

Engineered bacteriophage-defence systems in bioprocessing

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Abstract | Bacteriophages (phages) have the potential to interfere with any industry that produces bacteria as an end product or uses them as biocatalysts in the production of fermented products or bioactive molecules. Using microorganisms that drive food bioprocesses as an example, this review will describe a set of genetic tools that are useful in the engineering of customized phage-defence systems. Special focus will be given to the power of comparative genomics as a means of streamlining target selection, providing more widespread phage protection, and increasing the longevity of these industrially important bacteria in the bioprocessing environment.

Organoleptic

Being, affecting or relating to qualities (such as taste, colour, odour and feel) of a substance that stimulate the sense organs.

Backslopping

An artisanal practice whereby a small portion of a previous batch is used to inoculate subsequent batches. From the standpoints of safety and product consistency, backslopping is not recommended owing to the risk of transferring and enriching for pathogenic microorganisms or bacteriophages, respectively.

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Fermentation is one of the oldest and, arguably, most elegant techniques for the preservation of raw foodstuffs, including fruits, grains, meats, milk and vegetables. Fermentative lactic acid bacteria have been used for centuries during the bioprocessing of foodstuffs, well before the biochemical basis for these processes was studied scientifically. As a result of their growth and metabolism, these bacteria function as biocatalysts to alter the molecular and organoleptic characters of the fermentation substrate. These metabolic activities act to preserve the nutritional qualities of the substrate while inhibiting the increase of spoilage or pathogenic microorganisms that might also be present in the finished product. In many cases, these metabolic activities often augment the nutritional value of the finished product by increasing the bioavailability of otherwise indigestible nutrients, and through the production of various bioactive compounds, including vitamins.

Fermentation has a rich tradition of innovation and discovery in the microbial sciences. Prior to 1857, the conventional scientific belief was that alcoholic fermentation of grapes and wort was the result of complex chemical reactions of non-biological origin. By this time, it was already recognized that yeasts were present in wine; however, popular scientific belief was that they were a result (and not the cause) of alcoholic fermentation. During microscopic examination of wines, Louis Pasteur, a French chemist and pioneer microbiologist, noted that yeast cells were healthy and budding during successful fermentations in which optimal levels of ethanol were produced¹. By contrast, he noticed that smaller, rod-shaped microorganisms were also present in failed fermentations in which lactic acid was formed instead of ethanol. As a result of these and other findings, Pasteur

proposed that alcoholic fermentations were the result of biological, and not simply chemical, processes. These findings, although highly controversial at the time, would later be accepted as fact.

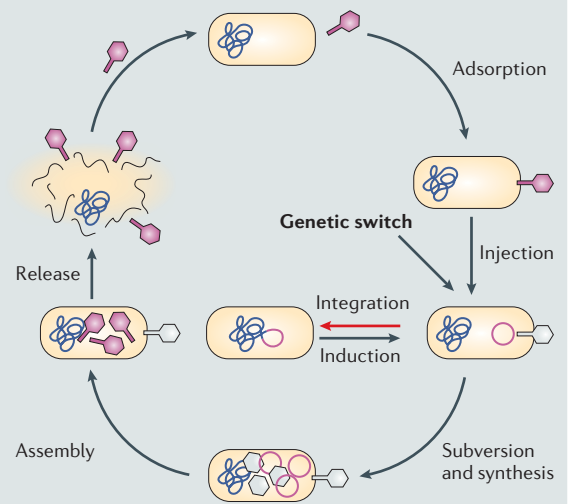
In a study of soured milk that was published in 1878, Joseph Lister, a student of Pasteur, used syringes to make serial dilutions in liquid medium to obtain the first pure bacterial culture². This mesophilic bacterium, which he named *Bacterium lactis*, was demonstrated to be the specific causative agent of milk souring by fermentation². Since its discovery, this microorganism has been renamed several times as techniques for species differentiation became more sensitive. *Bacterium lactis* was renamed *Streptococcus lactis* in 1909 (REFS 3,4), and was once again renamed *Lactococcus lactis* in 1985 (REF. 5).

Modern industrial-scale fermentations decreasingly rely on the native microflora that are present on the starting materials (spontaneous fermentation) or backslopping to drive product formation. Instead, the industry has developed more consistent inoculation strategies that are based on concentrated, well defined and product-optimized starter cultures to act as biocatalysts. The use of defined strain cultures has conferred a significant degree of control over fermentation systems, and has been widely adopted in large-scale production facilities. These starter cultures generally contain two to five phage-unrelated strains that exhibit complementary fermentation properties, such as rapid acidification, texturizing and the generation of compounds that are involved in flavour and aroma development⁶.

Bacteriophages (phages) have the potential to interfere with any industry that produces bacteria as an end product (such as starter cultures or live mucosal vaccines) or uses them as a biocatalyst for the production of

Box 1 | **Bacteriophage replication**

There are two types of bacteriophage (phage) life cycles, lytic and lysogenic. Virulent phages are restricted to a lytic life cycle that ultimately terminates by lysis of their host to release progeny phage particles (see figure). Most of the phages that are problematic to the fermentation and bioprocessing industries are obligately lytic in nature. After the phage has adsorbed to the surface of the cell, it injects its genome into the host cytoplasm, where the full complement of phage-encoded proteins is expressed by early, middle and late gene sets. Together, their action results in the catalysis of phage-genome replication and the synthesis of structural proteins. These polymers are then assembled into mature phage particles and released by a cell-lysis event that is coordinated by the action of two phage-encoded proteins, holin and endolysin^{30,31}. Some of these reactions rely heavily on the host's replication and biosynthetic systems. For instance, phages generally use pre-existing biosynthetic machinery (such as ribosomes) to draw from the host's pool of available building blocks (for example, dNTPs and amino acids). However, the degree to which a virus relies on host-encoded factors differs among phages. In addition to the lytic cycle, temperate phages are capable of undergoing a lysogenic life cycle (see figure, red arrow). In this case, the phage does not multiply lytically, but instead integrates its genome into the host chromosome to establish a prophage state. Therefore, the phage is effectively replicated once with every division of the bacterium in a quiescent state. The decision to enter the lytic or lysogenic life cycle by these temperate progeny phages is based on a genetic switch that senses the condition of the next host infected (see figure). Environmental triggers (for example, pH, temperature, salt concentration and nutritional deficiency) or cellular damage can induce the prophage into the lytic cycle and release progeny phages. Therefore, ideally, prophage-deficient or prophage-cured strains are best used for the development of starter cultures⁷³⁻⁷⁵, but in reality, most dairy starter cultures are lysogenic.



fermented foods or bioactive molecules (such as enzymes or metabolites). The significance of phage infections during the manufacture of dairy products was not recognized until 1935, some 20 years after the discovery of phages^{7,8}, when Whitehead and Cox discovered the first phages that were specific for strains of *L. lactis*⁹. It is now known that the presence of phages in bioprocessing environments can result in the lysis of the bacterial starter, which in turn results in reduced bioprocessing rates.

Phages continue to be problematic even today, because the dairy environment is a consistent reservoir for phage contamination. Viable phages are ubiquitously found in non-sterile fermentation substrates (in this case, milk) and might become fixed on industrial surfaces and equipment¹⁰⁻¹². Furthermore, most phages are not completely inactivated by standard processing conditions (including pasteurization) or equipment-sanitation regimes¹³⁻¹⁵. This problem is further compounded by phage biodiversity, which is driven by rapid growth rates, large burst size and genomic plasticity. These traits work synergistically to enable phages to rapidly evolve resistance to existing phage-defence systems by mutation and recombination.

Engineered defence systems

This review will describe genetic systems that have been designed to interfere with phage replication (BOX 1). In particular, we will highlight the power of information-based engineered defence systems to curb the propagation of virulent phages that are specific for the thermophilic dairy bacterium *Streptococcus thermophilus*, which is related to *L. lactis*. Examples from the mesophilic dairy

bacterium *L. lactis* will also be cited when appropriate to fill gaps in current knowledge and to provide a more comprehensive picture of the current defence systems that are available today.

S. thermophilus and *L. lactis* are used separately during the manufacture of various distinct fermented dairy products; however, they are often used together during the production of Cheddar cheese¹⁶. 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 phages. The availability of a growing number of phage and bacterial genomes has become an invaluable resource for the understanding of phage-host interactions. Accordingly, special focus will be given to the impact of genomic data on the development of information-based, genetically engineered solutions to industrial phage problems.

A wide variety of engineered phage-resistance systems that target different stages in the lytic life cycle have been constructed using information that has been garnered from the examination of sequence data (FIG. 1). Although this review will be focused on dairy microorganisms, the general principles for engineered phage-defence strategies can also be applied to other non-food bioprocessing systems, such as those involving *Bacillus subtilis* and *Escherichia coli*. It is important to note, however, that these systems have not been used in products for human consumption owing to legal restrictions and public concerns regarding the introduction of recombinant DNA into the food chain.

Starter culture

Concentrated preparations of viable microorganisms (usually strains of lactic acid bacteria or yeasts) that are added to bioprocessing systems to mediate the bioconversion of the substrate in an accelerated and more reproducible manner when compared to spontaneous fermentations. These preparations are product-optimized and are generally either freeze-dried or stored frozen.

Phage unrelated

Character of strains or species whereby the bacteria in question exhibit distinct phage-sensitivity profiles, meaning that they are attacked by different groups of phages.

Comparative genomics

When constructing engineered phage-defence systems for use in the dairy environment, the identification of target genes that are effective against various industrially relevant phages is of utmost importance. Genomic analyses expedite the identification of well conserved genes and *cis* regulatory elements that are shared among the various genomes, while conversely facilitating the elimination of poorly conserved targets without the need for experimentation. In addition, developmental pathways can also be modelled to anticipate potential evolutionary escape routes. These advantages are of crucial importance when engineering defence strategies that are intended for use in large-scale industrial settings, where protection is required against both the residing and potentially emerging phage populations.

Our understanding of these processes has progressed rapidly since the release of seven genome sequences for phages that are specific for *S. thermophilus*: **DT1** (REF. 17), **O1205** (REF. 18), **Sfi11** (REF. 19), **Sfi19** (REF. 20), **Sfi21** (REF. 20), **7201** (REF. 21) and **2972** (REF. 22). The genetic content and genomic organization of these seven phages exhibit significant similarities to other members of the λ -superfamily of phages²³. Bioinformatic analyses have provided significant insight into the evolution of *S. thermophilus* phages, revealing that their genomes are molecular mosaics that are assembled upon a relatively simple scaffold that consists of independently evolving modules, whereby each module directs distinct developmental processes (for example, genome replication and genome encapsidation)²⁴. Furthermore, functionally coupled genes are found arranged into conserved clusters within these modules, such that gene order was predicated by developmental order, or vice versa.

In some cases, two or more alternative and likely interchangeable modules, which are composed of distinct clusters of non-orthologous genes, have been found to catalyse these crucial developmental processes through very different mechanisms¹⁸. For example, comparative genomic analyses and Southern hybridization have shown the presence of two distinct gene clusters that direct phage capsid morphogenesis. This has enabled researchers to classify *S. thermophilus* phages into two distinct groups based on their method for genome encapsidation: *cos*-type or *pac*-type²⁵. The presence of either module correlates strongly with several other definable biomolecular characteristics that include the presence (*cos*-type) or absence (*pac*-type) of cohesive ends within the linear form of the phage genome, and the number of major structural proteins that are found in the mature phage particle²⁵.

These modules are generally, but not exclusively, found to be arranged into putative operons, so that they are co-transcribed and co-regulated^{26–29}. The first analyses of the complete transcriptomes of two *S. thermophilus* phages were recently conducted using DNA microarrays²⁹. These two phages (*cos*-type phage DT1 and *pac*-type phage 2972) were propagated on different hosts, but otherwise under the same experimental conditions. Phage gene expression was measured at nine time intervals over the course of an ongoing lytic infection; each phage gene was then classified into one of the three traditional transcription classes

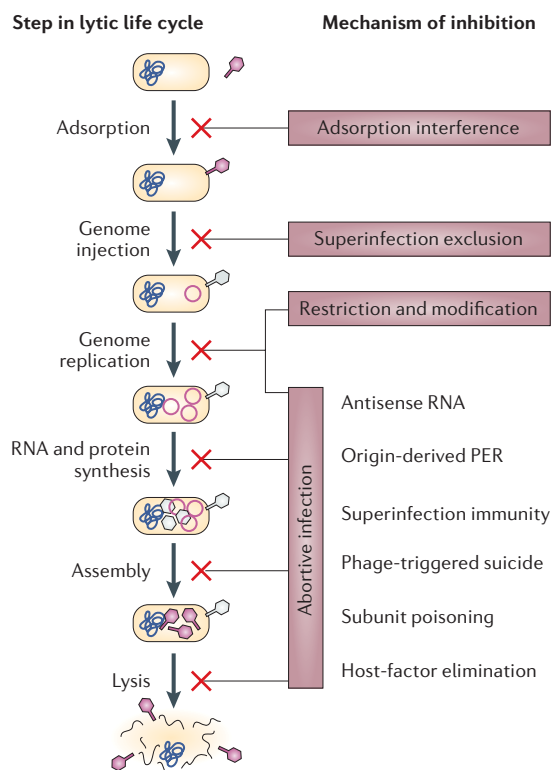


Figure 1 | Points of inhibition by specific phage-resistance mechanisms during a generalized phage lytic life cycle. Figure modified with permission from REF. 76 © (1995) Humana Press. PER, phage-encoded resistance.

— early-, middle- and late-expressed genes — based on the period of time during which the greatest change in transcript abundance occurred.

Although these phages belong to different phage groups, they displayed similar gene-expression patterns. Phage DT1 possesses 18 early-, 12 middle- and 12 late-expressed genes, whereas phage 2972 has 16 early-, 11 middle- and 14 late-expressed genes. In both phages, all of the early-expressed genes were adjacent to one another and comprised the putative lysogeny-replacement, genome-replication and transcription-regulation modules. The difference in the number of early-expressed genes is due to differences in the number of open reading frames in these modules. The middle-expressed genes encode proteins that are involved in genome encapsidation and capsid morphogenesis. The late-expressed genes code for proteins that are involved in tail morphogenesis. A fourth group of late-expressed genes (that is, late #2) was observed in both phages whereby expression was slowly but steadily increased over the course of the ongoing infection; these genes were considered late genes because their highest expression was at the end of the lytic cycle. The genes that were designated late #2 include those that encode proteins that are involved in host lysis (holin and endolysin)^{30,31}. It is important to note that not all phages that infect lactic acid bacteria have been found to encode genes that can be divided into the three distinct transcriptional classes³².

Holin

Small, membrane-spanning protein that accumulates in the cytosolic membrane during the lytic life cycle of a phage. Holin proteins act as a gateway to the cell wall for the phage-encoded endolysin.

Endolysin

Phage-encoded muralytic enzyme that degrades bacterial cell-wall polymers. During the lytic life cycle, endolysin accumulates in the cytosol and can cross the cytosolic membrane by the cooperative action of the phage-encoded holin.

In addition, two DNA-replication modules have been identified among *S. thermophilus* phages: Sfi21-type and 7201-type. The Sfi21-type replication module is composed of a single origin of DNA replication (*ori*) and several open reading frames that encode a putative helicase, a putative primase and several other proteins of

undetermined function²⁴. The phage 7201-type replication module, on the other hand, includes two distinct *oris* (*ori7201A* and *ori7201B*) and encodes a probable single-stranded DNA-binding protein, a putative replication protein, a putative DnaC homologue and several other proteins of undetermined function²¹. The variants of the Sfi21-type genome-replication module are found among most industrial phage isolates, and six of the seven sequenced phages^{22,33,34}.

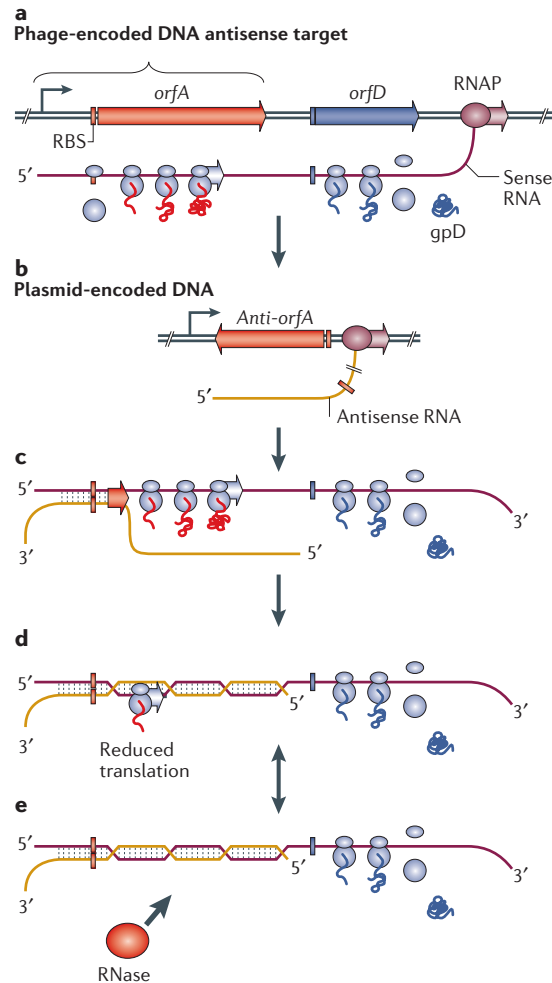


Figure 2 | The proposed mechanism of antisense-RNA-mediated gene silencing. Antisense RNAs interfere with phage development by inhibiting the translation of phage-encoded genes that are necessary for normal development. **a** | During an ongoing phage infection, the phage-encoded target (sense) RNA is co-expressed with **(b)** the gene-specific, host-encoded antisense RNA. **c** | Watson–Crick base pairings between the antisense RNA and its complementary, phage-encoded target mRNA(s) are initiated through several intermolecular nucleation events that occur within complementary unstructured regions^{35,77,78}. These associations are subsequently stabilized through progressive Watson–Crick base pairings that are proximal to the initial nucleation site(s) and results in **(d)** the formation of a translationally inactive double-stranded-RNA molecule, which is **(e)** subject to degradation. gpD, gene product D; *orfA*, open reading frame targeted by antisense RNA; *orfD*, downstream open reading frame; RBS, ribosome-binding site; RNAP, RNA polymerase; RNase, double-stranded-RNA-specific ribonuclease. Figure modified with permission from REF. 36 © (2005) Elsevier.

Antisense RNA

Mechanistically, antisense RNAs hybridize to the sense RNA strand and create a translationally inactive double-stranded RNA (dsRNA) molecule, which is subject to degradation³⁵ (FIG. 2). Antisense RNAs can be used to abort phage infection by inhibiting the translation of phage-encoded genes that are necessary for normal development. Using a genomic approach, the putative primase and putative helicase genes that are associated with the Sfi21-type genome-replication module were found to be among the best candidates for gene silencing through antisense RNA. This was due to their frequency of distribution in industrial phage isolates, striking sequence conservation between independent isolates and intrinsic strategic importance in early phage development³⁴ (see REF. 36 for a recent review of the antisense RNA constructs that have been tested so far in dairy lactic acid bacteria).

The phage κ3-derived putative helicase (*hel3*)³⁴ and putative primase (*pri3*)³⁷ genes have been cloned in the antisense orientation behind a strong, constitutive P6 promoter³⁸ and expressed from a high-copy-number vector, pNZ123 (REF. 39). These antisense RNAs consistently reduced the efficiency of plaquing (EOP) of phage κ3 to between 5×10^{-1} and 2×10^{-3} , depending on the gene and region of the gene that was targeted by antisense RNA. The expression of these antisense RNAs retarded phage-genome replication, and severely limited the number of progeny phages that were released from an infected cell, which indicated that the antisense RNAs acted to abort phage infection³⁷. Antisense RNAs generally reduced the abundance of the target transcript, and this correlated with the observed reductions in EOP. Furthermore, antisense RNAs that reduced the EOP also resulted in the synthesis of fewer phage genomes, which indicates a correlation between the lowered abundance of primase transcripts, lowered levels of genome replication and interference with progeny development. Reduced primase-transcript abundance was accompanied by a concomitant decrease in abundance of antisense RNA, indicating that both RNA species were degraded by ribonuclease(s).

The largest antisense RNAs (1.5 kb) were generally found to confer the greatest reductions in EOP; however, shorter antisense RNAs (0.5 kb) that were designed to the 5' region of the gene retained much of the inhibitory function. Larger antisense RNAs might have more opportunities over their length to maximize intermolecular base pairing and therefore exert their inhibitory effects, and/or might exhibit decreased stability when bound to the target RNA³⁷. No mutant phage were recovered that were insensitive to either primase- or

Efficiency of plaquing (EOP). Calculated by dividing the phage titre (in plaque forming units (pfu) per ml) on the test strain (phage-resistant) by the phage titre in pfu per ml on the parent strain (phage-sensitive indicator).

Prophage

The latent form of a temperate bacteriophage in which its genome is integrated into the bacterial chromosome without causing disruption of the bacterial cell.

Lysogen

A bacterium that contains a prophage integrated into its genome. Lysogens can induce the prophage into a lytic developmental cycle and cause cell lysis to release progeny phage. In the lysogen, the prophage remains quiescent and is effectively replicated once with every chromosomal division of the bacterium.

Superinfection

Any phage infection that occurs after an earlier one; often describing a secondary infection of a lysogenic bacterium.

helicase-targeted antisense RNA after many attempts to select or enrich for resistant mutants^{34,37}. Owing to the length of the antisense RNAs, it was proposed that the potential for multiple associations could limit the ease at which phages might overcome antisense inhibition through discrete mutation(s)^{37,40}. Antisense constructs that contained sequences complementary to the putative ribosome-binding site (RBS) generally reduced the EOP below the level of constructs that lacked them. This might be due to the formation of dsRNA over the length of the RBS, thereby preventing efficient ribosome loading and reducing translation of the target gene³⁵.

Origin-derived phage-encoded resistance

Phage-encoded *oris* are generally easily recognizable by examination of the genome sequences — they usually precede, or are located proximal to, the cluster of early-expressed genes; are located within intragenic regions but might also extend into, or be positioned within, open reading frames; are AT-rich regions; and contain several direct (iternons) and indirect repeats⁴¹. Origin-derived phage-encoded resistance (PER) was first reported to be effective in *L. lactis*⁴², but has since been extended to *S. thermophilus*^{21,34,43,44}. When a phage *ori* is provided in *trans* on a recombinant plasmid, the origin can act as a molecular decoy that competes for, and titrates away, both phage- and host-derived replication factors that catalyse phage-genome replication (FIG. 3). As a result, the number of phage genomes that are replicated over the course of the lytic infection is reduced. Conversely, the phage-associated replication factors might also catalyse 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 number⁴⁵ or the number of copies of the *ori* that are cloned within the same plasmid⁴⁶.

Foley *et al.* were the first to use origin-derived PER as a means of increasing phage resistance in *S. thermophilus*⁴³. They found that the phage-Sfi21-derived *ori* conferred strong resistance to related phages, as measured by a greater than seven-log reduction in the number of plaque forming units (pfu) per ml that were obtained when cultivated 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 indicated that the phage- and plasmid-encoded *oris* share a common ancestor.

More recently, Stanley *et al.* used Southern hybridization to classify *S. thermophilus* phages into three replication groups based on their origin of replication: group I, group II and non-I/non-II²¹. Phage O1205, which encodes a single origin of genome replication (*ori1205*), was found to be a member of replication group I. Phage 7201, which encodes two distinct *oris* (*ori7201A* and *ori7201B*), was found to be a member of replication group II. Thirty *S. thermophilus* phages were independently probed with *ori1205*, *ori7201A* and *ori7201B*. At least one of the phages in replication group I (phage O1205) is known to encode a complete Sfi21-type DNA-replication module, whereas at least one member of the replication group II phages (phage 7201) encoded a different genome-replication module. These results seem to be in agreement with the genomic data, however, it is not known if all of the group I or group II phages encoded a complete set of genes that were associated with the Sfi21-type or 7201-type replication modules, respectively.

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 phages when cloned onto the high-copy-number plasmid pNZ8048 (REF. 21). The level of protection that was provided by this plasmid was phage specific, and the EOP for these phages ranged from 10⁻³ to <10⁻⁷. Interestingly, the pORI1205 construct was also able to reduce the plaque diameter of three of the five group II phages that were tested, but did not have any impact on the EOP of any of these phages. These results indicated that a common host-encoded protein is required for the replication of the two phage groups, albeit to varying degrees.

Superinfection exclusion and immunity

Approximately half of the sequenced bacterial genomes contain prophage-associated sequences⁴⁷. Examination of these sequences reveals that prophage or prophage-remnant sequences comprise 3–10% of the total genomic content of lysogens⁴⁸. In the *S. thermophilus* temperate phage Sfi21, only two phage-specific regions were transcribed in the lysogenic state. One region comprised genes from the *cI*-like repressor to the superinfection-exclusion gene (*orf203*), which is located directly upstream of the phage integrase gene. The second region comprised four genes of unknown function that were located between the lysin gene and the *attR* site. Maintenance of this extra genetic information (prophage

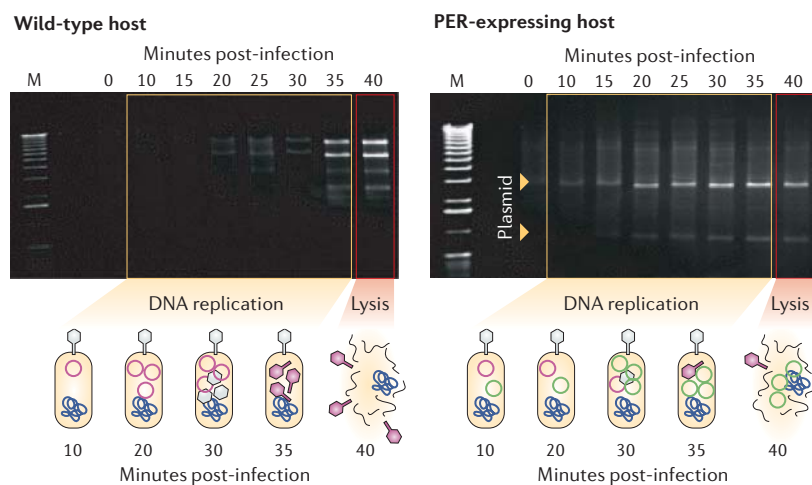


Figure 3 | The accumulation of phage DNA fragments over time. The impact of origin-derived phage-encoded resistance (PER) on the *in vivo* accumulation of restriction-endonuclease-digested phage DNA fragments over time and the explosive replication of PER plasmid DNA. The accumulation of phage DNA fragments over time in a PER⁻ (wild-type) strain (top left) and its associated phage lytic cycle (bottom left). Diminished accumulation of phage DNA fragments with concomitant increase of PER plasmid DNA over time in a PER⁺ (PER-expressing) strain (top right) and its associated phage lytic life cycle (bottom right). Phage genomic DNA is shown in pink, and PER plasmid DNA is shown in green.

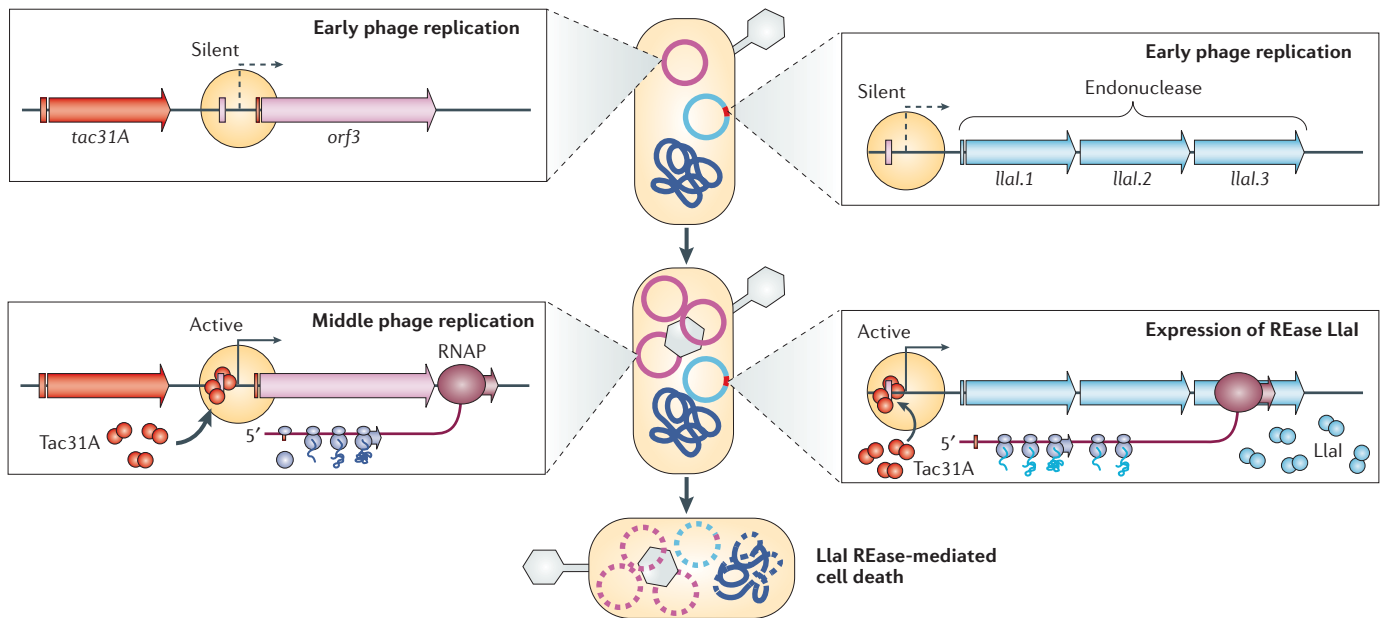


Figure 4 | **Phage-triggered suicide from an inducible cassette encoding a DNA endonuclease.** The yellow ovals depict the three bacterial states that exist during phage infection from adsorption and DNA injection to phage DNA replication (middle stage) and, finally, to cell lysis or phage-induced suicide death. Purple circles are phage genomes and blue circles are plasmids encoding the inducible suicide cassette (red). In a normal infection cycle (left panels), the transactivating regulator *tac31A* and its inducible promoter $\phi 31p$ (yellow circles) are expressed in the middle stages of the phage cycle. In either infection cycle, the $\phi 31p$ promoters are silent in the bacterial cell during early phage replication, but are activated after the expression of *tac31A* by the infecting phage during the middle stage of its developmental cycle. With the addition of the suicide cassette (right panels), which is based on the Llal restriction endonuclease (REase) in the absence of its cognate methyltransferase, *trans* activation of the plasmid-encoded $\phi 31p$ triggers the expression of the Llal REase suicide cassette, which digests the phage and host genomes, thereby degrading both the phage and host DNA. RNAP, RNA polymerase.

DNA) clearly increases the metabolic burden of the lysogen and should be expected to decrease its fitness relative to its non-lysogenic parent.

However, it is well known that some prophage-associated sequences can provide advantages to the lysogen by encoding one or more factors that increase the fitness of the bacterium. In *S. thermophilus*, the superinfection-immunity gene (mediated by the *ci*-like repressor) and the superinfection-exclusion gene are among the few that are expressed in the lysogenic state, and both confer a selective advantage to the lysogen by providing protection from subsequent phage infection. In one study, *orf203* conferred protection ($EOP \leq 10^{-3}$) from 12 of the 21 (57%) virulent phages that were tested when it was expressed in *S. thermophilus* from a high-copy-number plasmid⁴⁹. Superinfection-exclusion genes protect lysogens from becoming infected with additional phages by blocking genome injection. It has been proposed that *orf203* conferred phage resistance in this manner.

In a second study, the expression of the *CI*-like protein of phage Sfi21 (encoded by *orf127*) prevented the multiplication of the homologous temperate phage (EOP of approximately 10^{-7}), but failed to provide protection from 30 distinct virulent phages⁵⁰. The *CI*-like repressor protein expressed from the recombinant plasmid was shown to bind to an operator region essential for the expression of the lytic life cycle of the superinfecting phage Sfi21.

Repression of the lytic functions of a superinfecting phage by a *CI*-like repressor occurs by a process called superinfection immunity.

Phage-triggered suicide systems

Phage-triggered suicide systems put the expression of a toxic gene product under the control of a phage-inducible promoter^{51,52} (FIG. 4). One such system placed the expression of a restriction-endonuclease gene (*llaIR*)⁵³ under the control of $\phi 31p$, a middle-expressed inducible promoter that is isolated from the virulent lactococcal phage 31 (REF. 51). The expression of the suicide cassette, which was composed of the restriction endonuclease without its cognate methyltransferase gene (*llaIM*), from a high-copy-number vector reduced the EOP of phage 31 to approximately 10^{-4} . Once translated, the LlaIR restriction endonuclease killed the host and aborted phage infection by restricting both the unmethylated host and phage genomes. In the absence of phage infection, even a very low level of LlaIR expression can be toxic to the cell. As a result, these systems must be stringently regulated. Mutant phages that showed reduced sensitivity (EOP of ~ 0.4) to the original suicide system were then isolated and found to harbour mutations that mapped to the regulator, Tac31 (REF. 52). These phages exhibited lower levels of transcription from the $\phi 31p$ promoter, thereby decreasing the effectiveness of the phage-triggered suicide cassette expressed in *trans*.

Subunit poisoning

The expression in *trans* of mutant protein subunits can suppress, or poison, the function of native, multimeric proteins in a dominant-negative fashion⁵⁴ (FIG. 5). Subunit poisoning has recently been used as a novel phage-defence system in *S. thermophilus*³⁶. In this study, multiple alignments of related target protein sequences were used to identify essential amino-acid residues that were involved in enzyme catalysis and/or protein-subunit oligomerization. In this case, the putative primase (59 kDa), which is a component of the phage-Sfi21-type genome-replication module encoded by the *S. thermophilus* phage κ 3, was used as a model system. Directed by this approach, invariant and highly conserved amino acids within a phage primase consensus ATPase/helicase domain (pfam01057) were targeted by site-specific mutations. When expressed in *trans* from pTRK687, a high-copy-number vector, the K238A, K238T and RR340-341AA mutant proteins completely inhibited phage-genome replication and reduced the EOP of the homologous phage κ 3 and three other primase-encoding Sfi21-type phages by greater than nine logs. 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. Furthermore, host-encoded factors were unable to complement the deficiency caused by transdominant primase expression, indicating that the phage-encoded primase must have unique activities or associations that are essential for phage-genome replication.

The dominant-negative phenotype indicates that the plasmid-encoded mutant primase proteins must be structurally intact and can form stable interactions with the native phage-encoded primase proteins, thereby inhibiting their activity (FIG. 5). Alternatively, the mutant subunits might form other non-productive associations, such as substrate binding in the absence of catalysis and/or titrating away other phage- or host-encoded genome-replication factors. Amber mutations (N151*am*) that were introduced upstream of the transdominant RR340-341AA and K238T mutations restored phage-genome replication, parental-type EOP and completely suppressed phage resistance. These results indicated that translation of the transdominant mutant primase proteins was required to confer phage resistance. In a separate example, an E437A mutation, when introduced downstream of the transdominant K238T mutation, completely suppressed phage resistance as well. These results indicated that the E437A mutation precluded the association of the mutant primase protein from the native phage-encoded primase. Therefore, E437 was postulated to be a component of a C-terminal protein-oligomerization domain. The oligomerization domain of the gp4 primase from coliphage T7 is also located near the C-terminal region of the protein⁵⁵. Alternatively, the E437A mutation might preclude the association of the mutant primase protein with one or more as-yet-unknown host- or phage-encoded factor(s).

In a related form of resistance, Durmaz *et al.* recently cloned two derivatives of the *cI* repressor from the lactococcal P335-type phage 31 into the high-copy-number plasmid pTRKH2 (REF. 56). The first construct (pTRKH2::CI-per1) harboured 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 that were located between the ochre mutation and the downstream endogenous stop codon. The EOP of phage 31 was reduced to 10^{-6} in the presence of the pTRKH2::CI-per1 construct, whereas the EOP was further reduced to $<10^{-7}$ in the presence of the pTRKH2::CI-per2 construct. The authors went on to show that 12 of 16 heterologous virulent P335-type phages were completely inhibited by pTRKH2::CI-per2, whereas four phages were completely resistant to the defence system. Phage-31 mutants that were partially resistant to CI-per were isolated (EOPs for six phage mutants ranged between 0.1 and 0.9). Characterization of these mutant phages revealed mutations in two of the three putative operator sites for CI and Cro binding. During gel-mobility-shift experiments, both the wild-type and truncated CI proteins bound the two wild-type operators. However, the truncated CI failed to bind to the mutated operators. These data indicated that the truncated CI-per construct was able to inhibit phage replication by shutting down the expression of lytic functions by competitively binding the operator sites.

Host-factor elimination

The elimination of host-encoded factors that are essential for phage replication has great potential to block phage replication at one or more stages of development. Lucchini *et al.* described the use of pG⁺host9::ISS1-based insertional mutagenesis to identify genes that are involved in phage sensitivity in *S. thermophilus*⁵⁷. The plasmid pG⁺host9::ISS1 encodes

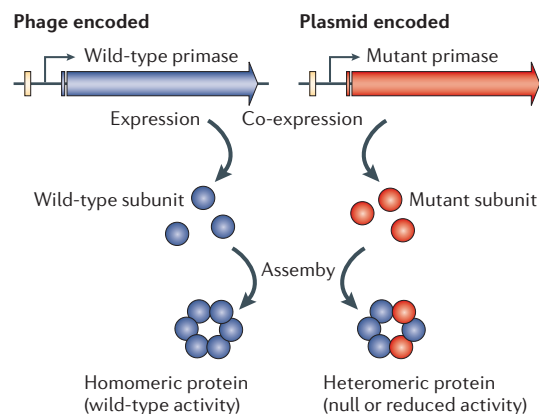


Figure 5 | The proposed mechanism of subunit poisoning in phage defence. Mutant subunits (red) of a multimeric protein (for example, primase) associate with the wild-type subunits (blue) to form functionally inactive heteromeric proteins with either reduced or null activity. Figure modified with permission from REF. 36 © (2005) Elsevier.

Amber mutation

A nonsense mutation that introduces a premature UAG translational stop codon in a gene.

Ochre mutation

A nonsense mutation that introduces a premature UAA translational stop codon in a gene.

an antibiotic-resistance marker, a temperature-sensitive replicon and a single copy of the insertion sequence *S1* (ISS1), which has previously been shown to integrate randomly into the genome of *S. thermophilus*⁵⁸. One of the principal advantages of plasmid-based mutagenesis systems over the use of spontaneous or chemically induced mutagenesis is that the genes that are interrupted by the integrated plasmid are readily cloned. Furthermore, the vector sequences can be removed from the chromosome by recombination, while leaving a single integrated copy of ISS1 in the chromosome. Using this approach, four distinct host-encoded loci that are involved in phage sensitivity have been identified⁵⁷. Among the most effective loci identified was an open reading frame (*orf394*) that encoded a putative transmembrane protein, gene product 394 (gp394). When mutated, gp394 conferred complete resistance to all 15 *S. thermophilus* phages that were tested. The modular composition of the phages was not tested; however, three *cos*-type phages that are known to encode variants of the Sfi21-type genome-replication module were among the phages tested (Sfi18, Sfi19 and Sfi21). 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 phages⁵⁹.

Another related phage-defence system involves the use of starter strains that are auxotrophic for the production or transport of nutrients that are essential for phage replication. One such system was developed in lactococci, based on mutants of the *thyA* gene that encodes thymidylate synthase^{60,61}. Thymidylate synthase is an enzyme that is used during the *de novo* synthesis of deoxythymidine triphosphate (dTTP), which is essential for DNA replication. In the absence of exogenous dTTP or its precursors (for example, thymidine), this enzymatic activity is essential for DNA replication and, therefore, growth of lactococci in milk, which is devoid of free dTTP⁶⁰. A Δ *thyA* mutant of *L. lactis* CHCC373 was constructed by gene replacement using the thermo-sensitive vector pGhost9 (REF. 58). This mutant, designated MBP71 Δ *thyA*, could be cultivated in medium that was supplemented with dTTP, washed to remove residual dTTP, concentrated to achieve a required activity level and then added to the fermentation substrate, milk. As the MBP71 Δ *thyA* is non-dividing, the authors found that the starter must be inoculated at higher levels relative to the parent to achieve a target pH in a certain timeframe. In this case, the inoculation level of MBP71 Δ *thyA* must be 17-fold higher than that for the parent strain if a final pH of 5.2 is to be reached in 6 hours. The addition of limiting thymidine (20 mg per litre) to milk, which temporarily restores DNA replication, was shown to increase the acidification rate such that the initial level of inoculum could be reduced to only 5-fold greater than that for the parent strain.

Importantly, the authors went on to show that the phage can inject its genome into the host cell, but is then unable to replicate. Therefore, the phage genome becomes entombed within the cellular confines of a host that cannot support its replication. The acidification rate

of MBP71 Δ *thyA* was completely unaffected by the presence of nine different phages that belong to two distinct phage groups (P335 and 936) at a multiplicity of infection of 0.1. At the population level, this type of defence system is advantageous because the non-replicating mutant acts to eliminate phages that are present in the medium. These phages might otherwise attack conventional, phage-related cultures that might be co-inoculated with the Δ *thyA* mutant strain, or inoculated later after an initial pre-incubation that is designed to eliminate phages that are present in the medium.

In a second study, the same group went on to show that, shortly after inoculation, the lactic acid flux of non-replicating MBP71 Δ *thyA* was 60% of that of exponentially growing MBP71 Δ *thyA*⁶¹. It was shown that increased ATPase activity was used to stimulate the rate of acidification by non-replicating MBP71 Δ *thyA* growing in milk. This stimulation was mediated through the plasmid-borne expression of the *atpAGD* genes from *L. lactis* MG1363. Using this system, non-replicating cultures in milk reached the target pH of 5.2 at a rate that was 40% faster than non-replicating cultures that lacked enhanced ATPase activity, but at a rate slower than that of the wild-type.

Rotation and conjugation

The utility of engineered phage-resistant strains in the dairy environment might be extended further through the use of the same rotation strategies that have been developed to protect traditional starter cultures. When using defined-strain starter systems, rotation is a process whereby individual strains or cultures are withdrawn from bioprocessing schedules and replaced with one or more non-isogenic and phage-unrelated strains or cultures with similar fermentative properties⁶². In practice, rotation can be done prophylactically on predetermined batch schedules or following the emergence of virulent phages that are specific for the individual strains or cultures being used⁶². These rotation strategies require the identification and characterization of many complementary strains of bacteria. Unfortunately, the identification of new starter strains is time-consuming and expensive. Furthermore, it is often difficult to identify non-isogenic strains with the appropriate fermentation characteristics.

Plasmid intracellular rotation^{63,64} has emerged as a novel alternative to traditional strain rotation that can increase the use of well characterized strains or ones that routinely 'phage out' during bioprocessing. This model was originally developed in lactococci, but should be equally useful in *S. thermophilus* and other strains of industrial relevance. During this process, several phage-resistant derivatives of a single specialized strain are constructed by introducing various phage-defence plasmids of different natures and specificity. When used properly, these rotation strategies using naturally occurring phage-resistance plasmids can significantly extend the longevity of strains in the dairy environment — especially when used in conjunction with effective methods to select for phage-insensitive mutants^{65–69}.

Phage out

The event whereby the starter culture inoculated in a bioprocessing system has been decimated by the lytic activity of phages to such a degree that the bioconversion is abandoned. Vats of partially cultured milk that have 'phaged out' are normally discarded.

Phage-insensitive mutant

Phage-resistant mutants that are derived from a parent strain that are insensitive to the phage(s) in question. Phage-insensitive mutants are generally the result of targeted phage-challenge assays, whereby a phage-sensitive parent strain is repeatedly challenged with a cocktail of two or more unrelated phages. This process is often referred to as phage hardening.

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^{70,71}. Conjugation is a natural process that involves the physical transfer of genetic material from one bacterial cell (donor) into another (recipient) and requires physical contact between the two bacteria involved, which is initiated by the donor cell. If DNA transformation and recombinant plasmids are considered, an additional requirement is that the complete vector system must be food grade in its composition, meaning that the vector must be composed of only DNA derived from the same type of microorganism that is normally used in the manufacture of food products, such as the lactic acid bacteria⁷².

Concluding remarks

The plasticity of phage genomes is crucial to their rapid evolution. The short generation time and large burst sizes of phages function cooperatively to accelerate the rate at which mutant phages might overcome a given defence. Unfortunately, phages have been able to evolve resistance to many of the naturally occurring defences that have been implemented in the industrial setting over the years. In fact, nearly 70 years after the discovery of phages that infect *L. lactis*, phages continue to be a leading cause of failure in industrial fermentations. Furthermore, as the demand for fermented food

products made with strains of lactic acid bacteria, including *S. thermophilus*, has increased, so has the incidence and severity of phage attacks against these thermophilic starter strains. With the expansion of fermentation and bioprocessing systems that are reliant on lactic acid bacteria, disruption by phages remains a growing concern. For example, strains of lactic acid bacteria are being exploited further for the manufacture of industrial chemicals (such as lactate) and used as vehicles for the delivery of biological agents (for example, vaccines and enzymes).

Together, these persistent pressures necessitate the continued development of starter cultures with enhanced phage-resistance properties. Over the years, the use of natural phage-defence systems has proved to be invaluable for the protection of strains that are expected to perform consistently and over extended time frames within industrial applications. The number of completely sequenced phage and bacterial genomes continues to grow rapidly, and has aided in the development of these systems. These emerging tools are invaluable for understanding the fundamental relationships that exist between the dairy lactic acid bacteria and their phages. When leveraged against a greater understanding of gene regulation, these sequences reveal the genetic content and comparative organization of phage genomes and facilitate the development of more efficient phage defences.

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Competing interests statement

The authors declare no competing financial interests.

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FURTHER INFORMATION

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