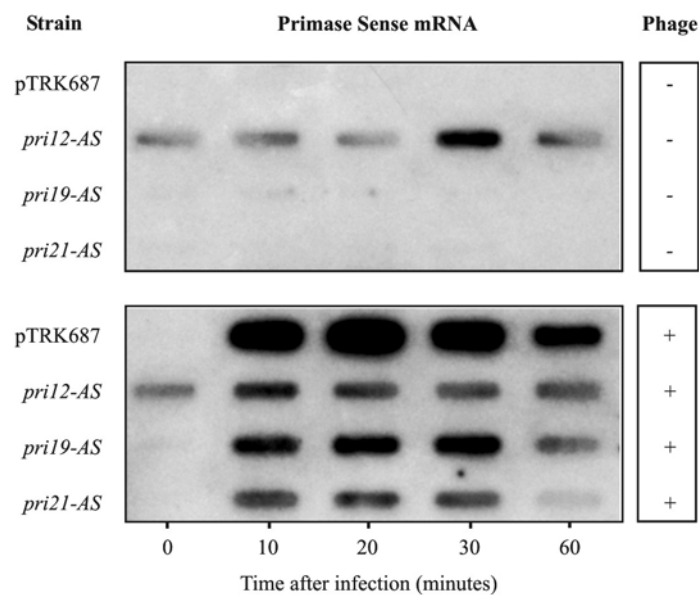


Figure 5. RNA-RNA slot blot hybridization of three antisense RNA expressing constructs and the vector control strain NCK1125 (pTRK687) with *detect-S*, a *pri3.12*-derived single-stranded RNA probe that was complementary to the sense strand of the phage $\kappa 3$ -encoded primase mRNA (Fig. 2). RNA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

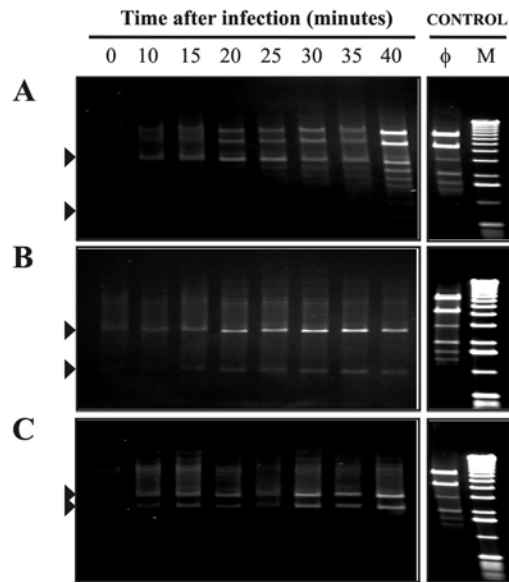


Figure 6. The impact of antisense RNA expression on the *in vivo* accumulation of *Hind*III-digested phage $\kappa 3$ -specific DNA fragments over time. (A), Vector control strain NCK1125 (pTRK687); (B) NCK1125 (pTRK789::*pri3.8-AS*); (C), NCK1125 (pTRK790::*pri3.10-AS*). Each plasmid encoded two *Hind*III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

CHAPTER IV

Inhibition of bacteriophage genome replication through the expression of transdominant mutant primase proteins in *Streptococcus thermophilus*

ABSTRACT

Invariant and highly conserved amino acids within a primase consensus sequence were targeted by site-specific mutations within the *S. thermophilus* phage κ 3-encoded putative primase. Residues within putative ATPase/helicase and oligomerization domains were identified that are suspected to be critical for primase function *in vivo*. PCR products containing the desired mutation(s) in the phage κ 3-encoded primase gene were cloned into a high-copy-number vector, pTRK687, and expressed in *S. thermophilus* NCK1125. The majority of the examined constructs (*i.e.* 10 of 14 constructs) remained sensitive to phage κ 3, however four constructs conferred strong phage resistance to the bacterial host. The co-expression *in trans* of the K238(A/T) and RR340-341AA mutant proteins suppressed the function of the native, phage-encoded primase protein in a dominant negative fashion via a proposed subunit poisoning mechanism. These constructs completely inhibited phage genome synthesis, reduced the efficiencies of plaquing more than nine log cycles, and lowered center of infection formation by 3.5-log cycles. Amber mutations introduced upstream of the transdominant RR340-341AA and K238(A/T) mutations restored phage genome replication and phage sensitivity of the host, indicating that translation was required to confer phage resistance. Introduction of an E437A mutation in a putative protein oligomerization domain located downstream of the transdominant K238T mutation also completely suppressed phage resistance. This study describes a novel, protein-based defense system effective against Sfi21-type *S. thermophilus* phages encoding variants of the Sfi21-type genome replication module. This study represents the first use of transdominant proteins to inhibit phage replication in the lactic acid bacteria.

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 substrate, 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 defense systems by mutation and recombination (Bouchard and Moineau, 2000; Durmaz and 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 strategies. 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. Interestingly, the functional components of these engineered systems have largely been phage-derived. Among the most successful of these strategies have been (i) the expression of antisense RNA specific for phage encoded transcripts; (ii) the presentation of phage origins of replication in *trans* to titrate away phage-encoded DNA replication factors; and (iii) the triggering of toxic suicide cassettes by phage-inducible promoters (for reviews, see Allison and Klaenhammer, 1998). 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 and Klaenhammer, 2002; Sturino and Klaenhammer, 2004). Identification of highly conserved phage-encoded genes, pathways, or processes can ensure that new defense systems exhibit efficacy against a wider variety phage strains, which is of critical importance when designing defense systems for industrial application.

S. thermophilus bacteriophages 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 of the phages for this species that have been 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 catalyzed 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; Desiere *et al.*, 2002). For several important reasons, the genes associated with the Sfi21-type genome replication module were previously found to be well suited for the expression of engineered phage-inhibitory defense strategies (Sturino and Klaenhammer, 2002). First, is their frequency of distribution in industrial isolates; the Sfi21-type gene cluster is found in the majority of industrial *S. thermophilus* phage isolates (Brüssow *et al.*, 1994; Sturino and Klaenhammer, 2002). Second, independently isolated variants exhibit striking sequence conservation at the nucleic acid level (*i.e.* greater than 99.9%) in this region. Third and lastly, genome replication

functions have an intrinsic strategic importance since they are expressed early in the lytic cycle, prior to the loss of control of host cellular processes and may allow for the recovery of the host after the infection is aborted (Sturino and Klaenhammer, 2002).

The Sfi21-type replication module is comprised of 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 and Desiere, 2001). The putative primase is known to be essential for the replication of genomes comprised of dsDNA (Frick and Richardson, 2001). Primases are hexameric DNA-dependant RNA polymerases that catalyze the *de novo* synthesis of short oligoribonucleotide primers at sequence-specific loci located across the lagging strand. These primers, which are generally between four and fifteen nucleotides in length, are subsequently elongated by the cognate DNA polymerase(s) to yield Okazaki fragments, which are finally ligated to complete the synthesis of the lagging strand during semidiscontinuous DNA replication.

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 primase. Expression of these mutant primases, *in trans*, was then examined for their impact on phage DNA replication and their ability to confer phage resistance to *St. thermophilus*.

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 (Sturino and Klaenhammer, 2004).

Bacteriophages and propagation assays. Phages used in this study are listed in Table 1. All of the phages used in this study were independently isolated from different dairy facilities across the continental United States and were propagated as described previously (Sturino and Klaenhammer, 2004). The efficiency of plaquing (EOP) was calculated by dividing plaque-forming units per milliliter (PFU/ml) on the test strain by the PFU/ml on the control strain, *S. thermophilus* NCK1125 (pTRK687). The diameters of phage plaques were measured using a caliper and represent the average 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) (Sing and Klaenhammer, 1990).

Polymerase chain reaction (PCR) mediated site-directed mutagenesis and plasmid construction. The plasmids and primers used in this study are listed in Table 1 and Table 2, respectively. Conventional PCR reactions, DNA sequencing, and sequence analyses were all performed as described previously (Sturino and Klaenhammer, 2004). When appropriate, PstI 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 (SOEing) during PCR as described elsewhere (Horton, 1995). All point mutations were confirmed by sequencing. Electrocompetent *E. coli* and *S. thermophilus* were prepared and electroporated as described previously (Sturino and Klaenhammer, 2004). The orientation of cloned PCR

products was confirmed by restriction digestion and PCR amplification using the primer P6 (Sturino and Klaenhammer, 2004) with either of the two primers used to amplify the PCR fragment (Table 2).

Plasmid and genomic DNA preparations. Plasmid DNAs and PCR fragments were isolated as described previously (Sambrook *et al.*, 1982). Genomic DNAs were isolated from (i) cultures 10, 20, 30, and 60 minutes after infection with phage $\kappa 3$ at a MOI of $1 \pm 20\%$ and (ii) uninfected control cultures grown in parallel as described previously (Hill *et al.*, 1991).

Southern hybridizations and alkaline gel electrophoresis. Alkaline transfer of *Hind*III-digested genomic DNA fragments and subsequent Southern hybridizations were performed as described previously (Sturino and Klaenhammer, 2002). The digoxigenin-11-dUTP labeled probes used during hybridization studies were derived from phage $\kappa 3$ genomic DNA and were purified using NucTrap Probe Purification Columns prior to use (Stratagene, La Jolla, CA). Intensities of ethidium bromide stained bands were determined using a Gel Doc 2000 gel documentation system (Bio-Rad Laboratories, Hercules, CA) using Quantity One software. When appropriate, undigested genomic DNAs were electrophoresed through alkaline agarose gels to better visualize single stranded DNA intermediates generated during DNA synthesis (Sambrook *et al.*, 1982).

Protein sequence manipulation and GenBank accession numbers. The nucleic acid and deduced amino acid sequences for the $\kappa 3$ -encoded putative primase proteins can be found in the GenBank database under the nucleotide accession number AY196178 (Sturino and Klaenhammer, 2004). Conserved domains were detected using the BlastP (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 ClustalX, which was obtained from <ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/> (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 1,515 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 and Klaenhammer, 2002).

A 1.5-kb fragment containing the native primase gene was amplified without its native promoter using primer set L001L (Table 2), 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 EOP, the expression of the native protein did not confer resistance to phage κ 3 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 impact the EOP of any of the phages tested, as was observed previously (Sturino and Klaenhammer, 2002).

Identification of protein domains and motifs common to prokaryotic primases and ATPases and site-directed mutagenesis. Using BlastP (Altschul *et al.*, 1997) in conjunction with the COG database (Tatusov *et al.*, 2001), a COG3378-type predicted ATPase domain (e-value of $2e^{-59}$) and a constituent conserved Parvo_NS1 domain (pfam01057) (e-value of $3e^{-3}$) were detected within the primary amino acid sequence of the putative phage $\kappa 3$ primase (Fig. 1B and 1C). Both DNA helicase and ATPase activities are associated with the pfam01057 domain, which is required for genome replication in double stranded DNA parvoviruses (Nuesch and Tattersall, 1993; Wang *et al.* 1998). This domain was found to contain a signature glycine-rich phosphate-binding loop of sequence GNGNDGKGT near the center of the protein. 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 e^{-3} were aligned using the ClustalX program (Thompson *et al.*, 1997). Regions of strong sequence conservation were identified (Fig. 1). The location of these motifs relative to the conserved domains described above may be found in Fig. 1A. In order to determine if the conserved amino acid residues identified by multiple alignments were critical for protein structure or function in *S. thermophilus* phage $\kappa 3$, thirteen mutant primase alleles derived from the native, primase-encoding plasmid pTRK809 were constructed by site-directed mutagenesis (Fig. 2).

Individual point mutations were introduced by SOEing PCR and the 1.5-kb mutant primase alleles were amplified using primer set L001L (Table 2), digested with PstI, and cloned into the PstI site of pTRK687 in the sense orientation relative to and downstream from the P6 promoter. In an effort to increase the efficiency of translation, some of the constructs were amplified using primer set L001M (Table 2), which mutated the putative leucine (L) alternative start codon (5'-TTG-3') to the standard, methionine (M) start codon (5'-ATG-3'). The resulting vectors were then electroporated into NCK1125 and tested for their ability to interfere with phage replication. Phage κ 3 was titered on all thirteen of the point mutant constructs during standard plaque assays (Fig. 2). The various primase constructs could easily be differentiated into two distinct populations based on their effects on relative EOP and plaque diameter: phage sensitive or phage resistant. The vast majority of the constructs (*i.e.* 10 of 14 constructs), including pTRK809, L::N151*am*, L::E437A, M::E437A, M::E437D, L::N151*am*::K238T, L::K238T::E347A, M::K238T::E347A, L::N326A, and M::N151*am*::RR340-341AA, were sensitive to phage κ 3, and exhibited parental-type EOP and plaque diameter during standard plaque assays. The remaining four constructs, L::K238T, M::K238T, M::K238A, and M::RR340-341AA exhibited strong resistance to phage κ 3; no plaques were visible at any dilution tested, corresponding to greater than a nine-log cycle reduction in EOP. The expression in *trans* of these four constructs suppressed the function of the native, phage-encoded primase protein in a dominant negative fashion. No phages resistant to the expression of transdominant L::K238T, M::K238T, M::K238A, or M::RR340-341AA have been isolated to date.

Center of infection assays. NCK1125 (pTRK809) and the four constructs that exhibited significant resistance to phage $\kappa 3$ during EOP assays (*i.e.* L::K238T, M::K238T, M::K238A, and M::RR340-341AA) were also tested for their ability to inhibit the formation of phage $\kappa 3$ infective centers relative to the frequency of COI formation of the NCK1125 (pTRK687) indicator strain (Fig. 3A). The strain harboring the pTRK809 vector exhibited parental type ECOI and plaque sizes, however the four strains harboring phage resistance constructs exhibited large (*i.e.* 3.5 log cycle) reductions in the ECOI formation indicating that most infected cells did not produce viable progeny. In addition, plaque size of the L::K238T construct during COI assays was reduced relative to the pTRK687 indicator strain by approximately 80% (Fig. 3B). The M::K238T, M::K238A, and M::RR340-341AA constructs also showed reductions in plaque size identical to those exhibited by L::K238T.

Phages from plaques generated during COI experiments were titered on the sensitive indicator host NCK1125 (pTRK687) and the four hosts expressing the transdominant primase proteins. The phages plaqued normally on NCK1125 (pTRK687), however no plaques were detected on L::K238T, M::K238T, M::K238A, or M::RR340-341AA at any dilution tested. Similar results were obtained when the resuspended plaques were repropagated on NCK1125 prior to titering. These data indicate that plaques formed during COI assays are not initiated by progeny phages that are resistant to the expression of mutant primase proteins.

Transdominant proteins interfere with intracellular bacteriophage genome synthesis *in vivo*. NCK1125 (pTRK687) and strains expressing L::K238T, L::N151am::K238T, and M::RR340-1AA were infected with phage $\kappa 3$ at a MOI of

approximately 1.0. Genomic DNAs were then isolated from phage-infected cells over the course of the lytic cycle in order to determine if the expression of transdominant primase proteins retarded or abolished the accumulation of phage $\kappa 3$ -specific DNA bands over time (Fig.4). The genomic DNAs were digested with *HindIII* and the fragments were separated by agarose gel electrophoresis prior to being transferred to nylon membranes for Southern hybridization. In the NCK1125 (pTRK687) parent strain (Figs. 4A and 4E), phage-specific dsDNA fragments began to accumulate 10 minutes post-infection, accrued maximally at 30 minutes post infection, and were reduced following host lysis, which occurred approximately 40 minutes post-infection. In contrast, phage-specific DNA bands failed to accumulate over time in hosts expressing primase proteins with the transdominant mutations K238T (Figs. 4B and 4F) and RR340-341AA (Fig. 4D). Interestingly, the transdominant phenotype was completely abolished in the T::N151*am*::K238T-expressing host and phage genome replication was restored when the N151*am* mutation was introduced upstream of the K238T mutation (Fig. 4C).

Reduced accumulation of phage-specific ssDNA. Genomic DNAs were isolated from the NCK1125 (pTRK687) control strain and the strain expressing transdominant mutant primase K238T over the course of the phage $\kappa 3$ lytic cycle (Fig. 5). Undigested genomic DNAs were subjected to alkaline denaturing agarose gel electrophoresis, transferred to a nylon membrane and hybridized with total phage DNA in order to visualize the accumulation of high- and low molecular weight (MW) ssDNA intermediates, which primarily represents the leading- and lagging strands, respectively that are generated during semidiscontinuous DNA synthesis of the phage DNA. The phage $\kappa 3$ -specific signal intensity increases over time in the NCK1125 (pTRK687)

control strain, and a wide range of both high- and low MW genomic intermediates were visible. In contrast, the levels of high MW intermediates are greatly reduced in the K238T strain and fewer genome-derived low MW intermediates are visible. Three diffuse bands of primase-specific plasmid-associated low MW intermediates were visible, which based on size corresponded to plasmid monomers, dimers, and trimers.

Inhibition of heterologous phages encoding Sfi21-type replication modules.

The transdominant primase constructs L::K238T, M::K238T, M::K238A, and M::RR340-341AA were then tested for their ability to inhibit the replication of three additional phages encoding variants of the Sfi21-type genome replication module and a single phage encoding a variant of the 7201-type genome replication module (Table 3). The expression of L::K238T, M::K238T, M::K238A, and M::RR340-341AA all similarly inhibited the replication of Sfi21-type phages $\kappa 3$, $\kappa 4$, $\kappa 9$, and $\kappa 10$, but failed to provide any protection from the 7201-type phage, $\kappa 6$.

DISCUSSION

The replication modules of six *S. thermophilus* phages were compared previously to identify genes that could be targeted by engineered phage defense systems with potentially widespread efficacy (Sturino and Klaenhammer, 2002). The genes associated with the Sfi21-type genome replication module, including a putative primase, were found to be among the best candidates due to their frequency of distribution in industrial phage isolates, striking sequence conservation between independent isolates, and intrinsic strategic importance in early phage development. 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 mutations. Characterizations of these mutant proteins lead 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 primase protein in a dominant negative fashion. The inhibition of phage genome replication and the subsequent failure of the phage lytic cycle suggested that the plasmid-encoded mutant primase proteins were structurally intact and formed stable interactions with the native, phage-encoded primase proteins, thus inhibiting their activity. Alternatively, the mutant primase proteins could have formed other non-productive associations, such as substrate binding (*e.g.* origin of replication) and/or inhibit 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-type plaquing efficiency and completely suppressed phage 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, indicating that the phage-encoded primase

must have unique activities and/or associations that are essential for phage genome replication. It is important to note that the phage $\kappa 3$ -encoded putative primase has not been definitively characterized biochemically.

Several phage-encoded primase proteins, including gene product 4 (gp4) encoded by coliphage T7 (*Podoviridae*; Notarnicola and Richardson, 1993) and gp α encoded by coliphage P4 (*Myoviridae*; Ziegelin *et al.*, 1995,) have been extensively characterized at the genetic and biochemical levels. Interestingly, the putative primase from phage $\kappa 3$ (family *Siphoviridae*) and its related putative orthologs lack several key functional motifs conserved among these *E. coli* DnaG-like primase proteins (Ilyina *et al.* 1992). For instance, the phage $\kappa 3$ -encoded putative primase lacks the N-terminal zinc-finger motif (CX₂CX₁₈C X₂C), which is required for DNA template recognition (Frick and Richardson, 2001; Ilyina *et al.* 1992). In addition, this protein also lacks the putative primase active center (EGYATA) (Frick and Richardson, 2001; Ilyina *et al.* 1992; Pansegrau and Lanka, 1992). These deficiencies notwithstanding, the phage $\kappa 3$ -encoded enzyme described in this study has been designated as a variant class of putative primase since it possesses significant regions along its carboxy terminus that exhibit weak similarities to other phage-encoded and prokaryotic polymerases, especially primases. For example, the carboxy-terminal ATPase domain (COG3387) shows similarities to the putative primase from *Lactobacillus gasseri* phage ϕ adh (Altermann *et al.*, 1999) and the characterized primase from *E. coli* satellite phage P4 (Ziegelin *et al.*, 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 located within the putative ATPase 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 and Richardson, 1993).

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 and Richardson, 1993). NBS mutants of the hexameric gp4, including those with alterations at the conserved lysine (K318) residue, suppressed the function of the native gp4 in a dominant negative fashion (Notarnicola and Richardson, 1993; Notarnicola *et al.* 1995). It was found that one mutant subunit present in a hexamer containing five native subunits lead to an inactive oligomer (Notarnicola and Richardson, 1993). Like gp4 of phage T7, the multifunctional gp α protein of phage P4 catalyzes both helicase and primase activities. Unlike gp4, gp α , has also been shown to be responsible for target sequence recognition (*e.g. ori-binding*) (Ziegelin *et al.*, 1993). Interestingly, transcomplementation studies using meridioid strains (*e.g. primase-null and helicase-null*) revealed that the various gp α domains function independently of one another (Ziegelin *et al.*, 1995). Further, transdominant negative interference was not observed when the K507(A/D) gp α mutant was expressed in *trans* during infection of the wild-type phage P4, which is in striking contrast to the results described here and in the T7 studies. From these observations, it was concluded that gp α might not act as an oligomer (Ziegelin *et al.*, 1995).

When expressed in *trans*, proteins encoding the N326A mutation did not have any effect on phage replication, however this mutation was not characterized in conjunction with any other mutation (Fig. 2). All of the plasmid constructs described in this study were readily constructed in *E. coli*, however several unsuccessful attempts were made to construct pTRK687::F409A in *E. coli* and *Lactococcus lactis* (data not shown). We suspect that this invariant amino acid is essential for oligomerization and/or protein stability, and that the misfolded mutant protein products were toxic to the host when overexpressed. As a result, residues downstream of F409 were targeted for site-specific mutation in order to identify non-toxic residues involved in protein oligomerization. Non-conservative E437A and conservative E437D mutant constructs were readily cloned, but had no effect on the replication of phage $\kappa 3$. However, when the E437A mutation was introduced downstream of the transdominant K238T mutation, the E437A mutation completely suppressed phage resistance. These results indicated that the E437A mutation precluded the association of the mutant primase protein from the native, phage-encoded primase. Hence, E437 might be a component of the protein oligomerization domain. The oligomerization domain of gp4 from coliphage T7 is also located near the carboxy-terminal region of the protein (Notarnicola *et al.*, 1995).

In a previous study, antisense RNAs directed against the phage $\kappa 3$ -encoded putative primase gene severely retarded phage genome replication and limited the number of progeny phages released in *S. thermophilus* (Sturino and Klaenhammer, 2004). The expression of *pri3.10-AS*, one of the largest and most effective antisense RNAs, resulted in a 2.7-log cycle 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 clearly much more effective than the use of even the most effective primase-specific antisense RNA. The transdominant K238(A/T) and RR340-1AA proteins completely inhibited phage genome replication and reduced the EOP by greater than 9 log cycles.

Significantly, no bacteriophages resistant to transdominant primase proteins have been isolated to date. This may be due to the fact that protein structure is tightly associated with protein function and strong conservation is found at the tertiary and quaternary structural levels of related proteins. As a result, target proteins encoded by heterologous phages will, by in large, contain variations in the primary amino acid sequences that result in subtle changes in their overall protein structure. Hence, unless these mutations specifically alter the oligomerization domain, these mutations would likely have marginal impacts on the efficacy of overexpressed transdominant proteins.

In conclusion, this study describes a novel, protein-based defense system effective against *S. thermophilus* phages encoding variants of the Sfi21-type genome replication module. Multiple alignments of related target proteins, in this case putative primase proteins, were examined for invariant and highly conserved amino acid residues within the primary sequences. These residues were targeted by site-specific mutations using the *S. thermophilus* phage κ 3-encoded putative primase as a model system. These characterizations enabled the identification of residues within the putative ATPase/helicase and oligomerization domains that are critical for primase function. When the K238(A/T) and RR340-341AA mutant derivatives were expressed in *trans*, the mutant proteins suppressed the function of the native, phage-encoded primase protein in a

dominant negative fashion. This study represents the first use of transdominant mutant proteins to inhibit phage replication via subunit poisoning in the lactic acid bacteria.

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REFERENCES

1. **Allison, G., and T. R. Klaenhammer.** 1998. Phage resistance mechanisms in lactic acid bacteria. *Int. Dairy J.* **8**:207-226.
2. **Altermann, E., J. R. Klein, and B. Henrich.** 1999. Primary structure and features of the genome of the *Lactobacillus gasseri* temperate bacteriophage ϕ adh. *Gene.* **236**:333-46.
3. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST, a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389-3402.
4. **Bouchard, J.D., and S. Moineau.** 2000. Homologous recombination between a lactococcal bacteriophage and the chromosome of its host strain. *Virology.* **270**:65-75.
5. **Brüssow, H., and F. Desiere.** 2001. Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. *Mol. Microbiol.* **39**:213-222.

6. **Brüssow, H., A. Probst, M. Fremont, and J. Sidoti.** 1994. Distinct *Streptococcus thermophilus* bacteriophages share an extremely conserved DNA fragment. *Virology*. **200**:854-857.
7. **Bruttin, A., F. Desiere, N. d'Amico, J. P. Guerin, J. Sidoti, B. Huni, et al.** 1997. Molecular ecology of *Streptococcus thermophilus* bacteriophage infections in a cheese factory. *Appl. Environ. Microbiol.* **63**:3144-3150.
8. **Coffey, A., and R. P. Ross.** 2002. Bacteriophage resistance systems in dairy starter strains: molecular analysis to application. *Antonie Van Leeuwenhoek*. **82**:303-321.
9. **Desiere, F., S. Lucchini, C. Canchaya, M. Ventura, and H. Brüssow.** 2002. Comparative genomics of phages and prophages in lactic acid bacteria. *Antonie Van Leeuwenhoek*. **82**: 73-91.
10. **Djordjevic, G., B. Bojovic, N. Miladinov, and L. Topisirovic.** 1997. Cloning and molecular analysis of promoter-like sequences isolated from the chromosomal DNA of *Lactobacillus acidophilus* ATCC 4356. *Can. J. Microbiol.* **43**:61-69.
11. **Durmaz, E., and T. R. Klaenhammer.** 2000. Genetic analysis of chromosomal regions of *Lactococcus lactis* acquired by recombinant lytic phages. *Appl. Environ. Microbiol.* **66**:895-903.

12. **Forde, A., and G. F. Fitzgerald.** 1999. Bacteriophage defense systems in lactic acid bacteria. *Antonie Van Leeuwenhoek*. **76**:89-113.
13. **Frick, D., and C. C. Richardson.** 2001. DNA primases. *Annu. Rev. Biochem.* **70**: 39-80.
14. **Hill, C., Massey, L.J., and T. R. Klaenhammer.** 1991. Rapid method to characterize lactococcal bacteriophage genomes. *Appl. Environ. Microbiol.* **57**:283-288.
15. **Horton, R. M.** 1995. PCR-Mediated recombination and mutagenesis: SOEing together tailor-made genes. *Mol. Biotechnol.* **3**:93-99.
16. **Huynh, T. V., R. A. Young, and R. W. Davis.** 1985. Construction and screening cDNA libraries in λ gt10 and λ gt11. In *DNA cloning, vol. I*. D. M. Glover (ed.). Oxford: IRL Press Ltd., pp. 49-78.
17. **Ilyina, T. V., A. E. Gorbalenya, and E. V. Koonin.** 1992. Organization and evolution of bacterial and bacteriophage primase-helicase systems. *J. Mol. Evol.* **34**:351-357.

18. **Lucchini, S., F. Desiere, and H. Brüssow.** 1999. Comparative genomics of *Streptococcus thermophilus* phage species supports a modular evolution theory. *J. Virol.* **73**:8647-8656.
19. **Moineau, S., M. Borkaev, B. J. Holler, S. A. Walker, J. K. Kondo, E. R. Vedamuthu, and P. A. Vandenberg.** 1996. Isolation and characterization of lactococcal bacteriophages from cultured buttermilk plants in the United States. *J. Dairy Sci.* **79**:2104-2111.
20. **Notarnicola, S. M., K. Park, J. D. Griffith, and C. C. Richardson.** 1995. A domain of the gene 4 helicase/primase of bacteriophage T7 required for the formation of an active hexamer. *J. Biol. Chem.* **270**:20215-24.
21. **Notarnicola, S. M., and C. C. Richardson.** 1993. The nucleotide binding site of the helicase/primase of bacteriophage T7. Interaction of mutant and wild-type proteins. *J. Biol. Chem.* **268**:27198-27207.
22. **Nuesch J. P., and P. Tattersall.** 1993. Nuclear targeting of the parvoviral replicator molecule NS1: evidence for self-association prior to nuclear transport. *Virology.* **196**:637-651.
23. **Pansegrau, W., and E. Lanka.** 1992. A common sequence motif among prokaryotic DNA primases. *Nucleic Acids Res.* **20**:4931.

24. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.
25. **Sing, W. D., and T. R. Klaenhammer.** 1990. Characteristics of phage abortion conferred in lactococci by the conjugal plasmid pTR2030. *J. Gen. Microbiol.* **136**:1807-1815.
26. **Sturino, J. M., and T. R. Klaenhammer.** 2002. Expression of antisense RNA targeted against *Streptococcus thermophilus* bacteriophages. *Appl. Environ. Microbiol.* **68**:588-596.
27. **Sturino, J. M., and T. R. Klaenhammer.** 2004. Antisense RNA targeting primase interferes with bacteriophage replication in *Streptococcus thermophilus*. *Appl. Environ. Microbiol.* In Press.
28. **Tatusov, R. L., D. A. Natale, I. V. Garkavtsev, T. A. Tatusova, U. T. Shankavaram, B. S. Rao, B. Kiryutin, M. Y. Galperin, N. D. Fedorova, and E. V. Koonin.** 2001. The COG database: new developments in phylogenetic classification of proteins from complete genomes. *Nucleic Acids Res.* **29**:22-28.

29. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **24**:4876-4882.
30. **Wang, D., W. Yuan, I. Davis, and C. R. Parrish.** 1998. Nonstructural protein-2 and the replication of canine parvovirus. *Virology.* **240**:273-281.
31. **Ziegelin, G., N. A. Linderoth, R. Calendar, and E. Lanka.** 1995. Domain structure of phage P4 alpha protein deduced by mutational analysis. *J. Bacteriol.* **177**:4333-41.
32. **Ziegelin, G., E. Scherzinger, R. Lurz, and E. Lanka.** 1993. Phage P4 alpha protein is multifunctional with origin recognition, helicase and primase activities. *EMBO J.* **12**:3703-8.

TABLE 1
Bacterial strains, bacteriophages and plasmids

Bacterial strain, bacteriophage or plasmid	Relevant characteristic(s) ^a	Source or Reference
<i>Streptococcus thermophilus</i>		
NCK1125	Industrial isolate; sensitive to phages κ3, κ4, κ6, κ9, κ10; Cm ^S	Sturino <i>et al.</i> , 2002
<i>Escherichia coli</i> MC1061		
Transformation host		Huynh <i>et al.</i> , 1985
Bacteriophages		
κ3	Encodes Sfi21-type replication module; <i>cos</i> -type encapsidation module	Sturino <i>et al.</i> , 2002
κ4	Encodes Sfi21-type replication module; <i>cos</i> -type encapsidation module	Sturino <i>et al.</i> , 2002
κ6	Encodes 7201-type replication module; <i>pac</i> -type encapsidation module	Sturino <i>et al.</i> , 2002
κ9	Encodes Sfi21-type replication module; <i>cos</i> -type encapsidation module	Sturino <i>et al.</i> , 2002
κ10	Encodes Sfi21-type replication module; <i>cos</i> -type encapsidation module	Sturino <i>et al.</i> , 2002
Plasmids		
pTRK686	2.4 kb; deletion derivative of pNZ123; Cm ^R	Sturino <i>et al.</i> , 2002
pTRK687	3.0 kb; pTRK686 containing the P6 promoter in α-orientation	Sturino <i>et al.</i> , 2002
pTRK809	4.5 kb; pTRK687 encoding native protein; TTG start; φ ^S	This study
pTRK810	4.5 kb; pTRK809 encoding N151 <i>am</i> 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 N151 <i>am</i> truncated RR340-1AA 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 N151 <i>am</i> truncated K238T protein; TTG start; φ ^S	This study

^a 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

TABLE 2
Primers used in this study

Mutation	Mutagenic Oligonucleotides ^{a b}
N151 _{am}	5' -TTATTCTCGTTAA ATA GGCATCTACGATAAACGAAAGAAGAAGC-3' 3' -AATAAGTGCAATTT TAT CCGTAGATGCTATTTGCTTCTTCTTCG-5'
K238T	5' -TGGTAACGGTAACGATGGT AC GGGTACGTTCAACAGTTGATTAGC-3' 3' -ACCATTGCCATTGCTACCA TGCC CATGCAAGTTGTCAACTAATCG-5'
K238A	5' -TGGTAACGGTAACGATGGT CCG GGTACGTTCAACAGTTGATTAGC-3' 3' -ACCATTGCCATTGCTACCA CGCC CATGCAAGTTGTCAACTAATCG-5'
N326A	5' -ACGGTTATCCAGTCTAC CGCT GCTATGCCAGTGTTTAAGAATAAG-3' 3' -TGCCAATAGGTCAGATGG CGA CGATACGGTCACAAATCTTATTC-5'
RR340AA	5' -AATAAGTCAAACGGTACATAT GCAGCT ATCGTGATTATCCCATTC-3' 3' -TTATTCAGTTTGCCATGTATA CGT CGA ATAGCACTAATAGGGTAAG-5'
E437A	5' -CCGCTCATGGTGT CGCG GAAATCATACTATACCTAAAAATC-3' 3' -GGCGAGTACCACAGT ACGCC CTTTAGTATGATATGGATTTTTTAG-5'
E437D	5' -CCGCTCATGGTGT GAC GGAATCATACTATACCTAAAAATC-3' 3' -GGCGAGTACCACAGT ACTG CCTTTAGTATGATATGGATTTTTTAG-5'
L001L	5' -aaactgcagATAAGGAGGATTGG ACTT GACAACGATTGATTTCG-3' 5' -aaactgcagGTTACTGAGTAACCATAACCACTACTGCTGCTTTC-3'
L001M	5' -aaactgcagATAAGGAGGATTGG ACT GACAACGATTGATTTCG-3' 5' -aaactgcagGTTACTGAGTAACCATAACCACTACTGCTGCTTTC-3'

^a Tags with PstI (5'-CTGCAG-3') restriction sites are in lower case, when appropriate

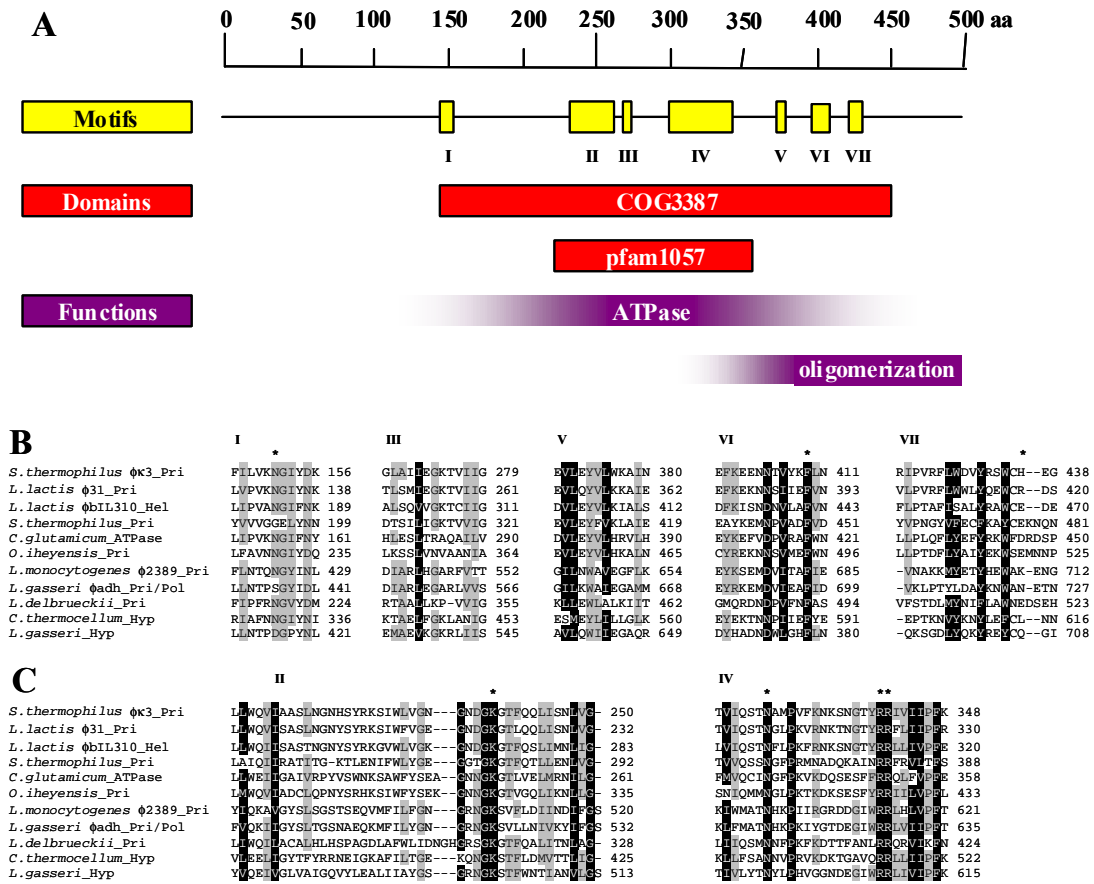
^b Mutated codons are bolded and altered nucleotide(s) is/are underlined, when appropriate

TABLE 3Impacts transdominant mutant proteins on the EOP^a of various *S. thermophilus* phages

Plasmid	Start	Mutation(s)	Sfi21-type		7201-type	
			κ4	κ9	κ10	κ6
pTRK687 ^b	-	-	1.0	1.0	1.0	1.0
pTRK905	TTG	K238T	< 10 ⁻⁹	< 10 ⁻⁹	< 10 ⁻⁹	0.9 ± 0.2
pTRK906	ATG	K238T	< 10 ⁻⁹	< 10 ⁻⁹	< 10 ⁻⁹	1.0 ± 0.1
pTRK907	TTG	K238A	< 10 ⁻⁹	< 10 ⁻⁹	< 10 ⁻⁹	0.8 ± 0.1
pTRK911	ATG	RR340-1AA	< 10 ⁻⁹	< 10 ⁻⁹	< 10 ⁻⁹	0.9 ± 0.1

^a Where appropriate, reductions in EOP are expressed as the mean ± standard deviation relative to pTRK687^b vector control, no primase protein encoded

Figure 1. 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 location of conserved regions and protein domains are demarcated with yellow and red bars, respectively. Putative functions attributed to specific protein regions are demarcated by purple bars. Panels B and C show amino acid sequence comparisons of putative primase orthologues derived from a diverse collection of bacteria and phages that when mutated in the *S. thermophilus* primase failed to confer (B) and conferred (C) phage resistance, respectively. Conserved residues and motifs are labeled 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 of panels B and C indicate the amino acid sequence coordinates.



Construct	Start	Mutation	EOP	RPD(%)
pTRK809	TTG		1.0 ± 0.2	100 ± 10
pTRK810	TTG	N151am	0.9 ± 0.2	100 ± 10
pTRK816	TTG	N326A	1.0 ± 0.3	100 ± 10
pTRK812	TTG	E437A	1.1 ± 0.1	100 ± 10
pTRK813	ATG	E437A	1.0 ± 0.2	100 ± 10
pTRK811	ATG	E437D	1.0 ± 0.1	100 ± 10
pTRK815	TTG	K238T	< 10 ⁰	-
pTRK814	ATG	K238T	< 10 ⁰	-
pTRK820	ATG	K238A	< 10 ⁰	-
pTRK823	TTG	N151am K238T	0.9 ± 0.1	100 ± 10
pTRK822	TTG	K238T E437A	0.9 ± 0.2	100 ± 10
pTRK819	ATG	K238T E437A	0.9 ± 0.2	100 ± 10
pTRK817	ATG	R340A R341A	< 10 ⁰	-
pTRK818	ATG	N151am R340A R341A	1.0 ± 0.1	100 ± 10

Figure 2. Map of the phage-encoded primase. Graphical depiction of point mutations (○) present in various derivatives of the phage-encoded putative primase. EOP and relative plaque diameter (RPD) values are expressed as the mean ± standard deviation relative to plaques formed on NCK1125 (pTRK687), the control indicator strain. RPD values were rounded to the nearest 10%. Residues downstream of the N151am point mutation are not translated and are denoted by a dotted line.

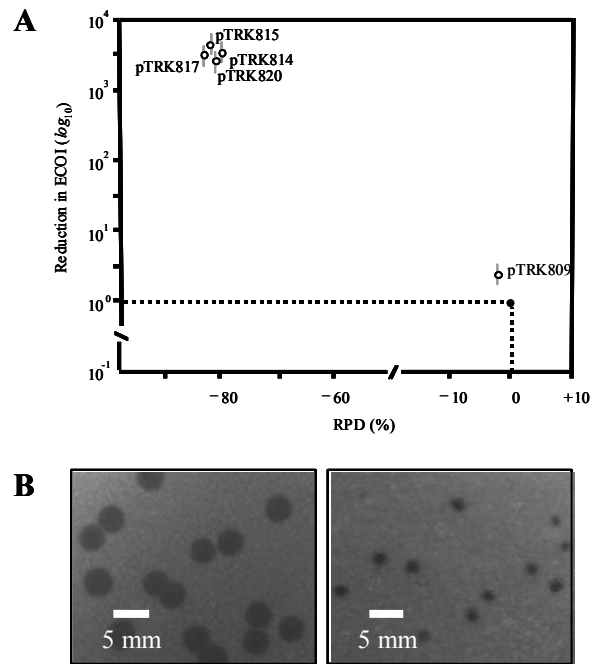


Figure 3. Center of infection experiments. (A) The effect of various protein overexpression constructs on the ECOI and relative plaque diameter of phage $\kappa 3$ during COI studies relative to propagation on the NCK1125 (pTRK687) control strain (●) located in at the junction of horizontal and vertical dotted lines. Error bars for ECOI were calculated from three independent experiments. (B) Plaque morphology and size of phage $\kappa 3$ on NCK1125 (pTRK687) (left) and L::K238T (right).

Figure 4. Accumulation of HindIII-digested phage-specific genomic DNA in sensitive and resistant hosts. Agarose gel electrophoresis of phage genomic DNAs isolated from (A), control strain NCK1125 (pTRK687); (B), transdominant primase strain L::K238T; (C), primase truncated strain L::N151*am*.K238T; and (D) transdominant primase strain M::RR340-1AA. Southern hybridization of *Hind*III-digested phage DNA accumulated on (E), control strain NCK1125 (pTRK687); and (F), transdominant primase strain L::K238T. Abbreviations: ϕ , purified phage κ 3 genomic DNA; M, digoxigenin-labeled molecular weight marker I (Invitrogen, Inc.). Where appropriate, pTRK687-specific DNA bands are denoted by white triangles (Δ), while plasmid-associated primase-specific fragments or hybridization signals are denoted by gray triangles (\blacktriangle).

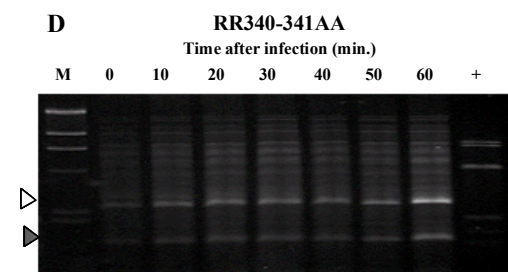
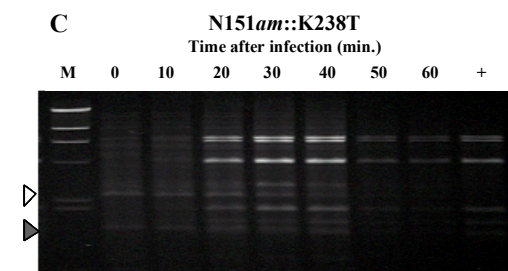
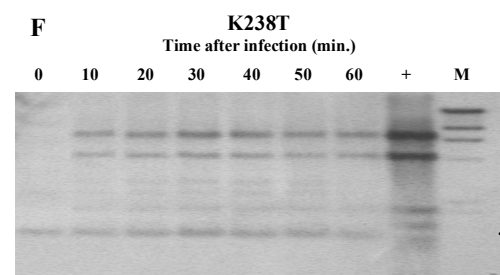
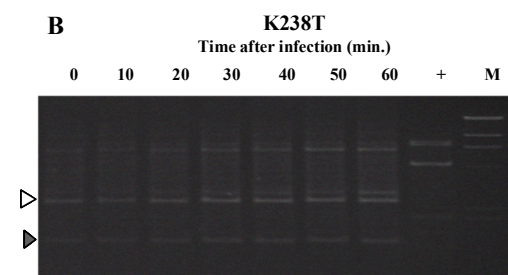
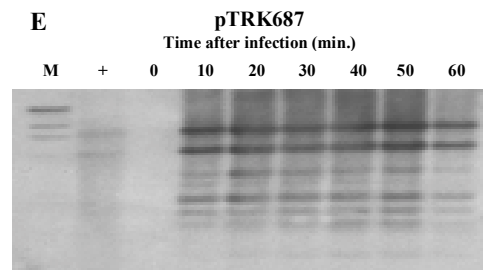
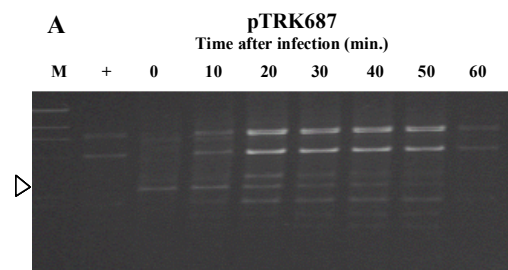
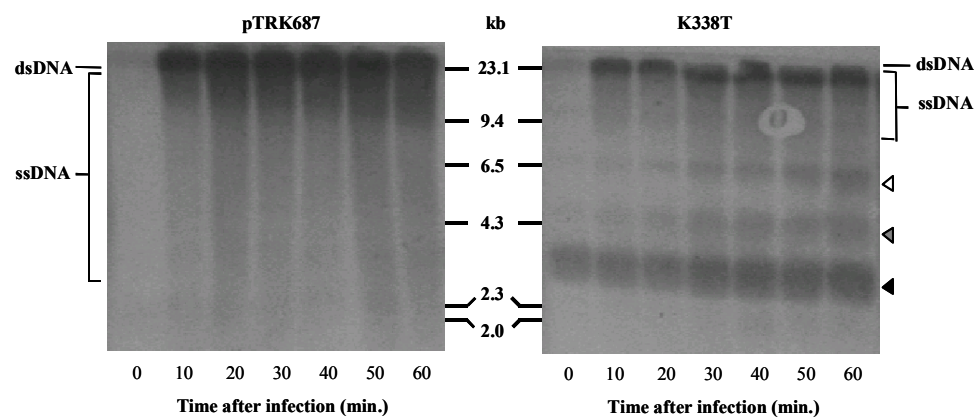


Figure 5. Monitoring the *in vivo* accumulation of single stranded DNA molecules. Total genomic DNA was isolated from the NCK1125 (pTRK687) control strain and the transdominant primase strain L::K238T prior to infection with phage κ 3 (time 0), and then 30, 40, and 50 minutes post infection with phage κ 3. Undigested DNAs were subjected to alkaline denaturing agarose gel electrophoresis, transferred to a nylon membrane and probed with total phage DNA in order to visualize leading and lagging strand synthesis of the phage DNA. Monomeric (\blacktriangle), dimeric (\blacktriangle), and trimeric (Δ) plasmid-associated primase-specific hybridization signals are denoted in the L::K238 derivative.



SUMMARY

- The genomes of six *S. thermophilus* phages were compared to identify genes that could be targeted by engineered phage defense systems with potentially widespread efficacy. The genes associated with the Sfi21-type genome replication module, including a putative primase and helicase, were found to be among the best candidates due to their frequency of distribution in industrial phage isolates, striking sequence conservation between independent isolates, and intrinsic strategic importance in early phage development.
- The phage κ 3-derived putative helicase (*hel3*) and putative primase genes (*pri3*) were cloned in the antisense orientation behind a strong promoter and expressed from a high-copy number vector. These antisense RNAs consistently reduced the EOP of phage κ 3 to between 5×10^{-1} and 2.0×10^{-3} depending on the (i) gene targeted and (ii) region of the gene that was targeted by antisense RNA (Table 1). The expression of these antisense RNAs also retarded phage genome replication and severely limited the number of progeny phages released from an infected cell, which indicated that the antisense RNAs acted to abort phage infection.
- The largest antisense RNAs were generally found to confer the largest reductions in EOP, however shorter antisense RNAs designed to the 5' region of the gene retained much of the inhibitory function. Larger antisense RNAs may (i) have more opportunities over their length to maximize intermolecular base pairing and thus exert their inhibitory effects or (ii) exhibit decreased stability when bound to the target RNA.

- No mutant phages were recovered that were insensitive to either primase- or helicase-targeted antisense RNA after numerous attempts to select or enrich for derivatives. Due to the length of the antisense RNAs, we suspect that the potential for multiple associations could limit the ease at which phages might overcome antisense inhibition via point mutation(s).
- Antisense RNAs generally reduced the abundance of the target transcript in a manner consistent with the observed EOP reductions. Further, antisense RNAs that strongly reduced EOP also resulted in the synthesis of fewer phage genomes, indicating a correlation between the lowered abundance of primase transcripts, lowered levels of genome replication, and interference with progeny development.
- Antisense constructs that contained sequences complementary to the putative RBS generally reduced the EOP below the level of constructs that lacked them. This may be due to the formation of a dsRNA over the length of the RBS, thus preventing efficient ribosome loading and reducing translation of the target gene.
- The combination of a phage origin of replication (*ori3.1*) with a high expression antisense RNA cassette (*hel3.1-AS*) provided protection from phages at significantly higher levels than for either mechanism alone.
- The putative primase from phage $\kappa 3$ lacks several key functional motifs conserved among *E. coli* DnaG-like primase proteins, however it possesses significant regions along its carboxy terminus that exhibit weak similarities to other phage-encoded and prokaryotic polymerases, especially primases, including a putative ATPase/helicase domain.

- Multiple alignments of related primase proteins were used to identify critical amino acid residues potentially involved in enzyme catalysis and/or protein subunit oligomerization. Invariant and highly conserved amino acids within a phage primase consensus sequence were targeted by site-specific mutations and the mutant proteins overexpressed.
- These characterizations enabled the identification of residues within a putative ATPase/helicase domain (*i.e.* RR340-341 and K238) and a putative oligomerization domain (*i.e.* E437) that are critical for primase function.
- The K238(A/T) and RR340-341AA mutant derivatives completely inhibited phage genome replication and reduced the EOP by greater than nine log cycles. In addition, they suppressed the function of the native, phage-encoded primase in a dominant negative fashion, a process called subunit poisoning. This represents the first application of subunit poisoning to inhibit phage replication in the LAB.
- The inhibition of phage genome replication suggested that the plasmid-encoded mutant primase subunits were structurally intact and formed stable interactions with the native, phage-encoded primase proteins, thus inhibiting their activity (Figure 1). Alternatively, the mutant primase proteins could have formed other non-productive associations, such as substrate binding and/or inhibit replication by titrating away other phage- or host-encoded genome replication factors.
- Given the magnitude of the resistance conferred, it was concluded that the putative primase is an essential enzyme required for genome replication in *S. thermophilus* Sfi21-type phages. Further, host-encoded factors were unable to complement the deficiency caused by transdominant primase expression.

- Amber mutations (N151*am*) introduced upstream of the transdominant mutations restored genome replication and parental-type plaquing efficiency and completely suppressed phage resistance. These results indicated that translation of the transdominant mutant primase proteins was required to confer phage resistance.
- Various amino acid residues were targeted for site-specific mutation in order to identify those involved in protein oligomerization. The E437A mutant construct was readily cloned, but had no effect on the replication of phage κ 3. When introduced downstream of the transdominant K238T mutation, however, the E437A mutation completely suppressed phage resistance. This indicated that E437A precluded the association of the mutant subunits from the native, phage-encoded subunits. Hence, E437 is believed to be a component of the protein oligomerization domain.
- Significantly, no phages resistant to transdominant primase proteins have been isolated. This may be due to the fact that protein structure is tightly associated with protein function and strong conservation is found at the tertiary and quaternary structural levels of related proteins. Hence, unless a mutation specifically alters the oligomerization domain of the phage primase, it would be unlikely to reduce the efficacy of subunit poisoning as a phage defense mechanism.

TABLE 1
Antisense RNA-based phage defense strategies effective (EOP < 1) in *Streptococcus thermophilus*

Antisense Construct	Base Vector	Promoter	Phage	Predicted Target	Function	Length ^a	ORF ^b	anti-RBS ^c	EOP	Reference
pTRK787::pri3.4-AS	pNZ123	P6	κ3	primase	early	587	P	yes	1.4×10^{-1}	Sturino & Klaenhammer (2004)
pTRK788::pri3.7-AS	pNZ123	P6	κ3	primase	early	1,023	P	yes	3.2×10^{-2}	Sturino & Klaenhammer (2004)
pTRK789::pri3.8-AS	pNZ123	P6	κ3	primase	early	519	P	yes	3.8×10^{-2}	Sturino & Klaenhammer (2004)
pTRK790::pri3.10-AS	pNZ123	P6	κ3	primase	early	1,512	C	no	2.0×10^{-3}	Sturino & Klaenhammer (2004)
pTRK791::pri3.11-AS	pNZ123	P6	κ3	primase	early	1,008	P	no	1.1×10^{-1}	Sturino & Klaenhammer (2004)
pTRK792::pri3.12-AS	pNZ123	P6	κ3	primase	early	504	P	no	9.3×10^{-2}	Sturino & Klaenhammer (2004)
pTRK793::pri3.13-AS	pNZ123	P6	κ3	primase	early	1,506	P	no	3.2×10^{-3}	Sturino & Klaenhammer (2004)
pTRK794::pri3.14-AS	pNZ123	P6	κ3	primase	early	1,486	P	no	4.8×10^{-3}	Sturino & Klaenhammer (2004)
pTRK795::pri3.15-AS	pNZ123	P6	κ3	primase	early	982	P	no	7.1×10^{-2}	Sturino & Klaenhammer (2004)
pTRK796::pri3.16-AS	pNZ123	P6	κ3	primase	early	487	P	no	2.1×10^{-2}	Sturino & Klaenhammer (2004)
pTRK797::pri3.18-AS	pNZ123	P6	κ3	primase	early	1,008	P	no	1.5×10^{-1}	Sturino & Klaenhammer (2004)
pTRK798::pri3.19-AS	pNZ123	P6	κ3	primase	early	504	P	no	6.1×10^{-1}	Sturino & Klaenhammer (2004)
pTRK799::pri3.21-AS	pNZ123	P6	κ3	primase	early	504	P	no	4.0×10^{-1}	Sturino & Klaenhammer (2004)
pTRK689::hel3.1-AS	pNZ123	P6	κ3	helicase	early	1,431	?	no	5×10^{-1}	Sturino & Klaenhammer (2002)

^a Length in nucleotides unless otherwise indicated

^b C, complete open reading frame (start to stop codon); P, partial open reading frame

^c Presence or absence of sequences complementary for the predicted ribosome binding site (RBS)

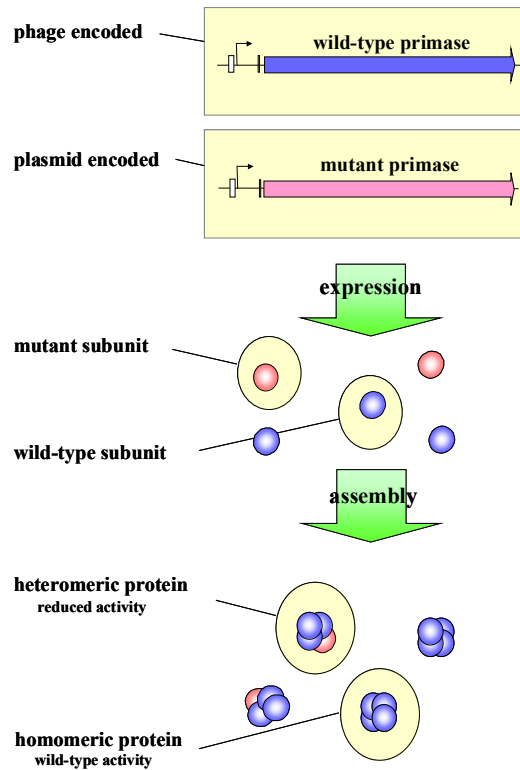


Figure 1. Illustration depicting the proposed mechanism of subunit poisoning in phage defense. Mutant subunits of an oligomeric protein (*e.g.* primase) associate with the wild-type subunits to form functionally inactive heteromeric proteins.