Ollscoil na hÉireann, Corcaigh

National University of Ireland, Cork

Discovery and characterization of antiphage systems in the lactococcal plasmidome

Thesis presented by

Andriana Grafakou

M.Sc. Food Science and Human Nutrition

for the degree of

PhD in Microbiology

University College Cork

School of Microbiology

Head of School: Dr. David Clarke

Supervisors: Prof. Douwe van Sinderen and Prof. Jennifer Mahony

2024

Table of contents

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

This thesis is dedicated to my parents,

Αντιγόνη και Κώστα

Abbreviations

aa: Amino acid

AAA: ATPases associated with diverse cellular activities

ABC: ATP-binding cassette

Abi: Abortive infection

AGS-C: Adenylyl, guanylyl & SMODS sensor C-terminal

ARE-ABCF: Antibiotic resistance ABC protein of the F subfamily

Cap: CD-NTase-associated protein

CARF: CRISPR-associated Rossman fold

Cas: CRISPR-associated

CBASS: Cyclic oligonucleotide-based antiphage signalling system

CDI: Contact-dependent growth inhibition

CD-NTase: cGAS/DncV-like NTase

CFU: Colony forming unit

COI: Centres of infection

CPEC: Circular polymerase extension cloning

CRISPR: Clustered regularly interspaced short palindromic repeats

Csp3: Copper storage protein 3

C_T: cycle threshold

CTD: C-terminal domains

DRT: Defence-associated Reverse Transcriptase

DSR: Defence-associated sirtuin

DUF: Domain of unknown function

ECOI: Efficiency of COI formation

EM: Escape mutant

EOP: efficiency of plaquing

Erf: Essential recombination function

FDA: Food and Drug Administration

FPPS: Farnesyl pyrophosphate synthase

GAF: cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA

GMO: Genetically modified organism

GRAS: Generally recognized as safe

HCL: Hierarchical clustering

HEPN: Higher eukaryotes and prokaryotes nucleotide-binding domain

LAB: Lactic acid bacteria

MC: Middle component

MCL: Markov clustering algorithm

MGEs: Mobile genetic elements

MOI: Multiplicity of infection

NAD: Nicotinamide adenine dinucleotide

NCBI: National Centre for Biotechnology Information

NTase: Nucleotidyltransferase

OD: Optical density

ORF: Open reading frame

OTE: Oolong Tea Extract

PADLOC: Prokaryotic antiphage defence locator

PAE: Predicted aligned errors

PARIS: Phage anti-restriction-induced system

PBP: Penicillin binding protein

PDB: Protein data bank

PFU: Plaque-forming unit

qPCR: quantitative Polymerase Chain Reaction

RM: Restriction-modification

RSM: Reconstituted skimmed milk

RT: Reverse transcriptase

Sak: Sensitivity to AbiK

SAVED: SMODS-associated and fused to various effector domains

SD: Standard deviation

Sie: Superinfection exclusion

Sir2: Silent information regulator 2

SIRTs: Sirtuins

SLATT: SMODS and LOG-associating two TM

SLFN-3: Schlafen group 3 proteins

SMC: Structural maintenance of the chromosome

SMODS: Second messenger oligonucleotide or dinucleotide synthetases

SNP: Single nucleotide polymorphisms

SSAP: Single-strand DNA annealing protein

SSB: Single-strand DNA binding

ssDNA: Single-stranded DNA

Stk2: Serine/threonine kinase

Sua-2TM: SMODS/Ubiquitin system-associated 2TM

TA: Toxin-antitoxin

TEM: Transmission electron microscopy

TM: Transmembrane

TOPRIM: Topoisomerase-primase

UG: Unknown group

Thesis Abstract

Lactococcus lactis and *Lactococcus cremoris* are important bacterial species that are widely used in the production of fermented dairy products such as buttermilk and cheese. However, the non-sterile dairy environment exposes them to (bacterio)phage infections, which can cause substantial economic losses. To counteract phage threats, bacteria have evolved various defence mechanisms, including abortive infection (Abi) systems. Abi systems are activated upon phage infection, and they typically interfere with essential cellular functions, thereby significantly limiting phage propagation.

In Chapter 1, we review the existing literature of 34 lactococcal Abi-like systems. Using the latest structural and functional prediction tools, we categorize certain Abi-like systems through structural superimposition and hypothesize their potential mechanisms based on predicted domain information. We also examine previously established mechanisms of action of certain Abi-like systems.

In Chapter 2, we use *Lactococcus* as a model to identify novel plasmid-encoded antiphage systems, leveraging the fact that many of the first Abi systems were discovered in these bacteria. A systematic evaluation of candidate defence systems led to the discovery of seven new plasmid-encoded antiphage systems (Rhea, Aristaios, Kamadhenu, Fliodhais, Audmula, Rugutis, Hesat), which seem to be widely distributed across bacteria, as well as five systems (PARIS, type I and II CBASS, Lamassu, Septu), homologues of which had previously been identified as antiphage systems in other bacteria. These systems confer resistance against the

most prevalent lactococcal phages, and all but one were shown to exhibit characteristics of abortive infection systems. Insights into their mechanisms of action were gained through structural and domain predictions.

In Chapter 3, functional insights into three novel plasmid-encoded lactococcal Abi-like antiphage systems are presented. Their effectiveness in milk-based media was established, supporting their application potential to enhance the reliability of dairy fermentations. Our findings indicate that two of these systems do not directly impede phage genome replication, transcription, or translation, while one was found to interfere specifically with phage transcription. Additionally, the plasmid carrying one of these systems was successfully transferred via conjugation to various lactococcal strains, each of which showed a significant increase in phage resistance.

In Chapter 4, mechanistic insights into the identified antiphage systems were established by isolating phage escape mutants that had overcome these systems. Genome analysis of these phage escape mutants revealed mutations in various genes, some of which encode proteins that appear to activate specific antiphage systems (terminase large subunit, major capsid protein, hypothetical proteins and major tail proteins), while others might act as targets (ssDNA annealing proteins). The cross-resistance of some phage escape mutants to different antiphage systems, as well as the activation of some systems by phage proteins with similar functions, indicates mechanistic commonalities among various antiphage systems. Additionally, we propose a novel mode of action for one antiphage system.

Our findings demonstrate that the co-evolution of *Lactococcus* species with their phages, spurred by their widespread use in dairy fermentation, has driven the acquisition of a diverse range of phage defence mechanisms, with plasmids serving as a rich source of such antiphage systems. Expanding our understanding of the lactococcal phage resistome not only offers practical solutions to the persistent phage challenges in industrial food fermentations but also holds potential for broader applications in biotechnology and biomedicine, while providing deeper insights into the dynamics of phage-host interactions.

Chapter I

Revisiting lactococcal abortive infection-like antiphage systems: Diversity, structures and mechanisms

This chapter will be submitted for publication as:

Grafakou A., Mosterd C., de Waal P.P., van Rijswijck I.M.H., van Peij N.N.M.E., Cambillau C., Mahony J. and van Sinderen D. Revisiting lactococcal abortive infection-like antiphage systems: Diversity, structures and mechanisms.

A.G, C.M, J.M. and D.v.S. were involved in the conceptualization of the study. A.G. and C.M. drafted the initial manuscript. C.C. performed the AlphaFold2 predictions and analysis. P.d.W., I.v.R. and N.v.P. contributed to meaningful discussion and interpretation of the results. J.M. and D.v.S. reviewed and edited the manuscript. All authors contributed to the final version of the manuscript.

1.1 Abstract

Lactococcus lactis and *Lactococcus cremoris* are cornerstone bacterial species employed in the production of fermented dairy products such as cheese. However, the non-sterile dairy processing environment exposes these bacteria to phage infection. To counteract the phage-imposed threat to their life, bacteria have evolved a range of defence mechanisms, including abortive infection (Abi) systems, of which many (AbiA to AbiZ) were originally discovered in *Lactococcus*. Recent discoveries have expanded the list of presumed Abi systems in *Lactococcus* species. In this review, we examine and revisit the antiphage activity spectrum, the interference with the phage lytic cycle, and the study of phage escape mutants associated with these systems. Since many of these antiphage systems were identified before the development of advanced functional and structural prediction tools, their functional domains and associated mechanisms have remained largely unexplored. Here, we use predicted domain information to postulate their mechanism of action, and we group various Abi systems based on structural superimposition (AbiA and AbiK; AbiC and AbiP; AbiD, AbiD1, AbiF and AbiD/F; PARIS and AbiL). Additionally, we discuss established mechanisms for a number of previously studied Abi systems. Our findings support the hypothesis that co-evolution of *Lactococcus* species with their phages, possibly enhanced by the extensive application of these bacteria in dairy fermentations, has resulted in the acquisition of a diverse array of phage defence mechanisms, including Abi systems.

1.2 Introduction

Lactococcus lactis and *Lactococcus cremoris* (previously two subspecies of *Lactococcus lactis;* (1)) are members of the lactic acid bacteria (LAB), many of which have a long history of application in the manufacture of various fermented foods, such as cheese, yoghurt and sauerkraut (2). Bacteriophages are the most abundant biological entities on the planet and their role in bacterial population dynamics is undeniable (3). The omnipresence of phages has forced bacteria to develop a plethora of antiphage systems for their protection (4). Lactococci are no exception to this, as their vulnerability to phage attack is exposed due to their widespread application as starter cultures in non-sterile dairy fermentation environments (5).

Bacterial defences against phage infection include diverse barriers that are effective at different stages of the phage cycle (4). For instance, bacteria can prevent phage adsorption, disable phage DNA injection or degrade the incoming phage nucleic acid molecule (6). As one of the last resorts, abortive infection (Abi) prevents completion of the phage infection cycle by blocking an essential cellular activity, thereby preventing phage proliferation and subsequent infection of neighbouring cells (7).

Due to the recurrent infection of starter cultures and associated risk of failed fermentations, the constant phage threat to *Lactococcus cremoris/lactis* strains during dairy fermentations has had and continues to have negative environmental and economic impacts (8). Therefore, it is not surprising that many of the first known Abi systems were discovered in these

species (reviewed by (9–11)), making Abi one the most abundant and most intensely studied lactococcal antiphage mechanisms. Lactococcal antiphage defences further comprise a variety of Type I, Type II and Type III Restriction-Modification (RM) (12–14) and five different Superinfection exclusion (Sie) systems (15, 16). Audmula and Fliodhais are two recently discovered antiphage systems that function through a non-Abi (Audmula) or unknown (Fliodhais) mechanism (17). CRISPR-Cas systems are typically not present in lactococcal genomes and do not appear to play a major role in their antiphage defence, although a type III-A CRISPR-Cas system has been described in *L. lactis* (18, 19). Interestingly, homologues of some of the Abi systems, including AbiD/F (or termed Abi2 by Tesson et al.) and AbiEi, are highly abundant in prokaryotes (20). Notably, most of the lactococcal Abi systems have been designated as such (i.e. being an Abi system) based on their phenotypic profile rather than on mechanistic characterization (21). Therefore, for the purpose of this review, they will hereafter be referred to as Abi-like systems.

A recent large-scale study of lactococcal plasmids revealed a considerable number of novel Abi-like systems (17), yielding a total of 34 distinct Abi-like systems identified in *Lactococcus.* Additionally, it was demonstrated that structure predictions and alignments of antiphage systems using advanced structural and functional analysis tools, such as AlphaFold2 and Dali, can assist in the prediction of their function and in their similarity-based classification, exemplified by the grouping of AbiD, AbiD1, AbiF and AbiD/F into the new AbiD/F group (17).

With currently 34 Abi-like systems identified in *Lactococcus* and the last review dedicated to lactococcal Abi systems dating back to 2005 (9), an update incorporating the use of recent functional prediction tools is highly desirable. The aim of this review is therefore to revisit and, where appropriate, reclassify some of the known lactococcal Abi-like systems by predicting and analysing their functions and structures using current bioinformatic tools.

1.3 The repertoire of known lactococcal Abi-like systems: A 35-year history

Since the discovery of AbiA in the early 1990s (22) and up until the identification of AbiZ in the late 2000s (23), 23 abortive infection-like systems have been described (named AbiA to AbiZ; notably there are no systems designated as AbiM, AbiW, AbiX and AbiY; Table 1, updated from Philippe et al. (11)). However, the search for new Abi systems in lactococci seemed to have slowed down for more than 15 years.

Recent reports on defence islands -genomic regions enriched with clustered antiphage systems- (24–26) have renewed interest in finding novel antiphage systems across various bacterial genera. Additionally, the ongoing issue of phage infection in dairy fermentations (8) highlights the need to develop (non-genetically modified) phage robust starter cultures that also encode other desirable traits (27, 28). The development of such phage robust strains can be facilitated by the natural transfer of conjugative or mobilizable plasmids carrying these traits (29–33).

The above led to a recent study (17) which identified 11 additional plasmid-encoded abortive infection-like systems in *Lactococcus* (Table 1). Five of the identified systems (Rhea, Aristaios, Kamadhenu, Hesat, Rugutis) were completely novel Abi-like systems and were named after ancient cheese and fermented food deities (following a nomenclature employed for antiphage systems discovered in *Escherichia coli* and *Bacillus subtilis* (24)). The remaining systems were similar to previously described abortive infection systems i.e. a homologue of a lactococcal Abi system (AbiD/F) and five Abi-like systems described in other bacteria but not previously (experimentally) confirmed in lactococci (PARIS, type I and II CBASS, Septu and Lamassu).

1.4 Components and genetic location of lactococcal Abi-like systems

Among the 34 confirmed Abi-like systems in *Lactococcus* (Table 1), the majority (25 systems) represent single-gene systems, while nine are two-component systems (AbiE, AbiG, AbiL, AbiQ, AbiT, AbiV, PARIS, type I CBASS, Septu; of which AbiQ and AbiV comprise a protein and an RNA molecule) and three are three-component systems (AbiR, type II CBASS, Lamassu). However, antiphage systems with more than three components may have been overlooked in *Lactococcus*. This hypothesis is supported by the identification of multi-component defence mechanisms in other bacteria, such as the four to eight-component BREX systems (34), and by the predominant selection of single- or two-gene candidate antiphage systems during the recent discovery of novel lactococcal antiphage systems (17).

Table 1. Antiphage activity, interference with the phage lytic cycle and phage escape mutant information of the experimentally confirmed abortive infection-like systems in *Lactococcus.*

System	Active against*			Interference with phage cycle	Phage escape mutants analysis	References
	S	***C***	**P**			
AbiA (Also Hsp)	+	+	+	DNA replication (P335 group)	Mutation(s) in putative ATP-binding protein; intergenic region; **ssDNA annealing protein and Holliday junction resolvase**	(22, 35–39, Chapter IV of this thesis)
AbiB **(Also abi-416)**	+	**-**	**-**	RNA transcription	**Mutation(s) in major capsid protein and hypothetical protein**	(17, 40, 41, Chapter IV of this thesis)
AbiC (Also Prf)	+	**-**	+	Major capsid production	N/A	(42, 43)
AbiD	**+**	**+**	**N/A**	**After DNA replication**	N/A	(44)
AbiD/F	**+**	**-**	**-**	**N/A**	**Mutation(s) in hypothetical proteins**	(17, Chapter IV of this thesis)
AbiD1	+	+	**N/A**	Degradation of sk1 RuvC endonuclease	Mutation(s) in hypothetical protein	(45–50)
AbiE	+	**-**	**-**	After DNA replication	N/A	(39, 51)

AbiF	**+/-**	**+/-**	**-**	DNA replication, delayed (*Skunavirus*)	N/A	(17, 39, 51, 52)
AbiG	+	+/−	**+/−**	RNA transcription (*Ceduovirus* & *Skunavirus*) DNA replication, delayed (P335)	**Mutation(s) in major tail protein**	(17, 39, 53, 54, Chapter IV of this thesis)
AbiH	+	+/−	**N/A**	N/A	N/A	(55)
AbiI	+	+/−	**N/A**	N/A	N/A	(56)
AbiJ **(Also abi-859)**	+/−	**+**	**N/A**	N/A	**Mutation(s) in ssDNA annealing protein**	(17, 57, Chapter IV of this thesis)
AbiK	+	+/−	+	After DNA replication (*Skunavirus*), before DNA replication (P335)	Mutation(s) in SaK ssDNA annealing protein; recombination with a prophage	(58–66)
AbiL	+	+/−	**-**	After transcription (*Ceduovirus*)	N/A	(67)
AbiN	+	+	**N/A**	N/A	N/A	(68)
AbiO	**+/−**	**+/−**	**N/A**	After DNA replication (*Ceduovirus*)	N/A	(69)
AbiP	+	**-**	**+/−**	DNA replication & temporal control (switch-off) of early transcripts	**Mutation(s) in major tail protein**	(17, 70, 71, Chapter IV of this thesis)

AbiQ	+	+	**-**	RNA transcription	Mutation(s) in hypothetical proteins	(72, 73)
AbiR	**N/A**	**+/−**	**N/A**	DNA replication, reduced	N/A	(74–76)
AbiS	+	**N/A**	**N/A**	N/A	N/A	(77)
AbiT	+	**-**	+	DNA replication (*Skunavirus* and P335 group) and capsid protein synthesis (*Skunavirus*)	Mutation(s) in major capsid protein and hypothetical proteins; recombination with prophage genes	(63, 78, 79)
AbiU	+/−	**+/−**	+/−	RNA transcription, delayed (*Skunavirus*, *Ceduovirus*)	N/A	(80)
AbiV	+	+	**-**	Protein synthesis and late gene transcription (***Skunavirus***)	Mutation(s) in a SaV protein	(81–83)
AbiZ	+/−	+/−	+	Premature lysis	Recombination with host genome	(23)
Rhea	**+**	**+/−**	**+/−**	**No effect on DNA replication, transcription, translation (*Skunavirus*)**	**Mutation(s) in terminase large subunit (ATPase domain)**	(17, Chapter IV of this thesis)
Aristaios	**+**	**-**	**-**	**Transcription (*Skunavirus*)**	**N/A**	(17, Chapter IV of this thesis)

Kamadhenu	**+**	**-**	**-**	**No effect on DNA replication, transcription, translation (*Skunavirus*)**	**Mutation(s) in ssDNA annealing protein**	(17, Chapter IV of this thesis)
Rugutis	**+**	**+/−**	**+/−**	**N/A**	**Mutation(s) in terminase large subunit (ATPase domain)**	(17, Chapter IV of this thesis)
Hesat	**+**	**-**	**N/A**	**N/A**	**N/A**	(17, Chapter IV of this thesis)
PARIS	**+**	**+/−**	**N/A**	**N/A in *Lactococcus***	**Mutation(s) in hypothetical protein; intergenic region**	(17, Chapter IV of this thesis)
Type I CBASS	**+**	**+/−**	**N/A**	**N/A in *Lactococcus***	**N/A**	(17, Chapter IV of this thesis)
Type II CBASS	**+**	**+**	**N/A**	**N/A in *Lactococcus***	**Mutation(s) in hypothetical protein; intergenic region**	(17, Chapter IV of this thesis)
Lamassu	**+**	**-**	**N/A**	**N/A in *Lactococcus***	**N/A**	(17, Chapter IV of this thesis)
Septu	**+**	**-**	**N/A**	**N/A in *Lactococcus***	**Mutation(s) in ssDNA binding protein**	(17, Chapter IV of this thesis)

*S, *Skunavirus*; C, *Ceduovirus*, P, P335 phage group; N/A, not available; +/−, indicates either lower/partial activity against phages of this group or activity against specific phages of this genus/group. Updated and adapted from Philippe et al. (11) changes/additions are found in bold-face text.

Notably, the majority of the lactococcal Abi-like systems are plasmid-encoded, with only a few, such as AbiH (55), AbiV (81) and AbiN (68), found on the host chromosome. This might lead to the incorrect assumption that lactococcal defences against phages are primarily plasmid-based. However, there is a discovery bias towards plasmid-encoded antiphage systems in *Lactococcus*, as plasmids are more frequently studied for phage-resistance associated genes due to the interest in transferring them by conjugation to strains of interest to enhance their phage robustness (9).

Nevertheless, it is perhaps unsurprising if defence systems are primarily plasmid-borne in these dairy lactococci, which are known to carry many plasmids (i.e. average of six plasmids per strain; (17)). These plasmids harbour genes associated with various beneficial traits (such as lactose utilization, casein metabolism, conjugation, bacteriocin production, exopolysaccharide production; (19)). Furthermore, a higher copy number of Abi-like-encoding genes on plasmids compared to the chromosome may be expected to result in increased levels of phage resistance (11).

1.5 The antiphage activity spectrum of lactococcal Abi-like systems

The activity of antiphage systems in *Lactococcus* has mainly been assessed against members of the three most prevalent and problematic phage genera in modern, large-scale dairy fermentation plants, i.e. *Skunavirus* (formerly 936 phage group), *Ceduovirus* (formerly c2 phage group) and P335 group phages (84, 85). Nonetheless, some systems have also been tested and proven effective against other lactococcal phage

genera, such as AbiT (which is effective against members of the *Audreyjarvisvirus, Questintvirus* and *Fremauxvirus* genera [formerly 949, Q54 and 1706 phage groups, respectively]; (86–88)) and AbiQ (which is effective against members of the *Audreyjarvisvirus* and *Questintvirus* genera, and coliphages; (73, 86, 87)).

It is evident that the antiphage activity spectrum of Abi-like systems is diverse (Table 1). Some systems are active only against phages of a single genus (e.g. AbiB; (17, 40, 41)), while others are active against phages belonging to multiple genera/groups (e.g. AbiA; (36, 39)). Additionally, some systems are active against specific members of a given phage genus (e.g. AbiO; (69)), and others seem to provide only modest protection against phages (e.g. AbiU, 10^{-1} to 10^{-2} reduction of the efficiency of plaquing of phages from all the three assessed groups; (80)). Interestingly, in all cases, each antiphage system was found to be active against at least one member of the *Skunavirus* genus, which are the most prevalent lactococcal phages (89), suggesting that these antiphage systems have evolved to target the most abundant and prevalent lactococcal phages. Notably, these Abi-like systems have been evaluated under different experimental conditions, such as diverse expression vectors and phage panels, complicating the comparison of their activity for practical applications and the assessment of their effectiveness in non-artificial conditions. This highlights the need for uniform testing approaches and a re-evaluation of the activity of the systems against a large panel of phages to establish and compare the spectrum of activity of these systems.

1.6 Effect of lactococcal Abi-like systems in the phage lytic cycle

Further characterisation of a lactococcal antiphage systems typically involves assessment of the effect of the antiphage system on specific steps of the phage lytic cycle (Table 1). Among the characterized lactococcal antiphage systems, seven were found to interfere with phage replication (i.e. AbiA; (35, 39), AbiF; (51), AbiG; (39), AbiK; (58), AbiP; (70), AbiR; (75), AbiT; (78)), six were shown to affect phage transcription (i.e. AbiB; (41), AbiG; (54), AbiQ; (73), AbiU; (80), AbiV; (83), Aristaios; (33)) while one was demonstrated to diminish phage protein production (AbiC; (43)). Two systems (Rhea and Kamadhenu) were found not to interfere with any of the above-mentioned steps. This implies an (direct or indirect) interference with other (and later) steps of the phage lytic cycle, such as DNA packaging or phage maturation.

1.7 Phage escape mutant analysis of lactoccocal Abi-like systems

Abi systems contain at least one functional module to sense (an aspect of) phage infection, upon which another domain becomes activated to elicit the bacteriostatic/bactericidal effect (7). Investigating the specific phage propagation step affected by activation of a particular Abi may provide general mechanistic information. However, this approach fails to differentiate if inhibition of this phage proliferation step is a direct or indirect target for the Abi system. Additionally, this approach generally provides less detailed mechanistic information compared to phage escape mutant

isolation and analysis, which may point to phage-derived proteins sensed or affected by the Abi system.

Phage escape mutants that bypass the activity of phage defence mechanisms, have been isolated and analysed for 16 of these 34 lactococcal systems (Table 1), in an attempt to define phage-derived proteins that may circumvent or trigger these antiphage systems. Phages typically acquire insensitivity to these systems through the acquisition of one (or occasionally multiple) point mutation(s). However, in some instances (AbiK; (59, 63), AbiT; (63), AbiZ; (23)), insensitivity has been achieved via recombination with a prophage or the host genome. Interestingly, escape mutants derived from five of these systems (AbiA; (Chapter IV of this thesis), AbiJ; (Chapter IV of this thesis), AbiK; (61), Kamadhenu; (Chapter IV of this thesis), Septu; (Chapter IV of this thesis)) were mutated in genes encoding a single-stranded DNA binding or DNA annealing protein, four in a structural protein (major tail or capsid protein; AbiB; (Chapter IV of this thesis), AbiG; (Chapter IV of this thesis), AbiP; (Chapter IV of this thesis), AbiT; (79)), two in the ATPase domain of the terminase large subunit (Rhea; (Chapter IV of this thesis), Rugutis; (Chapter IV of this thesis)), while eight were mutated in genes encoding hypothetical proteins (AbiB; (Chapter IV of this thesis), AbiD1; (47), AbiD/F; (Chapter IV of this thesis), AbiQ; (73), AbiT; (79), AbiV; (82), PARIS; (Chapter IV of this thesis), type II CBASS; (Chapter IV of this thesis)).

Based on recent reports of identified phage proteins that activate antiphage systems in bacteria other than *Lactococcus* (90–94), a recent study (Chapter IV of this thesis) confirmed lactococcal phage-derived

triggers (terminase large subunit for Rugutis, major capsid protein for AbiB, major tail protein for AbiG and AbiP, hypothetical proteins for AbiD/F) and possible targets (ssDNA annealing protein for AbiA and AbiJ) for some of these antiphage systems. Additionally, it was shown that AbiA escape mutants are also insensitive to AbiJ, however this was not observed for other combinations, where the same gene was mutated in escape mutant phages that bypass distinct antiphage systems.

The terminase large subunit, major capsid and major tail protein have previously been reported to activate antiphage systems present in other bacteria (93, 95, 96), showing that despite the diversity, antiphage systems are governed by some general activation/targeting principles regardless of the host origin and their sequence, structural and functional diversity.

1.8 Domains and structures of lactoccocal Abi-like systems

With AbiA discovered in the early 1990s (22) and AbiZ in the late 2000s (23), most of the lactococcal Abi-like systems were identified before the availability of advanced functional (HHpred and Interpro; (97, 98)) and structural prediction tools (AlphaFold2; (99)). Therefore, no similarity with proteins of known function was found for many of them (9), and with their domains mostly unexplored, the potential mechanisms of these systems remain largely unknown. To address this, we subjected all lactococcal Abi-like systems to HHpred, Interpro and AlphaFold2 analysis (Table 2; updated from (17)). Here, we group certain systems based on structure superimposition (summarized in Figure 1), while the predicted domain

information allows us to propose possible mechanisms of action. Additionally, we discuss established mechanisms for some Abi-like systems where these are already known (summarized in Figure 1).

Table 2. Functional analysis of lactococcal Abi-like systems

System name	Accession number/ Gene locus	InterPro Prediction	HHpred Prediction	AlphaFold2/Dali Prediction	InterPro/Pfam accession	HHpred PDB hit
AbiA	AAA65072.1	HEPN nuclease	DNA polymerase	Reverse transcriptase	IPR041026	8OZ7_D
AbiK	AAB53491.2	Reverse transcriptase	DNA polymerase	Reverse transcriptase	IPR000477	7R07_A
AbiB	AAA25158.1	-	-	HEPN	-	-
AbiJ	AAA99069.1	Abi-like	-	HEPN	IPR026001	-
AbiV	WP_011834704.1	HEPN nuclease	-	HEPN	PF18728	-
AbiC	AAA53569.1	Putative AbiC	-	Farnesyl pyrophosphate synthase	IPR031709	-
AbiP	AAC15900.1	Putative AbiC	-	Farnesyl pyrophosphate synthase	IPR031709	-
AbiD	AAA63619.1	AbiD/AbiF	-	Cas13d ribonuclease	IPR017034	-
AbiD1	AAA79209.2	AbiD/AbiF	-	Cas13d ribonuclease	IPR017034	-
AbiF	AAB52386.1	AbiD/AbiF	-	Cas13d ribonuclease	IPR017034	-

AbiD/F	LLA22_pB0040	AbiD/AbiF	-	Cas13d ribonuclease	IPR017034	-
AbiEi	AAB52382.1	-	AbiEi antitoxin	-	-	6Y8Q_D
AbiEii	AAB52383.1	NTase AbiEii toxin	NTase toxin	Guanylytransferase-like toxin	IPR014942	6Y5U_A
AbiQ	AAC98713.1	AbiQ toxin	AbiQ toxin	AbiQ toxin	IPR025911	4GLK_A
AbiLi	AAB53710.1	AAA_21 ATPase	SMC protein	Rad50 ATPase	IPR003959/ PF13304	6ZZ6_A
AbiLii	AAB53711.1	RloB/AbiLii-like	-	TOPRIM domain of OLD nucleases	IPR025591	-
PARIS-i	pUCCL620_250 PP556494.1	AAA_21 ATPase	SMC protein	Rad50 ATPase	IPR003959 PF13304	6QJ0_A
PARIS-ii	pUCCL620_249 PP556494.1	DUF4435	Hypothetical protein PH0156	TOPRIM domain of OLD nucleases	IPR029492	2P62_A
AbiGi	AAB38312.1	AbiGi	-	-	IPR021223	-
AbiGii	AAB38313.1	AbiGii	-	-	IPR031707	-
AbiH	CAA66252.1	AbiH	-	NAD-Dependent deacetylase sirtuin-3	IPR025935	-
AbiI	AAB66882.1	-	-	RNase HII	-	-

AbiN	CAA72648.1	-	-	Putative periplasmic ligand-binding sensor domain	-	-
AbiO	I61427.1 (714..2333 bp)	DNA/RNA helicase (SLFN_3-like)	Upf1 RNA helicase	DNA helicase II	IPR018647	2XZO_A
AbiRi	AAF75619.1	-	RNA polymerase sigma70 factor	RNA polymerase sigma70 factor	-	4LJZ_F
AbiRii	AAF75618.1	-	ParB	ParB	-	7BNR_B
AbiRiii	AAF75617.1	Phospholipase D-like and SNF2/RAD5-like	INO80 ATPase	INO80 ATPase	IPR025202 IPR000330 IPR049730	8EUF_Q
AbiTi	AAN60762.1	-	-	Csp3	-	-
AbiTii	AAN60763.1	AbiTii	-	-	IPR041304	-
AbiU	AAG17058.1	Abi	-	Restriction endonuclease	IPR018891	-
AbiZ	ABI93964.1	-	-	GTPase activating protein/kinase	-	-
Rhea	pUCCL617_141 PP556472.1	-	-	transposase TnsE	-	-
Aristaios	pUCCL641_199 PP589746.1	DUF2971	-	ADP-ribosyltransferase	IPR021352	-

Kamadhenu	pUCCL624_130 PP556517.1	YfbU	YfbU	YfbU	IPR005587	1WPB_F
Rugutis	pUCCL633_059 PP589699.1	SEC-C motif	SEC-C motif	SEC-C motif	IPR004027	2I9W_A
Hesat	pUCCL620_156 PP556493.1	-	-	colicin-D toxic domain	-	-
CBASS I-i	pUCCL631_167 PP589692.1	SLATT effector	-	SLATT effector	IPR040811	-
CBASS I-ii	pUCCL631_168 PP589692.1	NTase/AGS-C sensor	CD-NTase	CD-NTase	IPR006116 IPR040511	7LJO_A
CBASS II-i	pUCCL620_247 PP556494.1	SUa-2TM effector	-	Cap5 SAVED	IPR041502	-
CBASS II-i	pUCCL620_246 PP556494.1	NTase/SMODS	CD-NTase	CD-NTase	IPR006116 PF18144	7LJN_C
CBASS II-iii	pUCCL620_245 PP556494.1	-	Ubiquitin-conjugating enzyme E2 [a]	Cap2	-	7JZV_A
Lamassu-i	pUCCL630_136 PP589684.1	ABC-3C system CTD	Cap4 [a] endonuclease	Cap4 SAVED/CARF domain	IPR046920	7YIB_A
Lamassu-ii	pUCCL630_135 PP589684.1	ABC-3C system MC	-	CDI DNAse	IPR046905	-
Lamassu-iii	pUCCL630_134 PP589684.1	AAA_23 Rad50/SbcC ATPase	SMC protein	Rad50 ATPase	IPR038729 PF13476	7TVE_E
Septu-i	pUCCL618_274 PP556483.1	AAA_21 ATPase	ARE-ABCF protein	excinuclease ABC	IPR003959 PF13304	8BUU_9

Septu-ii	pUCCL618_273 PP556483.1	HNH endonuclease	Cas9 endonuclease	HNH endonuclease/GAF domain	IPR003615	7ENH_A

Table updated from Grafakou et al. (17); additions are presented in bold-face text. AbiS was not included in this analysis, as the gene responsible for the phage resistance has not been specified. The associated HHpred represents the highest probability hit (> 97%) with an E-value below 0.001 except for [a] (probability > 95%, 0.01 ≤ *E*-value ≤ 0.05). PDB (Protein Data Bank); HEPN (Higher Eukaryotes and Prokaryotes Nucleotide-binding domain); Cas (CRISPR associated); NTase (nucleotidyltransferase); AAA (ATPases associated with diverse cellular activities); SMC (structural maintenance of the chromosome); TOPRIM, (topoisomerase-primase); SLFN-3 (Schlafen group 3 proteins); Csp3 (Copper storage protein 3); DUF (domain of unknown function); PBP (penicillin binding protein); SLATT (SMODS [second messenger oligonucleotide or dinucleotide synthetases] and LOG-associating two TM [transmembrane]); AGS-C, (Adenylyl, Guanylyl & SMODS sensor C-terminal); CD-NTase (cGAS/DncV-like NTase; Sua-2TM (SMODS/Ubiquitin system-associated 2TM); Cap (CD-NTase-associated protein); SAVED (SMODS-associated and fused to various effector domains); ABC (ATP-binding cassette); CTD (C-terminal domains); CARF (CRISPR-associated Rossman fold); MC (Middle Component); CDI (contact-dependent growth inhibition); ARE-ABCF (antibiotic resistance ABC protein of the F subfamily); GAF (cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA)

1.8.1 Defence-associated reverse transcriptase (DRT) systems: AbiA and AbiK

A low level of amino acid sequence identity (23 %) was previously detected between AbiA and AbiK (9, 58). AbiA and AbiK share a reverse transcriptase (RT) catalytic domain (66, 100) (Figure 1), which superimposes well. These defence-associated RT (DRT) Abi-like systems have been classified under the unknown group (UG)/Abi RTs group, which also includes AbiP2 from *E. coli* and other predicted RTs whose antiphage activity and Abi mechanism has not (yet) been experimentally confirmed (101).

AbiA (originally Hsp) was the first discovered lactococcal Abi system (22), and is among those with the broadest antiphage activity spectrum (17, 36, 39). It was found to either interfere with or significantly reduce phage DNA replication (35, 39) (Figure 1). Recent crystallography studies revealed that AbiA contains three domains: the RT domain, a helical domain unique to Abi polymerases, and a C-terminal higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domain (100, 101). The RT polymerase and HEPN domain were both found to be essential for AbiA antiphage activity (100). While the HEPN domain was found to be enzymatically inactive, it was shown to be responsible for dimerisation of AbiA (100). Proteins containing a HEPN domain function as toxins and can be critical components of Abi/toxin-antitoxin systems in prokaryotes (102); see “Other HEPN-domain containing systems” paragraph).

The mode of action of AbiK appears to be phage species-dependent, interfering with the phage cycle during or prior to phage replication of P335 phages (58) or during the DNA packaging stage of skunaviruses (60) (Figure 1). AbiK was found to consist of two domains, a helical domain and an RT domain (66). Unlike AbiA, AbiK does not contain a HEPN domain and AbiK forms trimers instead of dimers (66, 100). The RT domain has previously been demonstrated to be essential for AbiK activity (62, 65). AbiK was found to synthesise DNA without a template, generating single stranded DNA sequences without any specificity (65). These DNA molecules remain covalently bound to AbiK. A role of the bound DNA in AbiK trimer formation has been proposed, but further investigation is required (66). Nevertheless, the synthesis of random DNA could lead to nucleotide depletion, potentially interfering with phage replication or other essential host processes, which in turn would explain the abortive infection phenotype.Interestingly, phage escape mutants of both AbiA and AbiK had mutations in genes involved in DNA repair or recombination or in single-strand annealing proteins (SSAPs) (37, 59, 61, 63, Chapter IV of this thesis), which further highlights the parallels between these two system. Genes mutated in AbiK escape mutants are structurally diverse SSAPs and have been called sensitivity to AbiK (*sak*) genes (61, 64). Notably, phage escape mutants of AbiA with mutations in SSAP- and Holliday junction resolvase-encoding genes were also found to be insensitive to AbiJ. This suggests a mechanistic similarity between AbiA and AbiJ despite the lack of structural resemblance (Chapter IV of this thesis).

1.8.2 Other HEPN-domain containing systems: AbiB, AbiJ and AbiV

While our functional analysis led to no predicted domains for AbiB and AbiJ based on InterPro and HHpred, AbiV contains a higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domain based on InterPro analysis. Interestingly, AlphaFold2 and Dali analysis revealed the presence of a HEPN domain in all three Abi proteins. HEPN domain-containing proteins function as toxins and can be essential toxin-antitoxin/Abi components in prokaryotes (102).

Toxin-antitoxin (TA) systems generally consist of two components, a toxin that suppresses cellular growth and an antitoxin that neutralizes the toxin's effects (103). The inactivation of the antitoxin and the subsequent activation of the toxin after phage infection can lead to abortive infection (6). Based on the nature of the TA system components and their interaction, there are eight different types of TA system (I–VIII) (104).

Interestingly, the function of the AbiV HEPN domain-containing protein was recently confirmed and was described to function as a type III TA system (Figure 1). The AbiV toxin is an RNase, which belongs to the HEPN superfamily and degrades ribosomal RNA (105). Its antitoxin is an RNA molecule that inhibits the RNase activity of the toxin (105). Since ribosomal RNAs are essential for cellular protein synthesis, degradation of host rRNAs would abort infection (105), This is consistent with previous findings that AbiV inhibits protein synthesis (83).

The activation of AbiB, upon phage infection, leads to mRNA degradation, thereby causing the arrest of phage development (41).

Although, no additional mechanistic information is currently available for AbiJ, the presence of the HEPN domain suggests that both AbiB and AbiJ likely function as TA Abi systems, with AbiB likely operating through a mechanism similar to AbiV (i.e. RNase activity). Notably, the major capsid protein of *Skunavirus* sv66901 was recently found to trigger AbiB toxicity (Chapter IV of this thesis).

1.8.3 Sequence and structural similarity of AbiC and AbiP

Amino acid sequence alignment of AbiC and AbiP reveals 24 % identity with a 57 % coverage of AbiC, indicating a relatively modest level of similarity. Based on AlphaFold2/Dali analysis, AbiC and AbiP exhibit similarity to Farnesyl pyrophosphate synthase (FPPS; Table 2). The extra-membrane (or ecto-domain) regions of AbiC and AbiP superimpose well; however, AbiC possess two transmembrane helices, whereas AbiP contains only one. This structural similarity justifies grouping these previously distinct systems into a single Abi group, henceforth referred to as AbiC/P. In bacteria, FPPS is involved in cell wall synthesis and cellular respiration (106). Although, to our knowledge, this domain has not previously been associated with phage defence and its role in bacterial phage immunity remains unclear, the structure predictions and superimpositions underscore the potential functional and evolutionary relationship between AbiC and AbiP.

Interestingly, AbiC and AbiP exhibit activity against the same phage groups (Table 1). Despite these similarities, their mechanisms seem to

differ. According to Durmaz et al., AbiC does not affect DNA replication (42), unlike AbiP (70), while AbiC has been shown to partially inhibit major capsid protein production of phage of the P335 group (43).

AbiP has been shown to contain a sequence-independent nucleic acid binding domain, with preferential binding to RNA over ssDNA, and an N-terminal transmembrane domain (both apparently essential for its Abi phenotype; (107)). Furthermore, the activity of AbiP has been demonstrated to result in higher levels of the early phage transcripts compared to the reference strain, interfering with their “switch off” which occurs 15 min post infection in the reference strain and also to result in lower levels of the middle and late phage transcripts (70).

AbiP has been shown to lack specificity for phage-originating nucleic acids, implying that it likely binds non-specifically to cellular nucleic acids (107). Nevertheless, AbiP was not toxic to the host cell, leading to the hypothesis that its activation requires a phage protein (107). This hypothesis is supported by recent findings (Chapter IV of this thesis), which demonstrated that the major tail protein of *Skunavirus* sk713 can trigger AbiP toxicity (Figure 1). This suggests that AbiP can abort infection by binding cellular nucleic acids once activated by a phage component.

1.8.4 Sequence and structural similarity of AbiD, AbiD1, AbiF and AbiD/F

Sequence similarity had previously been reported between AbiD, AbiD1 and AbiF (28-46 %) (9, 51). Antiphage system AbiD/F (previously called

AbiD-like) was later identified and was shown to share 59 % amino acid identity with AbiD (17). In this same study, structural analysis of AbiD, AbiD1, AbiF and AbiD/F revealed the high similarity of their protein structures and a resemblance to the Cas13d endonuclease domain, suggesting the classification of these systems into a single Abi group called AbiD/F (17).

AbiD, AbiD1 and AbiF were discovered within a very short time frame. It was determined that AbiD acts after phage DNA replication (44). Furthermore, it was demonstrated that transcription of the *abiD1* gene is very weak (46), although the presence of a strong promoter and adjacent terminator directly upstream of the gene was responsible for a short transcript which was considered to contribute to the antiphage effect (46). Recently it was determined that sequence identity upstream of the AbiD/F member genes (68-80 %) was higher than sequence identity between the genes themselves (46-65 %) (17). In addition, it was confirmed that this upstream sequence is essential for antiphage effect for AbiD/F and AbiF (17). Therefore, members of the AbiD/F group appear to consist of two component systems that comprise a derivative of CRISPR-Cas RNA-guided nuclease Cas13d and a short RNA molecule.

Phage escape mutants of AbiD1 and AbiD/F possess mutations in genes encoding hypothetical proteins (47, Chapter IV of this thesis). Spontaneous bIL66 escape mutants of AbiD1 with a single mutation in a gene encoding a hypothetical protein within the so-called “middle-expressed” region assumed to be associated with transcriptional regulation, provided partial resistance to AbiD1. The overexpression of another gene in the same

operon, which interestingly encodes a RuvC-like endonuclease (48), resulted in increased resistance of bIL66 against AbiD1 (47). Therefore, AbiD1 was proposed to be involved in degradation of the phage RuvC-like endonuclease upon activation by the hypothetical protein (48, 49). The hypothetical protein was found to activate translation of AbiD1. In contrast, the escape mutant version (same hypothetical protein with a single amino acid difference) was not found to promote AbiD1 translation, even though both the wild type and the escape mutant hypothetical protein were found to bind to AbiD1 mRNA (50). As for AbiD1, AbiD/F was found to be activated by two structurally unrelated proteins of unknown function (Chapter IV of this thesis) (Figure 1).

AbiF was found to interfere with the replication of phage 712 DNA (51). Furthermore, it was determined that chromosomally-encoded RecA was required for AbiF activity, a protein known to be involved in homologous recombination and the SOS response to DNA damage (108). The *cos* site of phage sk1 was found to provide resistance against the action of AbiF in phage 712, a phage normally sensitive to AbiF. This indicates that in the case of skunaviruses, the activity of AbiF involves the *cos* ends (52).

CRISPR-Cas systems are rarely found in *L. lactis* and *L. cremoris* (18, 109). Nevertheless, the AbiD/F group represents lactococcal antiphage systems reminiscent of a class 2 CRISPR-Cas system. AbiD1 and AbiD/F could only be introduced with their native signals (upstream sequence) due to toxicity issues (17, 46). Similarly, class 2 CRISPR-Cas systems are toxic at high expression (110). The toxicity of a class 2 CRISPR-Cas effector protein possibly explains not only why lactococcal strains are typically

devoid of CRISPR-Cas systems, but also their involvement in abortive infection.

1.8.5 TOPRIM/OLD family nuclease and Rad50/SMC family ATPase domain containing systems: PARIS and AbiL

The first component of AbiL (AbiLi) shares limited (34 %) amino acid similarity to the first component of PARIS (PARIS-i, 18 % query coverage) described in *Lactococcus* (17) and originally identified in *E. coli* (92). Based on previous analyses, PARIS-i resembles a Rad50 ATPase of the structural maintenance of chromosomes (SMC) family protein (17, 111). Here, AbiLi is predicted to contain a Rad50 ATPase domain as well (Table 2), and structural analysis reveals that the central β-barrel and α-helices of AbiLi and PARIS-i superimpose well, while differences in their structures are observed in the extensions, specifically between residues 250-323 and 195-319, respectively.

No significant sequence similarity was found between the second components of AbiL and PARIS (AbiLii and PARIS-ii, respectively). PARIS-ii contains a topoisomerase-primase (TOPRIM) domain of OLD family nucleases (17, 92, 111). Interestingly, despite the lack of sequence similarity between AbiLii and PARIS-ii, AbiLii is here predicted to contain the same domain as PARIS-ii based on AlphaFold2/Dali analysis (Table 2), however structure predictions showed only some weak superposition between AbiLii and PARIS-ii.

It was recently described that PARIS functions as a type II TA system in *E. coli* (111) in which expression of PARIS-ii (or AriB; effector) results in bacterial toxicity, while through protein-protein interactions, PARIS-i (or AriA; infection sensor) acts as an antitoxin. During phage infection, the phage-encoded anti-restriction protein (Ocr) binds to PARIS-i (AriA; sensor and anti-toxin) which undergoes a conformational change. This causes release of the PARIS-ii RNase (AriB; effector/toxin), which aborts infection by inhibiting protein translation through cleavage of a lysine tRNA (111) (Figure 1). However, phages can bypass PARIS activity by expressing lysine tRNA variants that are not cleaved by PARIS (112).

Interestingly, to overcome the activity of PARIS TA Abi system, lactococcal phages -where anti-restriction proteins have not been identified- exhibit mutations in a hypothetical protein (Chapter IV of this thesis). Beyond possessing a highly negatively charged cluster, this hypothetical protein bears no sequence or structural resemblance to Ocr (Chapter IV of this thesis), which was shown to activate PARIS Abi system in *E. coli* (92, 111). However, this hypothetical protein, at least on its own, has not been found to activate PARIS in *Lactococcus* (Chapter IV of this thesis).

Based on the modest structural similarity between AbiLii and PARIS-ii, it is possible that AbiL and PARIS share a similar sensor, but a different effector. Therefore, as previously suggested (111), further work will be needed to determine if all ATPase + TOPRIM/OLD phage defence systems, such as the lactococcal AbiL, the *Campylobacter* RloAB (113) and the unnamed two-gene system identified in *Nostoc* sp. (114), function via the same mechanism.

1.8.6 Other toxin-antitoxin systems: AbiQ and AbiE

In addition to AbiV and PARIS TA abortive infection systems (see above), some other lactococcal Abi systems, such as AbiE (51, 115)) and ToxIN/AbiQ (116, 117) are believed to function via a TA mechanism (Figure 1).

Interestingly, AbiE (and in particular the AbiEii component of this two-component system) is 4th most frequent system in prokaryotic genomes (20, 118). AbiE functions as a bacteriostatic, non-interacting type IV toxin-antitoxin (TA) system (115). In *Streptococcus agalactiae*, AbiEi antitoxin, which consists of an N-terminal DNA binding domain and C-terminal antitoxin domain, negatively autoregulates *abiE* expression by binding to inverted repeats within the promoter, thereby repressing its transcription (115, 118, 119). AbiEii toxin is a predicted DNA polymerase β-nucleotidyltransferase, that specifically binds GTP (115). Notably, the nucleotide specificity of AbiEii can vary among homologues; for instance, the activity of the *Mycobacterium tuberculosis* MenT3 toxin (AbiEii homologue) involves pyrimidines instead (120). Nevertheless, evidence suggests that AbiEii toxins transfers nucleotide(s) to tRNA(s), impairing translation (120–122). Additionally, AbiE not only provides phage resistance but also contributes to plasmid stability (115), as has previously been shown for other TA systems (123).

AbiQ is homologous to ToxIN Abi system found in *Pectobacterium atrosepticum* (formely *Erwinia carotovora* subspecies *atroseptica*) (72, 116). Both AbiQ and ToxIN are type III protein-RNA TA systems, with AbiQ being

the first TA system described and structurally characterized in *Lactococcus* (117, 124). AbiQ/ToxN are endoribonucleases (117, 124). The non-coding RNA antitoxin ToxI (ToxN inhibitor) inhibits toxicity by directly binding to the ToxN toxin (116, 124). Additionally, the *toxIN* operon is negatively autoregulated (125). AbiQ/ToxN are activated upon phage infection resulting in abortive infection (117, 124, 126). ToxN is activated by phage-induced host transcriptional inhibition in *E. coli*, leading to loss of the ToxI antitoxin and to the cleavage of the viral mRNAs by ToxN toxin (127). AbiQ activation affects phage transcription and results in the accumulation of nonmature forms of viral DNA (72, 73). Phages can escape ToxIN via RNA antitoxin mimics that inhibit ToxN toxicity (128).

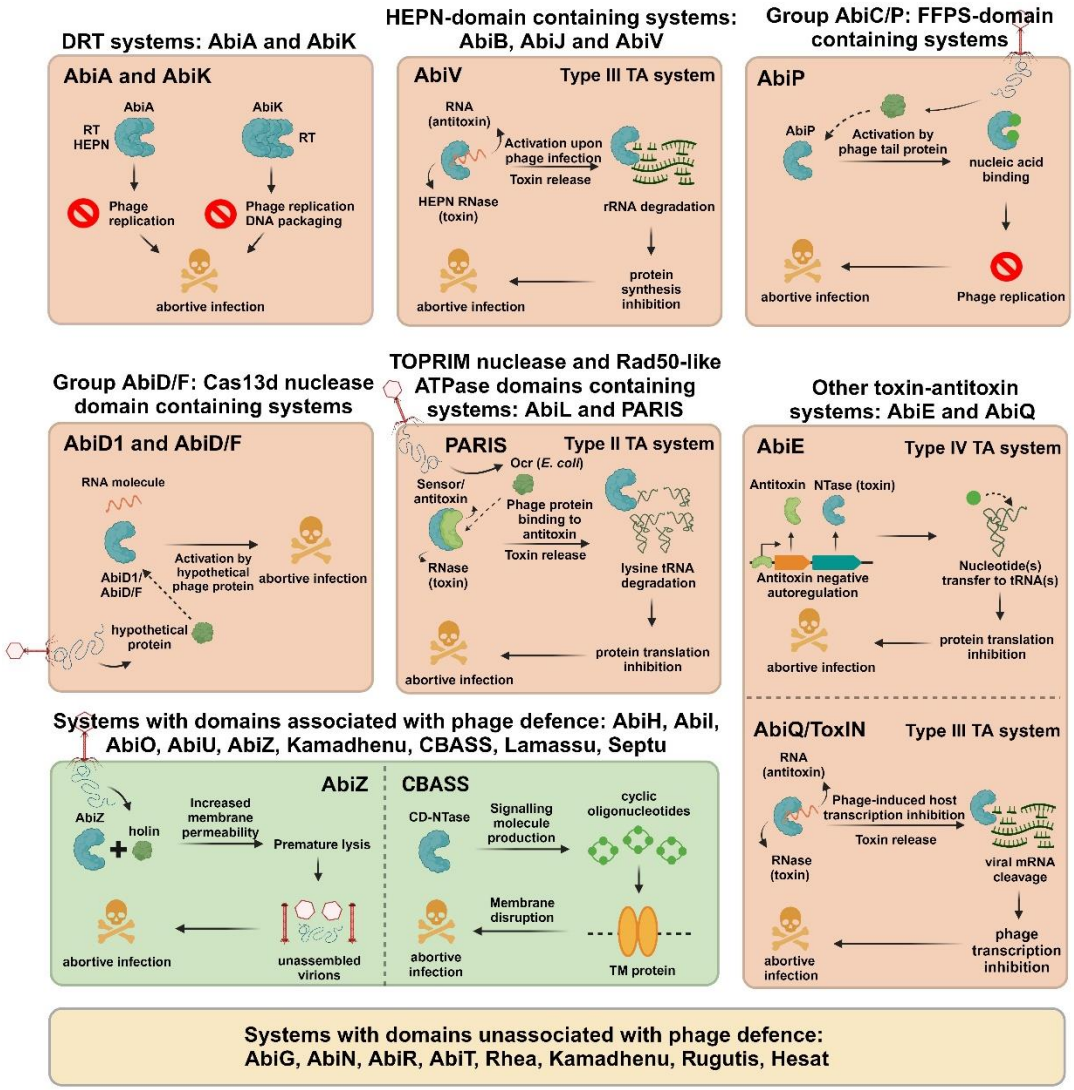


Figure 1. Summary of the domains, groups and mechanisms of lactoccocal Abi-like systems

1.8.7 Systems with domains associated with phage defence

Based on Alphafold2/Dali analysis AbiH resembles sirtuin 3 (SIRT3; Table 2). Sirtuins (SIRTs) are homologues of the Sir2 protein (silent information regulator 2) found in *Saccharomyces cerevisiae* and comprise nicotinamide adenine dinucleotide (NAD^+)-dependent enzymes typically functioning as deacetylases or ADP-ribosyltransferases (129–131). They are present in prokaryotic and eukaryotic organisms, with seven mammalian

homologues of Sir2 identified (SIRT1 to SIRT7) (130, 132). Bacterial sirtuins are involved in transcription, protein translation, carbon and nitrogen metabolism, and virulence (131). Recently, Sir2 domains have been shown to be associated with multiple bacterial phage defence systems, such as the prokaryotic argonautes (pAgo), Thoeris, AVAST, defence-associated sirtuin (DSR) and others (96). Several systems, such as Thoeris, DSR2, SIR2/pAgo and SIR2-HerA, have been shown to be responsible for depleting NAD+ once phage infection has been sensed thus aborting infection by depleting this essential electron transfer molecule from the cell (96, 133). Phage defence involving NAD+ depletion has been shown to eventually result in cell death, consistent with the concept of Abi systems (133–135). Notably, NADase activity of the DSR2 Abi system (DSRs family) has been shown to be activated by a phage tail tube protein (96). It is therefore possible that AbiH acts via a similar NAD-depleting pathway.

Based on Alphafold2/Dali analysis, AbiI contains a RNase HII domain (Table 2). RNases H can be found in all domains of life including viruses and cleave RNAs in RNA/DNA hybrids (136). In prokaryotes, RNases HI are RNases H with major activity, while RNases HII are related with minor activities (136). RNase H-like activity has previously been exhibited for CRISPR-Cas RNA-guided nuclease Cas13a (137). As with the AbiD/F group (CRISPR-Cas RNA-guided nuclease Cas13d), the presence of such domain in AbiI would be in accordance with the observed abortive infection phenotype (56).

AbiO contains a helicase domain based on functional and structural analysis. Helicases are known to be involved in antiviral immunity, either as

sensors or as effectors (138). At least 18 bacterial phage defence systems (e.g. Hachiman, Hna, DISARM, Gajiba, Zorya, BREX) are known to contain helicase domains, which have subsequently been grouped into seven subfamilies (139). For example, Hachiman in *E.coli*, is a two-component Abi antiphage system where the sensor is a DNA helicase (HamB) and the effector is a nuclease (HamA) activated upon DNA damage (139). Similarly, the activation of the Hna single-component antiphage system in *Sinorhizobium meliloti*, consists of a helicase and a nuclease domain, resulting in abortive infection (Hna; (140)). AbiO was previously shown to be toxic in *Lactococcus* when cloned in a high-copy number vector (69), supporting the abortive infection phenotype assigned to this phage defence system. Notably, based on InterPro analysis, AbiO contains a helicase domain found in group 3 of mammalian Schlafen proteins (SLFN_3-like; Table 2), which are associated with cell proliferation regulation, immune responses activation, and viral replication control (141), while based on HHpred analysis, AbiO resembles Upf1 helicase, which is involved in nonsense-mediated mRNA decay (142). Further studies are needed to determine whether AbiO has a second, computationally unpredicted domain that could function as the effector while the helicase acts as the sensor, or if the AbiO helicase itself has a dual role.

AbiU was initially reported to be a two-component antiphage system, shown to delay phage transcription (80). As the expression of AbiUi was sufficient to confer phage resistance, while the expression of AbiUii in addition to AbiUi resulted in lower antiphage activity (80), AbiU hereafter will be referred to as a single component antiphage system consisting of AbiUi.

Interestingly, AbiUii exhibits sequence similarity to AbiGii (31 % similarity; 19 % AbiGii query coverage) and has been predicted to contain a HEPN domain (80, 102). Here, based on AlphaFold2 analysis, AbiUi resembles a FokI restriction endonuclease, which is known to recognize a specific DNA sequence, but cleaves the DNA at a non-specific site located a short distance from that sequence (143).

AbiZ likely acts cooperatively with the phage holin to increase membrane permeability, leading to premature lysis of the infected cells (23) (Figure 1). This early cell lysis results in a reduced phage burst size, as more unassembled virions are released (23). Here, functional domain predictions for AbiZ reveal similarities with both a GTPase activating enzyme and a kinase. Kinases transfer phosphate groups between substrates, often regulating their structure and function (144), which may help to explain the previously suggested early activation of holin by AbiZ (23). While serine/threonine kinases are crucial for antiviral responses in eukaryotes, bacteria were not commonly known to use protein phosphorylation for phage defence until the discovery of a staphylococcal serine/threonine kinase (Stk2) that functions via abortive infection (90). Finally, since GTPases can be involved in membrane signalling pathways mainly in eukaryotes but also in prokaryotes (145, 146), the presence of a GTPase-activating enzyme domain in AbiZ may explain the observed increase in membrane permeability. However, both hypotheses related to these domains would require further investigation.

Kamadhenu is an Abi-like system recently described to resemble an ADP-ribosyltransferase (17). ADP-ribosyltransferases have been

associated with phage defence previously and are toxins known to inhibit essential host protein functions (147, 148), which is consistent with observations that Aristaios interferes with phage transcription (33).

Recently, several Abi-like systems have been identified in *L. lactis/cremoris* (i.e. CBASS type I, CBASS type II, Lamassu and Septu) that have been associated with phage defence in other species and whose domains have previously analysed extensively (17).

1.8.8 Systems with domains lacking phage defence associations

The structure of AbiN is predicted with high accuracy by AlphaFold2, though no significant matches were found in the PDB, except for a putative periplasmic ligand-binding sensor domain protein. Periplasmic binding proteins function as bacterial receptors that sense small molecules and undergo conformational changes upon ligand binding (149). This suggests that such a domain serves as the sensor in an Abi-like system, though further research is needed to clarify the mechanism of AbiN and if it functions via abortive infection.

AbiR was initially described to be encoded by two genetic loci, separated by the LlaKR2I RM system, and was found to interfere with phage DNA replication (75). AbiR consists of three genes and its function requires the methylase from the LlaKR2I RM system (76). Notably, in the absence of the LlaKR2I methylase, AbiR was toxic to *L. lactis* (76).

Based on HHpred and Alphafold2/Dali analysis, AbiRi (or AbiRa) resembles an RNA polymerase sigma70 factor, AbiRii (or AbiRb) resembles

partition system ParB and AbiRiii (or AbiRc) resembles a nucleosome-bound INO80 ATPase (Table 2), with a reliable complex being predicted between these three components. Sigma70 factors are involved not only in directing general transcription but also in activating specific genes in response to stress or other development and ancillary metabolism signals (150). Therefore, such a protein domain may act as the sensor of the system. ParB is involved in chromosome and low-copy number plasmid segregation during bacterial cell division (151). It is known to bind DNA and proteins such as the nucleoside triphosphatase ParA (151, 152). Interestingly, the ParB encoded by the RK2 plasmid exhibits nuclease activity (153), suggesting that this domain likely functions as the effector in the AbiR antiphage system. INO80 is a SNF2 family chromatin-remodelling complex with a core ATPase and has been shown to be involved in regulating transcription, DNA replication and repair (154, 155). Despite the possible interaction of the predicted AbiRii and AbiRiii domains with DNA or their involvement in DNA replication -a process known to be affected by AbiR activity- it remains unclear how the AbiR components interact to confer phage resistance.

Interestingly, AbiTi was found to contain a copper storage protein (Csp3) domain, initially identified in methanotrophs. Csps are involved in intracellular copper storage, which can be crucial for enzymatic reactions but can become cytotoxic at elevated levels (156). Csp3 homologues are widespread across diverse bacteria (156), and the presence of this domain in an Abi-like system suggests a novel mechanism for inducing (metal) cell toxicity upon infection. However, confirmation of this notion and *in silico*

prediction would require further studies. Notably, although the lactococcal AbiTii protein lacks a HEPN domain, its homologs in various bacteria are found to have two distinct HEPN domains at their C-terminal end (102).

Functional and structural analysis fail to predict any similarity with proteins of known function for some systems such as AbiG (Table 2), which was recently described to be activated by a phage major tail protein (Chapter IV of this thesis). The domains present in the recently identified Rhea (transposase TnsE), Kamadhenu (YfbU), Rugutis (SEC-C motif) and Hesat (colicin-D) could not be linked to other antiphage defence functions and have been described extensively elsewhere (17).

1.9 Conclusion

The recent discovery of lactococcal plasmid-encoded antiphage systems (17) has significantly increased the number of experimentally confirmed Abi-like systems in *Lactococcus*, from 23 to 34. These systems appear to be highly diverse, as evidenced by the range of phages they can target, the specific steps of the phage lytic cycle they interfere with, and the mutations that phages must acquire to overcome each system (Table 2). This diversity confirms that the co-evolution of these bacteria and their phages has driven the selection and exchange of a wide array of phage defence mechanisms (9).

The substantial expansion of the lactococcal Abi-like system repertoire, the development of strategies to uncover novel defence systems (26, 157) and the characterization of some of these systems have significantly

advanced our understanding of pan-bacterial immunity (4). These advancements hold the promise of discovering even more defence systems, whether they are unique to *Lactococcus* or widespread across different bacterial genera or species.

Most lactococcal Abi systems have been classified as such based on limited phenotypic observations associated with these systems. Direct evidence that the activity of the system upon recognition of phage infection results in cell death is often absent. Therefore, it is more appropriate to refer to them as Abi-like until further characterization proves their function as Abi systems.

Of the lactococcal Abi-like defence systems, the mechanisms of action of only a few have been extensively characterized as Abi: AbiV (105), AbiQ (117), AbiE (115, 120, 121), PARIS (111, 158), and CBASS (158). These are primarily identified as toxin-antitoxin (TA) Abi systems, with the exception of CBASS. Notably, apart from AbiV and AbiQ, the modes of action for these systems have been described in bacteria other than *Lactococcus*. Therefore, it is intriguing whether future studies will determine if the mechanisms of a given system are consistent across different bacterial species.

Additionally, the necessary components responsible for the antiphage activity of each identified system require further investigation, particularly for initially described systems (AbiA to AbiZ) (10, 11). For example, the component(s) responsible for AbiS activity remain(s) unknown (77), while

only recent studies have revealed that AbiV requires an RNA component for its activity (105), in addition to the previously known AbiV protein (81).

Despite the diversity of Abi-like antiphage systems confirmed in *Lactococcus*, some systems share sequence and structural similarities, allowing classification under the same group (i.e. DRT group for AbiA and AbiK, AbiC/P group, AbiD/F group). However, these similarities and the categorization under the same group do not automatically imply that they are mechanistically identical. This suggests that lactococci have evolved paralogous genes to enlarge the already substantial variety of available phage resistance mechanisms.

Furthermore, despite the lack of sequence similarity, some systems share common domains. For example, some systems contain a HEPN domain (AbiB, AbiV, AbiJ, AbiA, AbiTii in other bacteria than *Lactococcus*) while others a TOPRIM/OLD family nuclease and a Rad50/SMC family ATPase domain (PARIS and AbiL). In addition, some Abi-like systems are predicted to resemble proteins or contain domains commonly associated with phage defence, such as Sir2 (AbiH), helicase (AbiO), kinases (AbiZ), and nucleases (AbiI and AbiU). However, some systems either show similarities to proteins not previously linked to phage defence (AbiN, AbiR, AbiT) or have no clear similarity to known proteins (AbiG). These findings suggest that while certain functions are common in phage defence, there are also unique or specialized systems that might represent different mechanisms to combat phage infections.

Nevertheless, future studies on lactococcal and non-lactococcal Abi-like antiphage systems, along with other phage defence mechanisms, will continue to illuminate the landscape of bacterial phage immunity. This knowledge not only advances our understanding but can also guide the development of more robust phage-resistant starter cultures and has the potential to be applicable in other contexts.

1.10 References

1. Li,T.T., Tian,W.L. and Gu,C.T. (2019) Elevation of *Lactococcus lactis* subsp. *cremoris* to the species level as *Lactococcus cremoris* sp. nov. and transfer of *Lactococcus lactis* subsp. *tructae* to *Lactococcus cremoris* as *Lactococcus cremoris* subsp. *tructae* comb. nov. *Int J Syst Evol Microbiol*, 71.

2. Makarova,K., Slesarev,A., Wolf,Y., Sorokin,A., Mirkin,B., Koonin,E., Pavlov,A., Pavlova,N., Karamychev,V., Polouchine,N., *et al.* (2006) Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences*, 103, 15611–15616.

3. Naureen,Z., Dautaj,A., Anpilogov,K., Camilleri,G., Dhuli,K., Tanzi,B., Maltese,P.E., Cristofoli,F., De Antoni,L., Beccari,T., *et al.* (2020) Bacteriophages presence in nature and their role in the natural selection of bacterial populations. *Acta Biomed*, 91, e2020024.

4. Georjon,H. and Bernheim,A. (2023) The highly diverse antiphage defence systems of bacteria. *Nat Rev Microbiol*, 21, 686–700.

5. Hayes,S., Murphy,J., Mahony,J., Lugli,G.A., Ventura,M., Noben,J.P., Franz,C.M.A.P., Neve,H., Nauta,A. and van Sinderen,D. (2017) Biocidal inactivation of *Lactococcus lactis* bacteriophages: Efficacy and targets of commonly used sanitizers. *Front Microbiol*, 8, 107.

6. Labrie,S.J., Samson,J.E. and Moineau,S. (2010) Bacteriophage resistance mechanisms. *Nat Rev Microbiol*, 8, 317–327.

7. Lopatina,A., Tal,N. and Sorek,R. (2020) Abortive infection: Bacterial suicide as an antiviral immune strategy. *Annu Rev Virol*, 7, 371–384.

8. Ortiz Charneco,G., de Waal,P.P., van Rijswijck,I.M.H., van Peij,N.N.M.E., van Sinderen,D. and Mahony,J. (2023) Bacteriophages in the dairy industry: A problem solved? *Annu Rev Food Sci Technol*, 14, 367–385.

9. Chopin,M.C., Chopin,A. and Bidnenko,E. (2005) Phage abortive infection in lactococci: Variations on a theme. *Curr Opin Microbiol*, 8, 473–479.

10. Ainsworth,S., Stockdale,S., Bottacini,F., Mahony,J. and van Sinderen,D. (2014) The *Lactococcus lactis* plasmidome: much learnt, yet still lots to discover. *FEMS Microbiol Rev*, 38, 1066–1088.

11. Philippe,C., Cornuault,J.K., de Melo,A.G., Morin-Pelchat,R., Jolicoeur,A.P. and Moineau,S. (2023) The never-ending battle between lactic acid bacteria and their phages. *FEMS Microbiol Rev*, 47.

12. Nyengaard,N., Vogensen,F.K. and Josephsen,J. (1995) Restriction-modification systems in *Lactococcus lactis*. *Gene*, 157, 13–18.

13. Schouler,C., Clier,F., Lerayer,A.L., Ehrlich,S.D. and Chopin,M.C. (1998) A type IC restriction-modification system in *Lactococcus lactis*. *J Bacteriol*, 180, 407–11.

14. Su,P., Im,H., Hsieh,H., Kang'A,S. and Dunn,N.W. (1999) *Lla*FI, a type III restriction and modification system in *Lactococcus lactis*. *Appl Environ Microbiol*, 65, 686–93.

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

16. Mahony,J., McGrath,S., Fitzgerald,G.F. and van Sinderen,D. (2008) Identification and characterization of lactococcal-prophage-carried superinfection exclusion genes. *Appl Environ Microbiol*, 74, 6206–6215.

17. Grafakou,A., Mosterd,C., Beck,M.H., Kelleher,P., McDonnell,B., de Waal,P.P., van Rijswijck,I.M.H., van Peij,N.N.M.E., Cambillau,C., Mahony,J., *et al.* (2024) Discovery of antiphage systems in the lactococcal plasmidome. *Nucleic Acids Res*, 52, 9760–9776.

18. Millen,A.M., Horvath,P., Boyaval,P. and Romero,D.A. (2012) Mobile CRISPR/Cas-mediated bacteriophage resistance in *Lactococcus lactis*. *PLoS One*, 7, e51663.

19. Kelleher,P., Mahony,J., Bottacini,F., Lugli,G.A., Ventura,M. and van Sinderen,D. (2019) The *Lactococcus lactis* Pan-Plasmidome. *Front Microbiol*, 10.

20. Tesson,F., Hervé,A., Mordret,E., Touchon,M., d'Humières,C., Cury,J. and Bernheim,A. (2022) Systematic and quantitative view of the antiviral arsenal of prokaryotes. *Nat Commun*, 13, 2561.

21. Aframian,N. and Eldar,A. (2023) Abortive infection antiphage defense systems: separating mechanism and phenotype. *Trends Microbiol*, 31, 1003–1012.

22. Hill,C., Miller,L.A. and Klaenhammer,T.R. (1990) Nucleotide sequence and distribution of the pTR2030 resistance determinant (*hsp*) which aborts bacteriophage infection in lactococci. *Appl Environ Microbiol*, 56, 2255–2258.

23. Durmaz,E. and Klaenhammer,T.R. (2007) Abortive phage resistance mechanism AbiZ speeds the lysis clock to cause premature lysis of phage-infected *Lactococcus lactis*. *J Bacteriol*, 189, 1417–1425.

24. Doron,S., Melamed,S., Ofir,G., Leavitt,A., Lopatina,A., Keren,M., Amitai,G. and Sorek,R. (2018) Systematic discovery of antiphage defense systems in the microbial pangenome. *Science (1979)*, 359.

25. Gao,L., Altae-Tran,H., Böhning,F., Makarova,K.S., Segel,M., Schmid-Burgk,J.L., Koob,J., Wolf,Y.I., Koonin,E. V. and Zhang,F. (2020) Diverse enzymatic activities mediate antiviral immunity in prokaryotes. *Science (1979)*, 369, 1077–1084.

26. Millman,A., Melamed,S., Leavitt,A., Doron,S., Bernheim,A., Hör,J., Garb,J., Bechon,N., Brandis,A., Lopatina,A., *et al.* (2022) An expanded arsenal of immune systems that protect bacteria from phages. *Cell Host Microbe*, 30, 1556-1569.e5.

27. Cavanagh,D., Fitzgerald,G.F. and McAuliffe,O. (2015) From field to fermentation: The origins of *Lactococcus lactis* and its domestication to the dairy environment. *Food Microbiol*, 47, 45–61.

28. Mills,S., O'sullivan,O., Hill,C., Fitzgerald,G. and Ross,R.P. (2010) The changing face of dairy starter culture research: From genomics to economics. *Int J Dairy Technol*, 63, 149–170.

29. Mills,S., McAuliffe,O.E., Coffey,A., Fitzgerald,G.F. and Ross,R.P. (2006) Plasmids of lactococci-genetic accessories or genetic necessities? *FEMS Microbiol Rev*, 30, 243–273.

30. Derkx,P.M., Janzen,T., Sørensen,K.I., Christensen,J.E., Stuer-Lauridsen,B. and Johansen,E. (2014) The art of strain improvement of industrial lactic acid bacteria without the use of recombinant DNA technology. *Microb Cell Fact*, 13, S5.

31. Kohler,V., Keller,W. and Grohmann,E. (2019) Regulation of gram-positive conjugation. *Front Microbiol*, 10, 1134.

32. Ortiz Charneco,G., Kelleher,P., Buivydas,A., Streekstra,H., van Themaat,E.V.L., de Waal,P.P., Mahony,J. and van Sinderen,D. (2021) Genetic dissection of a prevalent plasmid-encoded conjugation system in *Lactococcus lactis*. *Front Microbiol*, 12, 680920.

33. Grafakou,A., Mosterd,C., de Waal,P.P., van Rijswijck,I.M.H., van Peij,N.N.M.E., Mahony,J. and van Sinderen,D. (2024) Functional and practical insights into three lactococcal antiphage systems. *Appl Environ Microbiol*, 90, e0112024.

34. Goldfarb,T., Sberro,H., Weinstock,E., Cohen,O., Doron,S., Charpak-Amikam,Y., Afik,S., Ofir,G. and Sorek,R. (2015) BREX is a novel phage resistance system widespread in microbial genomes. *EMBO J*, 34, 169–183.

35. Hill,C., Massey,I.J. and Klaenhammer,T.R. (1991) Rapid method to characterize lactococcal bacteriophage genomes. *Appl Environ Microbiol*, 57, 283–288.

36. Dinsmore,P.K. and Klaenhammer,T.R. (1994) Phenotypic consequences of altering the copy number of *abiA*, a gene responsible for aborting bacteriophage infections in *Lactococcus lactis*. *Appl Environ Microbiol*, 60, 1129–1136.

37. Dinsmore,P.K. and Klaenhammer,T.R. (1997) Molecular characterization of a genomic region in a *Lactococcus* bacteriophage that is involved in its sensitivity to the phage defense mechanism AbiA. *J Bacteriol*, 179, 2949–2957.

38. Dinsmore,P.K., O'Sullivan,D.J. and Klaenhammer,T.R. (1998) A leucine repeat motif in AbiA is required for resistance of *Lactococcus lactis* to phages representing three species. *Gene*, 212, 5–11.

39. Tangney,M. and Fitzgerald,G.F. (2002) Effectiveness of the lactococcal abortive infection systems AbiA, AbiE, AbiF and AbiG against P335 type phages. *FEMS Microbiol Lett*, 210, 67–72.

40. Cluzel,P.J., Chopin,A., Ehrlich,S.D. and Chopin,M.C. (1991) Phage abortive infection mechanism from *Lactococcus lactis* subsp. *lactis*, expression of which is mediated by an Iso-IS*S1* element. *Appl Environ Microbiol*, 57, 3547–3551.

41. Parreira,R., Ehrlich,S.D. and Chopin,M.C. (1996) Dramatic decay of phage transcripts in lactococcal cells carrying the abortive infection determinant AbiB. *Mol Microbiol*, 19, 221–230.

42. Durmaz,E., Higgins,D.L. and Klaenhammer,T.R. (1992) Molecular characterization of a second abortive phage resistance gene present in *Lactococcus lactis* subsp. *lactis* ME2. *J Bacteriol*, 174, 7463–7469.

43. Moineau,S., Durmaz,E., Pandian,S. and Klaenhammer,T.R. (1993) Differentiation of two abortive mechanisms by using monoclonal antibodies directed toward lactococcal bacteriophage capsid proteins. *Appl Environ Microbiol*, 59, 208–212.

44. McLandsborough,L.A., Kolaetis,K.M., Requena,T. and McKay,L.L. (1995) Cloning and characterization of the abortive infection genetic determinant *abiD* isolated from pBF61 of *Lactococcus lactis* subsp. lactis KR5. *Appl Environ Microbiol*, 61, 2023–2026.

45. Gautier,M. and Chopin,M.C. (1987) Plasmid-determined systems for restriction and modification activity and abortive infection in *Streptococcus cremoris*. *Appl Environ Microbiol*, 53, 923–927.

46. Anba,J., Bidnenko,E., Hillier,A., Ehrlich,D. and Chopin,M.C. (1995) Characterization of the lactococcal *abiD1* gene coding for phage abortive infection. *J Bacteriol*, 177, 3818–3823.

47. Bidnenko,E., Ehrlich,D. and Chopin,M.C. (1995) Phage operon involved in sensitivity to the *Lactococcus lactis* abortive infection mechanism AbiD1. *J Bacteriol*, 177, 3824–3829.

48. Bidnenko,E., Ehrlich,S.D. and Chopin,M. (1998) *Lactococcus lactis* phage operon coding for an endonuclease homologous to RuvC. *Mol Microbiol*, 28, 823–834.

49. Bidnenko,E., Chopin,M.C., Ehrlich,S.D. and Anba,J. (2002) *Lactococcus lactis* AbiD1 abortive infection efficiency is drastically increased by a phage protein. *FEMS Microbiol Lett*, 214, 283–287.

50. Bidnenko,E., Chopin,A., Ehrlich,S.D. and Chopin,M.C. (2009) Activation of mRNA translation by phage protein and low temperature: the case of *Lactococcus lactis* abortive infection system AbiD1. *BMC Mol Biol*, 10, 4.

51. Garvey,P., Fitzgerald,G.F. and Hill,C. (1995) Cloning and DNA sequence analysis of two abortive infection phage resistance determinants from the lactococcal plasmid pNP40. *Appl Environ Microbiol*, 61, 4321–4328.

52. Rincé,A., Tangney,M. and Fitzgerald,G.F. (2000) Identification of a DNA region from lactococcal phage sk1 protecting phage 712 from the abortive infection mechanism AbiF. *FEMS Microbiol Lett*, 182, 185–191.

53. O'Connor,L., Coffey,A., Daly,C. and Fitzgerald,G.F. (1996) AbiG, a genotypically novel abortive infection mechanism encoded by plasmid pCI750 of *Lactococcus lactis* subsp. *cremoris* UC653. *Appl Environ Microbiol*, 62, 3075–3082.

54. O'Connor,L., Tangney,M. and Fitzgerald,G.F. (1999) Expression, regulation, and mode of action of the AbiG abortive infection system of *Lactococcus lactis* subsp. *cremoris* UC653. *Appl Environ Microbiol*, 65, 330–335.

55. Prévots,F., Daloyau,M., Bonin,O., Dumont,X. and Tolou,S. (1996) Cloning and sequencing of the novel abortive infection gene *abiH* of *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* S94. *FEMS Microbiol Lett*, 142, 295–299.

56. Su,P., Harvey,M., Im,H.J. and Dunn,N.W. (1997) Isolation, cloning and characterisation of the *abiI* gene from *Lactococcus lactis* subsp. *lactis* M138 encoding abortive phage infection. *J Biotechnol*, 54, 95–104.

57. Deng,Y.M., Harvey,M.L., Liu,C.Q. and Dunn,N.W. (1997) A novel plasmid-encoded phage abortive infection system from *Lactococcus lactis* biovar. *diacetylactis*. *FEMS Microbiol Lett*, 146, 149–154.

58. Emond,E., Holler,B.J., Boucher,I., Vandenbergh,P.A., Vedamuthu,E.R., Kondo,J.K. and Moineau,S. (1997) Phenotypic and genetic characterization of the bacteriophage abortive infection mechanism AbiK from *Lactococcus lactis*. *Appl Environ Microbiol*, 63, 1274–1283.

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

60. Boucher,I., Émond,É., Dion,É., Montpetit,D. and Moineau,S. (2000) Microbiological and molecular impacts of AbiK on the lytic cycle of *Lactococcus lactis* phages of the 936 and P335 species. *Microbiology (N Y)*, 146, 445–453.

61. Bouchard,J.D. and Moineau,S. (2004) Lactococcal phage genes involved in sensitivity to AbiK and their relation to single-strand annealing proteins. *J Bacteriol*, 186, 3649–3652.

62. Fortier,L.C., Bouchard,J.D. and Moineau,S. (2005) Expression and site-directed mutagenesis of the lactococcal abortive phage infection protein AbiK. *J Bacteriol*, 187, 3721–3730.

63. Labrie,S.J. and Moineau,S. (2007) Abortive infection mechanisms and prophage sequences significantly influence the genetic makeup of emerging lytic lactococcal phages. *J Bacteriol*, 189, 1482–1487.

64. Scaltriti,E., Launay,H., Genois,M., Bron,P., Rivetti,C., Grolli,S., Ploquin,M., Campanacci,V., Tegoni,M., Cambillau,C., *et al.* (2011) Lactococcal phage p2 ORF35-Sak3 is an ATPase involved in DNA recombination and AbiK mechanism. *Mol Microbiol*, 80, 102–116.

65. Wang,C., Villion,M., Semper,C., Coros,C., Moineau,S. and Zimmerly,S. (2011) A reverse transcriptase-related protein mediates phage resistance and polymerizes untemplated DNA in vitro. *Nucleic Acids Res*, 39, 7620–7629.

66. Figiel,M., Gapińska,M., Czarnocki-Cieciura,M., Zajko,W., Sroka,M., Skowronek,K. and Nowotny,M. (2022) Mechanism of protein-primed template-independent DNA synthesis by Abi polymerases. *Nucleic Acids Res*, 50, 10026–10040.

67. Deng,Y.M., Liu,C.Q. and W. Dunn,N. (1999) Genetic organization and functional analysis of a novel phage abortive infection system, AbiL, from *Lactococcus lactis*. *J Biotechnol*, 67, 135–149.

68. Prévots,F., Tolou,S., Delpech,B., Kaghad,M. and Daloyau,M. (1998) Nucleotide sequence and analysis of the new chromosomal abortive infection gene *abiN* of *Lactococcus lactis* subsp. *cremoris* S114. *FEMS Microbiol Lett*, 159, 331–336.

69. Prevots,F. and Ritzenthaler,P. (1998) Complete sequence of the new lactococcal abortive phage resistance gene *abiO*. *J Dairy Sci*, 81, 1483–1485.

70. Domingues,S., Chopin,A., Ehrlich,S.D. and Chopin,M.C. (2004) The lactococcal abortive phage infection system AbiP prevents both phage DNA replication and temporal transcription switch. *J Bacteriol*, 186, 713–721.

71. Domingues,S., Chopin,A., Ehrlich,S.D. and Chopin,M.-C. (2004) A phage protein confers resistance to the lactococcal abortive infection mechanism AbiP. *J Bacteriol*, 186, 3278–3281.

72. Emond,E., Dion,E., Walker,S.A., Vedamuthu,E.R., Kondo,J.K. and Moineau,S. (1998) AbiQ, an abortive infection mechanism from *Lactococcus lactis*. *Appl Environ Microbiol*, 64, 4748–56.

73. Samson,J.E., Bélanger,M. and Moineau,S. (2013) Effect of the abortive infection mechanism and type III toxin/antitoxin system AbiQ on the lytic cycle of *Lactococcus lactis* phages. *J Bacteriol*, 195, 3947–56.

74. McKay,L.L., Bohanon,M.J., Polzin,K.M., Rule,P.L. and Baldwin,K.A. (1989) Localization of separate genetic loci for reduced sensitivity towards small isometric-headed bacteriophage sk1 and prolate-headed bacteriophage c2 on pGBK17 from *Lactococcus lactis* subsp. *lactis* KR2. *Appl Environ Microbiol*, 55, 2702–2709.

75. Twomey,D.P., De Urraza,P.J., McKay,L.L. and O'Sullivan,D.J. (2000) Characterization of AbiR, a novel multicomponent abortive infection

mechanism encoded by plasmid pKR223 of *Lactococcus lactis* subsp. *lactis* KR2. *Appl Environ Microbiol*, 66, 2647–2651.

76. Yang,J.M., Deurraza,P.J., Matvienko,N. and O'Sullivan,D.J. (2006) Involvement of the LlaKR2I methylase in expression of the AbiR bacteriophage defense system in *Lactococcus lactis* subsp. *lactis* biovar diacetylactis KR2. *J Bacteriol*, 188, 1920–1928.

77. Holubová,J. and Josephsen,J. (2007) Potential of AbiS as defence mechanism determined by conductivity measurement. *J Appl Microbiol*, 103, 2382–2391.

78. Bouchard,J.D., Dion,E., Bissonnette,F. and Moineau,S. (2002) Characterization of the two-component abortive phage infection mechanism AbiT from *Lactococcus lactis*. *J Bacteriol*, 184, 6325–6332.

79. Labrie,S.J., Tremblay,D.M., Moisan,M., Villion,M., Magadán,A.H., Campanacci,V., Cambillau,C. and Moineau,S. (2012) Involvement of the major capsid protein and two early-expressed phage genes in the activity of the lactococcal abortive infection mechanism AbiT. *Appl Environ Microbiol*, 78, 6890–6899.

80. Dai,G., Su,P., Allison,G.E., Geller,B.L., Zhu,P., Kim,W.S. and Dunn,N.W. (2001) Molecular characterization of a new abortive infection system (AbiU) from *Lactococcus lactis* LL51-1. *Appl Environ Microbiol*, 67, 5225–32.

81. Haaber,J., Moineau,S., Fortier,L.C. and Hammer,K. (2008) AbiV, a novel antiphage abortive infection mechanism on the chromosome of

Lactococcus lactis subsp. *cremoris* MG1363. *Appl Environ Microbiol*, 74, 6528–6537.

82. Haaber,J., Rousseau,G.M., Hammer,K. and Moineau,S. (2009) Identification and characterization of the phage gene *sav*, involved in sensitivity to the lactococcal abortive infection mechanism AbiV. *Appl Environ Microbiol*, 75, 2484–2494.

83. Haaber,J., Samson,J.E., Labrie,S.J., Campanacci,V., Cambillau,C., Moineau,S. and Hammer,K. (2010) Lactococcal abortive infection protein AbiV interacts directly with the phage protein SaV and prevents translation of phage proteins. *Appl Environ Microbiol*, 76, 7085–7092.

84. Deveau,H., Labrie,S.J., Chopin,M.C. and Moineau,S. (2006) Biodiversity and classification of lactococcal phages. *Appl Environ Microbiol*, 72, 4338–4346.

85. Mahony,J., McDonnell,B., Casey,E. and Van Sinderen,D. (2016) Phage-host interactions of cheese-making lactic acid bacteria. *Annu Rev Food Sci Technol*, 7, 267–285.

86. Samson,J.E. and Moineau,S. (2010) Characterization of *Lactococcus lactis* phage 949 and comparison with other lactococcal phages. *Appl Environ Microbiol*, 76, 6843–6852.

87. Fortier,L.C., Bransi,A. and Moineau,S. (2006) Genome sequence and global gene expression of Q54, a new phage species linking the 936 and c2 phage species of *Lactococcus lactis*. *J Bacteriol*, 188, 6101–6114.

88. Garneau,J.E., Tremblay,D.M. and Moineau,S. (2008) Characterization of 1706, a virulent phage from *Lactococcus lactis* with similarities to prophages from other Firmicutes. *Virology*, 373, 298–309.

89. Mahony,J., Murphy,J. and van Sinderen,D. (2012) Lactococcal 936-type phages and dairy fermentation problems: From detection to evolution and prevention. *Front Microbiol*, 3, 1–9.

90. Depardieu,F., Didier,J.P., Bernheim,A., Sherlock,A., Molina,H., Duclos,B. and Bikard,D. (2016) A eukaryotic-like serine/threonine kinase protects staphylococci against phages. *Cell Host Microbe*, 20, 471–481.

91. Millman,A., Bernheim,A., Stokar-Avihail,A., Fedorenko,T., Voichek,M., Leavitt,A., Oppenheimer-Shaanan,Y. and Sorek,R. (2020) Bacterial retrons function in anti-phage defense. *Cell*, 183, 1551-1561.e12.

92. Rousset,F., Depardieu,F., Miele,S., Dowding,J., Laval,A.-L., Lieberman,E., Garry,D., Rocha,E.P.C., Bernheim,A. and Bikard,D. (2022) Phages and their satellites encode hotspots of antiviral systems. *Cell Host Microbe*, 30, 740-753.e5.

93. Zhang,T., Tamman,H., Coppieters ’t Wallant,K., Kurata,T., LeRoux,M., Srikant,S., Brodiazhenko,T., Cepauskas,A., Talavera,A., Martens,C., *et al.* (2022) Direct activation of a bacterial innate immune system by a viral capsid protein. *Nature*, 612, 132–140.

94. Stokar-Avihail,A., Fedorenko,T., Hör,J., Garb,J., Leavitt,A., Millman,A., Shulman,G., Wojtania,N., Melamed,S., Amitai,G., *et al.* (2023) Discovery of phage determinants that confer sensitivity to bacterial immune systems. *Cell*, 186, 1863-1876.e16.

95. Gao,L.A., Wilkinson,M.E., Strecker,J., Makarova,K.S., Macrae,R.K., Koonin,E. V. and Zhang,F. (2022) Prokaryotic innate immunity through pattern recognition of conserved viral proteins. *Science (1979)*, 377.

96. Garb,J., Lopatina,A., Bernheim,A., Zaremba,M., Siksnys,V., Melamed,S., Leavitt,A., Millman,A., Amitai,G. and Sorek,R. (2022) Multiple phage resistance systems inhibit infection via SIR2-dependent NAD+ depletion. *Nat Microbiol*, 7, 1849–1856.

97. Söding,J., Biegert,A. and Lupas,A.N. (2005) The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res*, 33.

98. Paysan-Lafosse,T., Blum,M., Chuguransky,S., Grego,T., Pinto,B.L., Salazar,G.A., Bileschi,M.L., Bork,P., Bridge,A., Colwell,L., *et al.* (2023) InterPro in 2022. *Nucleic Acids Res*, 51, D418–D427.

99. Jumper,J., Evans,R., Pritzel,A., Green,T., Figurnov,M., Ronneberger,O., Tunyasuvunakool,K., Bates,R., Žídek,A., Potapenko,A., *et al.* (2021) Highly accurate protein structure prediction with AlphaFold. *Nature*, 596, 583–589.

100. Gapińska,M., Zajko,W., Skowronek,K., Figiel,M., Krawczyk,P.S., Egorov,A.A., Dziembowski,A., Johansson,M.J.O. and Nowotny,M. (2024) Structure-functional characterization of *Lactococcus* AbiA phage defense system. *Nucleic Acids Res*, 52, 4723–4738.

101. Mestre,M.R., Gao,L.A., Shah,S.A., López-Beltrán,A., González-Delgado,A., Martínez-Abarca,F., Iranzo,J., Redrejo-Rodríguez,M., Zhang,F. and Toro,N. (2022) UG/Abi: a highly diverse family of prokaryotic

reverse transcriptases associated with defense functions. *Nucleic Acids Res*, 50, 6084–6101.

102. Anantharaman,V., Makarova,K.S., Burroughs,A.M., Koonin,E. V and Aravind,L. (2013) Comprehensive analysis of the HEPN superfamily: identification of novel roles in intra-genomic conflicts, defense, pathogenesis and RNA processing. *Biol Direct*, 8, 15.

103. Page,R. and Peti,W. (2016) Toxin-antitoxin systems in bacterial growth arrest and persistence. *Nat Chem Biol*, 12, 208–214.

104. Singh,G., Yadav,M., Ghosh,C. and Rathore,J.S. (2021) Bacterial toxin-antitoxin modules: classification, functions, and association with persistence. *Curr Res Microb Sci*, 2, 100047.

105. Zhu,X., Morency,C., Picard,M.È., Mosterd,C., McAlister,J.A., Perrault-Jolicoeur,A., Geddes-McAlister,J., Shi,R. and Moineau,S. (2024) HEPN-AbiV is an RNase in the antiphage system AbiV. *bioRxiv*, 10.1101/2024.05.05.592566.

106. Dhar,M.K., Koul,A. and Kaul,S. (2013) Farnesyl pyrophosphate synthase: a key enzyme in isoprenoid biosynthetic pathway and potential molecular target for drug development. *N Biotechnol*, 30, 114–123.

107. Domingues,S., McGovern,S., Plochocka,D., Santos,M.A., Ehrlich,S.D., Polard,P. and Chopin,M.C. (2008) The lactococcal abortive infection protein AbiP is membrane-anchored and binds nucleic acids. *Virology*, 373, 14–24.

108. Garvey,P., Rince,A., Hill,C. and Fitzgerald,G.F. (1997) Identification of a RecA homolog ($RecA_{LP}$) on the conjugative lactococcal phage resistance plasmid pNP40: Evidence of a role for chromosomally encoded $RecA_L$ in abortive infection. *Appl Environ Microbiol*, 63, 1244–1251.

109. Couvin,D., Bernheim,A., Toffano-Nioche,C., Touchon,M., Michalik,J., Néron,B., Rocha,E.P.C., Vergnaud,G., Gautheret,D. and Pourcel,C. (2018) CRISPRCasFinder, an update of CRISRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. *Nucleic Acids Res*, 46, W246–W251.

110. Song,X., Liu,L., Liu,X.X., Xiong,Z.Q., Xie,C.L., Wang,S.J. and Ai,L.Z. (2021) Single-plasmid systems based on CRISPR-Cas9 for gene editing in *Lactococcus lactis*. *J Dairy Sci*, 104, 10576–10585.

111. Deep,A., Liang,Q., Enustun,E., Pogliano,J. and Corbett,K.D. (2024) Architecture and activation mechanism of the bacterial PARIS defence system. *Nature*, 10.1038/s41586-024-07772-8.

112. Burman,N., Belukhina,S., Depardieu,F., Wilkinson,R.A., Skutel,M., Santiago-Frangos,A., Graham,A.B., Livenskyi,A., Chechenina,A., Morozova,N., *et al.* (2024) A virally-encoded tRNA neutralizes the PARIS antiviral defence system. *Nature*, 10.1038/s41586-024-07874-3.

113. Miller,W.G., Pearson,B.M., Wells,J.M., Parker,C.T., Kapitonov,V. V. and Mandrell,R.E. (2005) Diversity within the *Campylobacter jejuni* type I restriction–modification loci. *Microbiology (N Y)*, 151, 337–351.

114. Makarova,K.S., Wolf,Y.I., Snir,S. and Koonin,E. V. (2011) Defense islands in bacterial and archaeal genomes and prediction of novel defense systems. *J Bacteriol*, 193, 6039–6056.

115. Dy,R.L., Przybilski,R., Semeijn,K., Salmond,G.P.C. and Fineran,P.C. (2014) A widespread bacteriophage abortive infection system functions through a Type IV toxin–antitoxin mechanism. *Nucleic Acids Res*, 42, 4590–4605.

116. Fineran,P.C., Blower,T.R., Foulds,I.J., Humphreys,D.P., Lilley,K.S. and Salmond,G.P.C. (2009) The phage abortive infection system, ToxIN, functions as a protein–RNA toxin–antitoxin pair. *Proceedings of the National Academy of Sciences*, 106, 894–899.

117. Samson,J.E., Spinelli,S., Cambillau,C. and Moineau,S. (2013) Structure and activity of AbiQ, a lactococcal endoribonuclease belonging to the type III toxin–antitoxin system. *Mol Microbiol*, 87, 756–768.

118. Beck,I.N., Usher,B., Hampton,H.G., Fineran,P.C. and Blower,T.R. (2020) Antitoxin autoregulation of *M. tuberculosis* toxin-antitoxin expression through negative cooperativity arising from multiple inverted repeat sequences. *Biochemical Journal*, 477, 2401–2419.

119. Hampton,H.G., Jackson,S.A., Fagerlund,R.D., Vogel,A.I.M., Dy,R.L., Blower,T.R. and Fineran,P.C. (2018) AbiEi binds cooperatively to the Type IV *abiE* toxin–antitoxin operator via a positively-charged surface and causes DNA bending and negative autoregulation. *J Mol Biol*, 430, 1141–1156.

120. Cai,Y., Usher,B., Gutierrez,C., Tolcan,A., Mansour,M., Fineran,P.C., Condon,C., Neyrolles,O., Genevaux,P. and Blower,T.R. (2020) A nucleotidyltransferase toxin inhibits growth of *Mycobacterium tuberculosis* through inactivation of tRNA acceptor stems. *Sci Adv*, 6.

121. Hampton,H.G., Smith,L.M., Ferguson,S., Meaden,S., Jackson,S.A. and Fineran,P.C. (2020) Functional genomics reveals the toxin–antitoxin repertoire and AbiE activity in *Serratia*. *Microb Genom*, 6.

122. Yu,X., Gao,X., Zhu,K., Yin,H., Mao,X., Wojdyla,J.A., Qin,B., Huang,H., Wang,M., Sun,Y.-C., *et al.* (2020) Characterization of a toxin-antitoxin system in *Mycobacterium tuberculosis* suggests neutralization by phosphorylation as the antitoxicity mechanism. *Commun Biol*, 3, 216.

123. Guglielmini,J. and Van Melderen,L. (2011) Bacterial toxin-antitoxin systems. *Mob Genet Elements*, 1, 283–306.

124. Blower,T.R., Pei,X.Y., Short,F.L., Fineran,P.C., Humphreys,D.P., Luisi,B.F. and Salmond,G.P.C. (2011) A processed noncoding RNA regulates an altruistic bacterial antiviral system. *Nat Struct Mol Biol*, 18, 185–190.

125. Blower,T.R., Fineran,P.C., Johnson,M.J., Toth,I.K., Humphreys,D.P. and Salmond,G.P.C. (2009) Mutagenesis and functional characterization of the RNA and protein components of the *toxIN* abortive infection and toxin-antitoxin locus of *Erwinia*. *J Bacteriol*, 191, 6029–6039.

126. Short,F.L., Akusobi,C., Broadhurst,W.R. and Salmond,G.P.C. (2018) The bacterial Type III toxin-antitoxin system, ToxIN, is a dynamic protein-

RNA complex with stability-dependent antiviral abortive infection activity. *Sci Rep*, 8, 1013.

127. Guegler,C.K. and Laub,M.T. (2021) Shutoff of host transcription triggers a toxin-antitoxin system to cleave phage RNA and abort infection. *Mol Cell*, 81, 2361-2373.e9.

128. Blower,T.R., Evans,T.J., Przybilski,R., Fineran,P.C. and Salmond,G.P.C. (2012) Viral evasion of a bacterial suicide system by RNA–based molecular mimicry enables infectious altruism. *PLoS Genet*, 8, e1003023.

129. Rine,J., Strathern,J.N., Hicks,J.B. and Herskowitz,I. (1979) A suppressor of mating-type locus mutations in *Saccharomyces cerevisiae*: evidence for and identification of cryptic mating-type loci. *Genetics*, 93, 877–901.

130. North,B.J. and Verdin,E. (2004) Sirtuins: Sir2-related NAD-dependent protein deacetylases. *Genome Biol*, 5, 224.

131. Gallego-Jara,J., Ortega,Á., Lozano Terol,G., Sola Martínez,R.A., Cánovas Díaz,M. and de Diego Puente,T. (2021) Bacterial sirtuins overview: An open niche to explore. *Front Microbiol*, 12.

132. Wu,Q.J., Zhang,T.N., Chen,H.H., Yu,X.F., Lv,J. Le, Liu,Y.Y., Liu,Y.S., Zheng,G., Zhao,J.Q., Wei,Y.F., *et al.* (2022) The sirtuin family in health and disease. *Signal Transduct Target Ther*, 7, 402.

133. Ofir,G., Herbst,E., Baroz,M., Cohen,D., Millman,A., Doron,S., Tal,N., Malheiro,D.B.A., Malitsky,S., Amitai,G., *et al.* (2021) Antiviral activity of

bacterial TIR domains via immune signalling molecules. *Nature*, 600, 116–120.

134. Tang,J.Y., Bullen,N.P., Ahmad,S. and Whitney,J.C. (2018) Diverse NADase effector families mediate interbacterial antagonism via the type VI secretion system. *Journal of Biological Chemistry*, 293, 1504–1514.

135. Morehouse,B.R., Govande,A.A., Millman,A., Keszei,A.F.A., Lowey,B., Ofir,G., Shao,S., Sorek,R. and Kranzusch,P.J. (2020) STING cyclic dinucleotide sensing originated in bacteria. *Nature*, 586, 429–433.

136. Lai,L., Yokota,H., Hung,L.-W., Kim,R. and Kim,S.-H. (2000) Crystal structure of archaeal RNase HII: a homologue of human major RNase H. *Structure*, 8, 897–904.

137. Abudayyeh,O.O., Gootenberg,J.S., Konermann,S., Joung,J., Slaymaker,I.M., Cox,D.B.T., Shmakov,S., Makarova,K.S., Semenova,E., Minakhin,L., *et al.* (2016) C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science (1979)*, 353.

138. Ahmad,S. and Hur,S. (2015) Helicases in antiviral immunity: Dual properties as sensors and effectors. *Trends Biochem Sci*, 40, 576–585.

139. Tuck,O.T., Adler,B.A., Armbruster,E.G., Lahiri,A., Hu,J.J., Zhou,J., Pogliano,J. and Doudna,J.A. (2024) Hachiman is a genome integrity sensor. *bioRxiv*, 10.1101/2024.02.29.582594.

140. Sather,L.M., Zamani,M., Muhammed,Z., Kearsley,J.V.S., Fisher,G.T., Jones,K.M. and Finan,T.M. (2023) A broadly distributed predicted

helicase/nuclease confers phage resistance via abortive infection. *Cell Host Microbe*, 31, 343-355.e5.

141. Mavrommatis,E., Fish,E.N. and Platanias,L.C. (2013) The schlafen family of proteins and their regulation by interferons. *J Interferon Cytokine Res*, 33, 206–10.

142. Chakrabarti,S., Jayachandran,U., Bonneau,F., Fiorini,F., Basquin,C., Domcke,S., Le Hir,H. and Conti,E. (2011) Molecular mechanisms for the RNA-dependent ATPase activity of Upf1 and its regulation by Upf2. *Mol Cell*, 41, 693–703.

143. Wah,D.A., Hirsch,J.A., Dorner,L.F., Schildkraut,I. and Aggarwal,A.K. (1997) Structure of the multimodular endonuclease FokI bound to DNA. *Nature*, 388, 97–100.

144. Cheng,H.-C., Qi,R.Z., Paudel,H. and Zhu,H.-J. (2011) Regulation and function of protein kinases and phosphatases. *Enzyme Res*, 2011, 1–3.

145. March,P.E. (1992) Membrane-associated GTPases in bacteria. *Mol Microbiol*, 6, 1253–1257.

146. Caldon,C.E. and March,P.E. (2003) Function of the universally conserved bacterial GTPases. *Curr Opin Microbiol*, 6, 135–139.

147. Simon,N.C., Aktories,K. and Barbieri,J.T. (2014) Novel bacterial ADP-ribosylating toxins: structure and function. *Nat Rev Microbiol*, 12, 599–611.

148. LeRoux,M., Srikant,S., Teodoro,G.I.C., Zhang,T., Littlehale,M.L., Doron,S., Badiee,M., Leung,A.K.L., Sorek,R. and Laub,M.T. (2022) The

DarTG toxin-antitoxin system provides phage defence by ADP-ribosylating viral DNA. *Nat Microbiol*, 7, 1028–1040.

149. Borrok,M.J., Zhu,Y., Forest,K.T. and Kiessling,L.L. (2009) Structure-based design of a periplasmic binding protein antagonist that prevents domain closure. *ACS Chem Biol*, 4, 447–456.

150. Paget,M.S. and Helmann,J.D. (2003) The sigma70 family of sigma factors. *Genome Biol*, 4, 203.

151. Osorio-Valeriano,M., Altegoer,F., Das,C.K., Steinchen,W., Panis,G., Connolley,L., Giacomelli,G., Feddersen,H., Corrales-Guerrero,L., Giammarinaro,P.I., *et al.* (2021) The CTPase activity of ParB determines the size and dynamics of prokaryotic DNA partition complexes. *Mol Cell*, 81, 3992-4007.e10.

152. Schumacher,M.A., Tonthat,N.K., Lee,J., Rodriguez-Castañeda,F.A., Chinnam,N.B., Kalliomaa-Sanford,A.K., Ng,I.W., Barge,M.T., Shaw,P.L.R. and Barillà,D. (2015) Structures of archaeal DNA segregation machinery reveal bacterial and eukaryotic linkages. *Science (1979)*, 349, 1120–1124.

153. Johnson,E.P., Mincer,T., Schwab,H., Burgin,A.B. and Helinski,D.R. (1999) Plasmid RK2 ParB protein: Purification and nuclease properties. *J Bacteriol*, 181, 6010–6018.

154. Conaway,R.C. and Conaway,J.W. (2009) The INO80 chromatin remodeling complex in transcription, replication and repair. *Trends Biochem Sci*, 34, 71–77.

155. Wu,H., Muñoz,E.N., Hsieh,L.J., Chio,U.S., Gourdet,M.A., Narlikar,G.J. and Cheng,Y. (2023) Reorientation of INO80 on hexasomes reveals basis for mechanistic versatility. *Science (1979)*, 381, 319–324.

156. Vita,N., Platsaki,S., Baslé,A., Allen,S.J., Paterson,N.G., Crombie,A.T., Murrell,J.C., Waldron,K.J. and Dennison,C. (2015) A four-helix bundle stores copper for methane oxidation. *Nature*, 525, 140–143.

157. Vassallo,C.N., Doering,C.R., Littlehale,M.L., Teodoro,G.I.C. and Laub,M.T. (2022) A functional selection reveals previously undetected anti-phage defence systems in the *E. coli* pangenome. *Nat Microbiol*, 7, 1568–1579.

158. Millman,A., Melamed,S., Amitai,G. and Sorek,R. (2020) Diversity and classification of cyclic-oligonucleotide-based anti-phage signalling systems. *Nat Microbiol*, 5, 1608–1615.

Chapter II

Discovery of antiphage systems in the lactococcal plasmidome

This chapter was published in:

Grafakou A., Mosterd C., Beck M.H., Kelleher P., McDonnell B., de Waal P.P., van Rijswijck I.M.H., van Peij N.N.M.E., Cambillau C., Mahony J. and van Sinderen D. (2024) Discovery of antiphage systems in the lactococcal plasmidome. *Nucleic Acids Res*, 10.1093/nar/gkae671.

A.G., C.M., J.M. and D.v.S. led the study. A.G. and C.M. performed the experiments and the analyses unless otherwise indicated. C.C. performed the AlphaFold2 predictions and analysis. M.B. contributed to the discovery and the preliminary analysis of some antiphage systems. P.K. was responsible for the genomic assembly and annotation of the lactococcal plasmid sequences and the grouping of the plasmid genes into families. B.M. contributed to the generation of some constructs and to the initial project administration. P.d.W., I.v.R. and N.v.P. contributed to meaningful discussion and interpretation of the results. J.M. and D.v.S. supervised and examined the results of the experiments. J.M. and D.v.S. conceived the project and contributed to the selection of candidate systems. The initial concept manuscript was written by A.G., C.M. and C.C., and all authors contributed to the final version of the manuscript.

2.1 Abstract

Until the late 2000s, lactococci substantially contributed to the discovery of various plasmid-borne phage defence systems, rendering these bacteria an excellent antiphage discovery resource. Recently, there has been a resurgence of interest in identifying novel antiphage systems in lactic acid bacteria owing to recent reports of so-called “defence islands” in diverse bacterial genera. Here, 321 plasmid sequences from 53 lactococcal strains were scrutinised for the presence of antiphage systems. Systematic evaluation of 198 candidates facilitated the discovery of seven not previously described antiphage systems, as well as five systems, homologues of which had been described in other bacteria. All described systems confer resistance against the most prevalent lactococcal phages, and act post phage DNA injection, while all except one behave like abortive infection systems. Structure and domain predictions provided insights into their mechanism of action and allow grouping of several genetically distinct systems. Although rare within our plasmid collection, homologues of the seven novel systems appear to be widespread among bacteria. This study highlights plasmids as a rich repository of as yet undiscovered antiphage systems.

2.2 Introduction

Lactococcus lactis and *Lactococcus cremoris* (formerly assigned as two subspecies of *Lactococcus lactis*) (1) are bacterial species of substantial economic and industrial importance due to their extensive use in the production of fermented food products, such as cheese, buttermilk and sour cream (2–4). These lactococcal species are characterised as Gram-positive, non-spore forming, micro-aerophilic coccoid bacteria that belong to the lactic acid bacteria (LAB) (5).

The widespread and intensive application of lactococcal strains is associated with the emergence of host-specific bacteriophages, which are ubiquitous in the dairy environment and which represent a persistent challenge to fermentation processes (6). Phage infection may cause (partial) elimination of the starter culture, resulting in delayed or even failed fermentations with severe economic consequences for producers (7). Among the described lactococcal phage groups/genera (8, 9), members of the *Skunavirus* genus (formerly 936 phage group), *Ceduovirus* genus (formerly c2 phage group) and P335 group are particularly prevalent and problematic in modern, large-scale dairy fermentation plants (10).

To defend themselves against phages, bacteria have adopted multiple phage resistance strategies that interfere with different stages of the phage infection cycle, such as preventing phage adsorption or phage DNA injection, restriction of incoming phage nucleic acids (Restriction-Modification [RM] and Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein [CRISPR-Cas] systems) and

abortive infection (Abi) (11, 12). CRISPR-Cas systems are typically not present in *Lactococcus* with very limited reports of their presence in recent studies (13, 14). Abi systems instead represent one of the most common lactococcal antiphage mechanisms. They are activated upon phage infection preventing phage proliferation and subsequent infection of neighbouring cells (12, 15). To date, 23 genetically distinct and mechanistically diverse lactococcal Abi systems have been reported, designated AbiA to AbiZ (12, 16). The majority of the currently defined lactococcal Abi systems are plasmid-encoded, typically by one or two genes, and with diverse modes of action interfering with critical stages of the phage lytic cycle, including DNA replication, transcription and protein production (5, 16).

Despite intense research efforts focused on antiphage systems in lactococci until the 2000s (12), the recent discovery of many previously unknown antiphage systems using computational and high-throughput methods in other bacteria demonstrates that the phage resistance landscape is far more extensive and varied than previously imagined. More specifically, a multitude of studies have reported on the exploration of genomic data sets for the identification of novel antiphage systems in primarily *Escherichia coli* and *Bacillus subtilis* as model and test organisms (17–22). Furthermore, most of the recent studies have mined whole genome sequences and are largely based on the genomic co-localisation with known antiphage systems (so-called “defence islands”) for high-throughput bioinformatic prediction and discovery of antiphage systems (17–19, 22). However, a recent experimental screening approach based on random

cloning of genome fragments has also allowed discovery of antiphage systems, indicating their ubiquitous nature (20).

Although it has recently been suggested that mobile genetic elements (MGEs) may frequently carry antiphage systems and represent major contributors to their horizontal transfer (19–21, 23, 24), plasmids have not thus far been systematically searched for antiphage systems. Dairy-associated lactococcal strains typically harbour several plasmids, with a report of an isolate containing as many as twelve (25). In a large-scale analysis of lactococcal plasmids, an average of 5 plasmids per strain was identified, ranging in size from 0.9 kb to 193 kb and accounting for up to 355 kb of extra-chromosomal DNA (14), which is a remarkable feature considering that the genome size of individual lactococcal strains can range between 2,242 to 2,688 kb (26). Many biotechnologically relevant traits, such as casein degradation, lactose utilisation and citrate metabolism are plasmid-encoded (14, 16). However, the function of most lactococcal plasmid-associated gene products remains unknown and it may well be that some of these confer antiphage activity.

The aim of the current study was to survey the combined plasmidome of 53 lactococcal strains for encoded antiphage systems and to provide insights into the structure and function of the identified systems, thus expanding our understanding of the bacterial immunity landscape and generating a phage resistance-encoding plasmid library that can be applied in the dairy industry for improved strain robustness or beyond.

2.3 Materials and methods

2.3.1 Strains, media and growth conditions

Bacterial strains, plasmids and bacteriophages used in this study are listed in Table S1. Lactococcal strains used in this study were grown at 30 °C in M17 broth (Oxoid) supplemented with 0.5 % (w/v) glucose (GM17) for 16-20 h. GM17 was supplemented with either chloramphenicol (5 µg/ml, Sigma-Aldrich, to select for strains carrying pNZ44 or its derivatives), tetracycline (5 µg/ml, Sigma-Aldrich, to select for strains harbouring pPTPi or its derivatives) or erythromycin (10 µg/ml, Sigma-Aldrich, for pPEPi- or derivative-containing strains). Nisin from *L. lactis* (1-10 ng/ml, Sigma-Aldrich) was added to growing cultures when an OD_{600nm} of 0.2-0.3 was reached for the induction of the P*nis*A promoter of pPTPi-derivative plasmids. Where appropriate, GM17 agar plates were also supplemented with nisin as described above.

Escherichia coli strains were grown overnight at 37 °C with agitation (150 rpm, Multitron Standard, Infors HT) in LB broth (Thermo Fisher Scientific) supplemented with 10 µg/ml tetracycline, to select for strains harbouring pPTPi or its derivatives. For the preparation of GM17 or LB agar plates 1.5 % (w/v) bacteriological agar was added.

2.3.2 Bacteriophage propagation

Bacteriophages used in this study were propagated by adding 2-5 % (v/v) of a fresh overnight culture of the appropriate lactococcal host strain, $CaCl_2$ (10 mM) and 1 % (v/v) of the phage lysate (10^8-10^{10} PFU/ml) or a single

plaque in GM17 broth. Incubation was continued at room temperature or 30 °C until lysis occurred. The lysates were filtered (pore size 0.45 µm, Sarstedt AG & Co. KG) and stored at 4 °C. The phages used in this study belong to one of four genera of lactococcal phages which have previously been shown to be genetically distinct (9). The *Skunavirus* members employed in this study share over 90 % nucleotide sequence similarity over at least 70 % of their genomes highlighting the intra-genus diversity of these phages based on BLASTN alignments, while the P335 phages used have previously been shown to be highly genetically diverse (27).

2.3.3 DNA extraction, sequencing, assembly and annotation of lactococcal strains

Genomic DNA of 53 lactococcal strains was isolated from a fresh 10 ml overnight culture of each strain using a NucleoBond® DNA extraction kit with Buffer set III (Macherey-Nagel) according to the manufacturer's instructions, with the exception of the addition of mutanolysin (Sigma-Aldrich; 50 U/mL) to Buffer G3 prior to an extended (16 – 18 h) incubation at 37 °C. DNA was stored at -20 °C prior to shipment to the sequencing facility.

Genome sequencing was performed by a combined SMRT (long read) and Illumina (short read) approach on a Pacific Biosciences RS II sequencing platform (executed by Eurofins Genomics, Germany) and an Illumina MiSeq platform (executed by GenProbio s.r.l., Italy), respectively. *De novo* hybrid assemblies using both datasets were performed using the

Unicycler v0.5.0 hybrid assembly pipeline (28). Open Reading Frame (ORF) prediction was performed with Prodigal v2.5 prediction software (29) and confirmed using BLASTX v2.2.26 alignments (30). ORFs were automatically annotated using BLASTP v2.2.26(30) analysis against the non-redundant protein databases curated by the National Centre for Biotechnology Information (NCBI). Artemis v18 genome browser (31) and annotation tool was used to manually curate ORFs and for the combination and inspection of ORF results. Final ORF annotations were refined where necessary using Pfam (32) and/or HHpred (33). Extrachromosomal contigs were annotated (as above) and manually curated (using BLAST) to confirm the presence of one or more previously described lactococcal plasmid-borne *rep* genes. Contigs of appropriate sizes containing at least one *rep* gene were determined to be plasmids.

2.3.4 Extracting gene families from plasmid sequences

All-against-all, bi-directional BLAST alignments were performed for all sequence comparisons at protein level. An alignment cut-off value of >50 % amino acid identity across 50 % of the sequence length was used (with an associated E-value of <0.0001). The Markov Clustering Algorithm (MCL) was applied for analysis and clustering of these results in the mclblastline pipeline v12-0678. TM4 MeV, MultiExperiment Viewer v4.9 was employed to view MCL clustering data and to conduct hierarchical clustering (HCL) (https://webmev.tm4.org/about) (Table S2).

2.3.5 Selection criteria and analysis of the candidate systems

At least one representative gene sequence of each family (Table S2) was subjected to comparative sequence analysis (BLASTN and BLASTP) (30) against available sequence data on the National Center for Biotechnology Information (NCBI) database (34) and to protein homology prediction analysis using HHpred (33) and InterPro (35). TMHMM v.2.0 software was used for the prediction of transmembrane regions in these proteins using the hidden Markov model (HMM) (36). The selection criteria for putative antiphage systems were rather loose and based on properties of previously identified lactococcal Abi systems (12). Specifically, single or pairs of genes with a high AT % content (preferably ≥ 70 %) and encoding proteins, predominantly of unknown function and preferably with no transmembrane domains, were manually selected. Additionally, all plasmid sequences were submitted into DefenseFinder v1.0.9 (37, 38) and PADLOC v1.1.0 (39) (databases v1.2.1 and v1.4.0, respectively) to search for established antiphage systems.

2.3.6 Cloning of candidate antiphage systems

DNA fragments to be cloned were amplified using Phusion Green High-Fidelity DNA Polymerase (Thermo Fisher Scientific), Phusion High-Fidelity DNA Polymerase, Q5 High-Fidelity DNA Polymerase or OneTaq DNA Polymerase (all New England Biolabs) according to the manufacturer's instructions and with relevant primers (Table S2; Eurofins Genomics). The initial denaturation step was performed for 3-10 min to allow cell disruption

and release of the template DNA. The high-copy, constitutive-expression lactococcal plasmid pNZ44 was used as a cloning vector (Table S2), although in some cases DNA fragments, which appeared to be recalcitrant to cloning in pNZ44, were instead cloned in the low-copy, nisin-inducible vector, pPTPi (Table S2).

Nine candidates that could not be amplified by PCR were ordered as synthetic genes from Eurofins Genomics. Candidate genes were excised using restriction digestion from synthetic constructs and restricted DNA fragments were manipulated as described below.

FastDigest restriction enzymes, including PstI, KpnI, XbaI, HindIII, SalI, SacI, EcoRI, BamHI (Table S2; Thermo Fisher Scientific) and T4 DNA ligase (Promega) were used according to the manufacturer's instructions. Restricted DNA fragments and corresponding cloning vector backbones were purified prior to ligation using the GenElute PCR Clean-up Kit (Sigma-Aldrich). Ligation mixtures were introduced into *L. lactis* NZ9000 by electroporation (see next paragraph) and transformants were selected based on either chloramphenicol, tetracycline or erythromycin (for the presence of [derivatives of] pNZ44, pPTPi or pPEPi respectively). Two candidates (Table S2, no 129 and 130) were cloned by circular polymerase extension cloning (CPEC, Quan and Tian, 2011), using the primers indicated at the end of Table S2 to prepare the linearised vectors and *E. coli* EC101 as the host.

Transformants carrying the desired recombinant plasmids were screened by colony PCR using vector-specific primers (pNZ44_F and pNZ44_R or

pPTPi_F and pPTPi_R, Table S2). Plasmid DNA was extracted from the positive clones using the GeneJET Plasmid Miniprep/Maxiprep Kit (Thermo Scientific) according to the manufacturer's instructions with the following modifications. Harvested cells were resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing 25 % sucrose and 30 mg/mL lysozyme (Sigma Aldrich) and incubated for 30 min at 37 ^{o}C prior to the plasmid DNA extraction procedure to facilitate the degradation of the cell wall. Sequence integrity of the recombinant plasmids was verified by Sanger sequencing (Eurofins Genomics).

Lactococcal electrocompetent cells were prepared by inoculating 40 ml of GM17 supplemented with 0.5 M sucrose and 1 % (for *L. cremoris* 3107) or 1.5 % glycine (for *L. cremoris* NZ9000 and *L. lactis* IL1403) with up to 5 % of fresh or pre-adapted overnight culture. The culture was incubated at 30 ^{o}C until an OD_{600nm} of 0.5 was reached. Cells were harvested, washed, aliquoted and stored as previously described. 45 µl of competent cell suspension was mixed with 5 µl of the ligation mixture and electroporated at 2.0 kV, 200 Ω and 25 µF in 0.2 cm cuvettes using an ECM 630 Exponential Decay Wave Electroporation System (BTX). Following electroporation, cells were recovered in 950 µl GM17 broth containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$ and incubated at 30 °C for 2.5 h. Bacteria were then plated on GM17 supplemented with relevant antibiotics for selection of pNZ44 or pPTPi and incubated at 30 °C for 24-48 h.

E. coli EC101 electrocompetent cells were prepared by inoculating 500 ml of LB broth with 5 ml of overnight culture. The culture was incubated at 37 ^{o}C with shaking until an OD_{600nm} of 0.8 was reached. Cells were

harvested at 4500 rpm at 4 ^{o}C for 10 min and were then washed twice with 100 ml of ice-cold ELGA water and once with 50 ml of 10 % ice-cold glycerol. The pellet was resuspended in 2.5 ml of 10 % glycerol and aliquots of 100 µl were stored at -80 ^{o}C. *E. coli* competent cells were electroporated as described for the lactococcal cells except using a voltage of 2.5 kV and were recovered in LB broth with no supplementations. Bacteria were plated on LB agar supplemented with 10 µg/mL tetracycline for the selection of pPTPi derivatives and incubated at 37 ^{o}C for 24 h.

2.3.7 Spot and plaque assays

Spot and plaque assays were performed using the double agar method of Lillehaug (40) with some modifications. Solid (bottom layer) and semi-solid agar (top layer) were prepared using GM17 medium supplemented with $CaCl_2$ (10 mM); 1 % bacteriological agar was incorporated in the solid layer, and 0.4 % agar was used in the semi-solid agar. To enlarge the plaque size, semi-solid agar was supplemented with 0.2 % agarose instead of agar or 0.4 % glycine was added to both layers where required. SM buffer (10 mM $CaCl_2$, 100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl at pH 7.5) was used as the diluent in all bacteriophage assays. The efficiency of plaquing (EOP) was determined by dividing the titre (PFUs/ml) of the test strain by that of the control strain. An antiphage system was considered to contribute to plaque size reduction if the plaque size range of the phage plated on the strain expressing the antiphage system was substantially smaller compared to the plaque size range of the same phage plated on the wild-type strain under the same conditions. An example of plaque counts,

EOP calculation with standard deviation and plaque size range measurements can be found in Table S3.

2.3.8 Adsorption and transduction assays

Adsorption assays were carried out following the protocol described by Mosterd and Moineau (41) with some modifications. Specifically, host cells were grown at 30 ^{o}C and $CaCl_2$ was added to a final concentration of 10 mM.

Transduction assays were based on the protocol of McGrath et al. (42). To prepare sk1 and c2 transduction lysates, phages sk1 and c2 were propagated on *L*. *cremoris* MG1363 harbouring pPTPL-sk1*cos* or on *L*. *cremoris* NZ9000 carrying pPTPi-c2*cos*, respectively (Table S2). At an OD_{600nm} of 0.2, strains to be tested were infected with transduction lysate at a multiplicity of infection (MOI) of 1. $CaCl_2$ was added to a final concentration of 10 mM and after 10 min of incubation at 30 ^{o}C cultures were plated on solid growth medium supplemented with 5 µg/ml tetracycline for the selection of the transductants. Transduction frequencies were calculated as number of transductants divided by the total number of phage particles applied.

2.3.9 Lysis-in-broth assay

Overnight cultures of *L. cremoris* NZ9000 carrying pNZ44/pPTPi and their antiphage system derivatives were subcultured (2 %) in GM17 broth medium and incubated at 30 ^{o}C until an OD_{600nm} of 0.2 was reached. $CaCl_2$

was added to a final concentration of 10 mM and an equal volume of phage sk1 or c2 was added to a final MOI of 0.05 and 5. For the uninfected control culture, an equal volume of spun-down and filtered overnight culture was used. 200 µL of each culture was then transferred into a 96 wells plate. Plates were incubated at 30 ^{o}C and OD_{600nm} measurements were recorded every 30 min for a total of 300 min with a MULTISKAN FC plate reader (Thermo Fischer Scientific).

2.3.10 Alphafold2 structure and domain predictions

We performed predictions with either a Colab notebook running AlphaFold v2.3.1 (43, 44) (https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb) or HPC resources from GENCI-IDRIS running AlphaFold v2.3.1 (45). The pLDDT values (Figure S5) of predicted structures were stored in the PDB files as B-factors and plotted with the Colab or the IDRIS resources. The final predicted protein or domain structures were submitted to the Dali server (46) to identify the closest structural homologues in the PDB. Visual representations of the structures were prepared with ChimeraX (47).

2.3.11 Assessing prevalence by BLASTP and HMMER searches

Antiphage systems were subjected to NCBI's non-redundant protein sequence database (BLASTP (48)). A cut-off value of >70 % amino acid identity with an associated E-value of <0.001 was used to factor the distinct

sequence hits or organisms. Similarly, the new antiphage sequences were subjected to HMMER (49). NCBI's non-redundant protein sequence database (34) filtered for a maximum pairwise sequence identity of 70 % was selected with a cut-off E-value of <0.001 for the target sequences, while a maximum number of 10000 hits was selected to be displayed.

2.4 Results

2.4.1 Selection of candidate plasmid-encoded antiphage systems

To identify previously unknown, non-RM antiphage systems encoded by lactococcal plasmids, a total of 321 plasmid sequences derived from 53 sequenced lactococcal strains in our collection (University College Cork and dsm-firmenich) were analysed. These strains were found to harbour four to 10 plasmids, with an average of six plasmids per strain. Based on hierarchical clustering (HCL) analysis, the 819,589 identified plasmid-borne coding sequences were grouped into 1,019 distinct genes families (Table S2). For a gene sequence to be selected as a candidate antiphage system, relaxed criteria were applied based on the generic properties of previously identified lactococcal Abi systems (see methods section).

Genes that encode proteins identical (AbiA, AbiE, AbiF, AbiG, AbiJ, AbiP and AbiZ) or similar (11 AbiB-like, AbiC-like, AbiD-like and AbiR-like; ranging from 38.4 to 99.8 % amino acid identity) to the 23 previously established lactococcal Abi systems (12) were also identified during the candidate gene selection and were included in our experimental approach to validate our identification methodology and to provide a more complete

overview of all non-RM antiphage systems present in our surveyed plasmidome. In addition to these 11 systems, a homologue encoding the AbiQ toxin (50) was present in our plasmid collection, though apparently in the absence of its associated antitoxin and was therefore not included in our experimental approach. Interestingly, for 11 out of the 23 currently known lactococcal Abi systems, no homologues were found within the 1,020 analysed gene families representing our plasmid collection.

To identify additional antiphage systems not previously confirmed or described in *Lactococcus*, the lactococcal plasmidome was also subjected to bioinformatic scrutiny using PADLOC (Prokaryotic Antiphage Defence LOCator) and DefenseFinder (37–39). This resulted in the prediction of eight antiphage systems previously shown to be active in bacteria other than *Lactococcus lactis*/*cremoris* (i.e. Bunzi, Cyclic oligonucleotide-based antiphage signaling system [type I and II CBASS], DUF2290/Helicase, Lamassu, phage anti-restriction-induced system [PARIS], RnlAB and Septu type I).

In total, we selected 198 candidate antiphage-encoding systems (153 of which represent a single gene, while the remaining 45 consist of more than one gene), together representing 232 different gene families (Table S2).

2.4.2 Identification of plasmid-encoded antiphage systems

The 198 candidate antiphage systems were cloned in pNZ44 (high copy vector) or pPTPi (low copy vector) and introduced into *L. cremoris* NZ9000. Following sequence verification of the resulting recombinant plasmids,

plaque assays were performed against a selection of lactococcal phages to determine if the presence of a given recombinant plasmid was associated with phage resistance.

Of these 198 tested candidates, 20 were shown to provide resistance against the tested phages (discussed in detail below). Eight of these were homologues of the previously established lactococcal Abi systems (AbiA, AbiB-like, AbiD-like, AbiF, AbiG, AbiJ, AbiP, and AbiZ with AbiB-like and AbiD-like exhibiting 94 % and 59 % amino acid sequence identity to AbiB and AbiD, respectively). Interestingly, AbiF-mediated phage resistance activity was initially not observed when *abiF* was cloned with the native or an artificial Shine Dalgarno sequence derived from pNZ8048 (Table S2). However, homology at amino acid sequence level had previously been reported between AbiD, AbiD1 and AbiF (51–53). For AbiD1, it had been shown that a short upstream sequence containing a strong promoter and terminator was required for its antiphage activity (52). When analysing if *abiF* and *abiD-like* are preceded by a similar sequence structure, the sequence upstream of *abiF* and *abiD-like* was found to be 68 % and 80 % identical, respectively, to that upstream of *abiD1*. Cloning *abiF* and *abiD-like* with their respective upstream promoter and terminator sequences resulted in constructs which upon testing were shown to exhibit antiphage activity in the particular set-up as assessed here. Interestingly, several systems similar to established Abi systems showed no antiphage activity. In more detail, only one out of the eleven AbiB-like constructs (94 % amino acid identity to AbiB) was shown to elicit anti-phage activity. The remaining (ten) assessed and apparently inactive AbiB-like systems shared 92 to 99

% identity at amino acid level though with varying coverage which in many cases appeared to be due to gene truncations. AbiC-like shares just 38 % amino acid sequence identity to the previously described AbiC and provided no resistance against the tested phages. The absence of AbiE activity is probably due to its phage specificity, as it had previously been shown to be active only against one particular phage (out of several tested) (54), an exact copy of which we did not have in our collection. AbiR-like was not analysed due to cloning issues.

Among the eight systems identified by PADLOC and DefenseFinder (i.e. Bunzi, type I and II CBASS, DUF2290/Helicase, Lamassu, PARIS, RnlAB and Septu, Table S2), antiphage activity of five lactococcal homologues (PARIS, type I and II CBASS, Lamassu and Septu) was confirmed in our *Lactococcus* test set-up. Bunzi, DUF2290/Helicase and RnlAB did not offer any protection activity against tested lactococcal phages.

Based on comparative sequence analysis (BLASTN and BLASTP) and on functional and structural analysis (HHpred and InterPro) the remaining seven antiphage systems (out of the twenty identified) did not display significant homology to any previously described antiphage system, thus rendering them novel antiphage systems (Table 1). These systems were named after cheese, cattle or fermentation related deities (Rhea, Aristaios, Kamadhenu, Fliodhais, Audmula, Rugutis and Hesat), following a similar naming approach as established by Doron et al. (17). Except for Hesat, which was cloned in pPTPi with the addition of its native signals, all novel systems were cloned in the high-copy number vector pNZ44 (Table S2). All discovered systems, except Fliodhais, are single gene antiphage systems.

Both genetic components are necessary for Fliodhais activity; the described antiphage activity (see below) was not observed when the two components were cloned and tested separately.

Table 1. Names, genes loci and predicted domains of the discovered antiphage systems.

	System name	Gene locus	Protein size (aa)	InterPro Prediction	HHpred Prediction	AlphaFold2/Dali Prediction	Associated InterPro/Pfam	Associated HHpred
New antiphage systems	Rhea	pUCCL617_141	303	-	-	transposase TnsE (62)	-	-
	Aristaios	pUCCL641_199	316	DUF2971	-	ADP-ribosyltransferase (64)	IPR021352/ PF11185	-
	Kamadhenu	pUCCL624_130	180	YfbU (66)	YfbU	YfbU	IPR005587/ PF03887	1WPB_F
	Fliodhais	pUCCL632_186	41	-	lipoprotein	membrane protein	-	5CYB_A
		pUCCL632_185	255	-	-		-	-
	Audmula	pUCCL630_365	392	PBP transglycosylase	PBP	transglycosylase	IPR036950	2JCH_A
	Rugutis	pUCCL633_059	359	SEC-C motif	SEC-C motif	SEC-C motif	IPR004027/ PF02810	2I9W_A
	Hesat	pUCCL620_156	213	-	-	colicin-D toxic domain (68)	-	-
Systems new in *Lactococcus*/ -like systems	PARIS	pUCCL620_250	453	AAA_21 ATPase (19)	SMC protein	Rad50 ATPase	IPR003959/ PF13304	6QJ0_A
		pUCCL620_249	280	DUF4435 (19)	Hypothetical protein PH0156	TOPRIM domain of OLD nucleases (19)	IPR029492/ PF14491	2P62_A
	Type I CBASS	pUCCL631_167	190	SLATT effector (72)	-	SLATT effector	IPR040811/ PF18186	-
		pUCCL631_168	408	NTase/AGS-C sensor (70)	CD-NTase (72)	CD-NTase	IPR006116/ IPR040511/ PF18134	7LJO_A

Type II CBASS	pUCCL620_247	286	SUa-2TM effector (72)	-	Cap5 SAVED (71)	IPR041502/ PF18179	-
	pUCCL620_246	383	NTase/SMODS (72)	CD-NTase	CD-NTase	IPR006116/ PF18144	7LJN_C
	pUCCL620_245	155	-	Ubiquitin-conjugating enzyme E2[a]	Cap2 (72)	-	7JZV_A
Lamassu	pUCCL630_136	427	ABC-3C system CTD	Cap4 endonuclease[a] (22)	Cap4 SAVED/CARF domain	IPR046920/ PF20276	7YIB_A
	pUCCL630_135	242	ABC-3C system MC	-	CDI DNAse	IPR046905/ PF20289	-
	pUCCL630_134	883	AAA_23 Rad50/SbcC ATPase	SMC protein (22)	Rad50 ATPase	IPR038729/ PF13476	7TVE_E
Septu	pUCCL618_274	537	AAA_21 ATPase (17)	ARE-ABCF protein	excinuclease ABC	IPR003959/ PF13304	8BUU_9
	pUCCL618_273	443	HNH endonuclease (17)	Cas9 endonuclease	HNH endonuclease/GAF domain	IPR003615/ PF01844	7ENH_A
AbiD-like	LLA22_pB0040	345	AbiD/AbiF-like	-	Cas13d ribonuclease (75)	IPR017034/ PF07751	-

The associated HHpred represents the highest probability hit (> 97 %) with an E-value below 0.001 except for [a] (probability > 95 %, 0.01 ≤ E-value ≤ 0.05). DUF (domain of unknown function); PBP (penicillin binding protein); AAA (ATPases associated with diverse cellular activities); SMC (structural maintenance of the chromosome); TOPRIM, (topoisomerase-primase); SLATT (SMODS [second messenger oligonucleotide or dinucleotide synthetases] and LOG-associating two TM [transmembrane]); AGS-C, (Adenylyl, Guanylyl & SMODS sensor C-terminal); CD-NTase (cGAS/DncV-like nucleotidyltransferase; Sua-2TM (SMODS/Ubiquitin system-associated 2TM); Cap (CD-NTase-associated protein); SAVED (SMODS-associated and fused to various effector domains); ABC (ATP-binding cassette); CTD (C-terminal domains); CARF (CRISPR-associated Rossman fold); MC (Middle Component); CDI (contact-dependent growth inhibition); ARE-ABCF (antibiotic resistance ABC protein of the F subfamily); Cas (CRISPR-associated); GAF (cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA)

2.4.3 Antiphage spectrum of the verified antiphage systems

The antiphage activity spectrum of the twenty antiphage systems when present in *L. cremoris* NZ9000, *L. cremoris* 3107 or *L. lactis* IL1403 was evaluated by challenging these strains with various lactococcal bacteriophages (representing the *Skunavirus*, *Ceduovirus*, P335 group and *Teubervirus* genera).

Figure 1 (Detailed information is presented in Table S3) summarises the efficiency of plaquing (EOP) of each tested phage on the lactococcal strain harbouring a given antiphage system compared to the corresponding wild-type strain carrying the empty vector (pNZ44 or pPTPi). In this experimental setup, all 20 systems, except Fliodhais and Audmula, provided (varying levels of) resistance against at least one member of the *Skunavirus* genus, which are the most frequently encountered phages in the dairy industry (55). Furthermore, the majority of the systems was shown to exhibit a similarly high level of resistance (over four log EOP reduction) against all assessed skunaviruses. Varying levels of resistance across assessed skunaviruses were observed for Rugutis, AbiG, AbiJ and AbiF. The anti-*Skunavirus* activity of Rugutis, AbiG and AbiJ is strain-dependent, whereas the activity of AbiF was shown to be phage-specific in our tests. Eleven out of the 18 systems that provide resistance against skunaviruses also cause noticeable plaque size reduction (Figure 1).

Ten systems were shown to provide varying levels of resistance against the *Ceduovirus* c2, another dominant lactococcal phage genus (56). Fliodhais was associated with smaller c2 plaques surrounded by a halo (<1

mm clear-centred plaques with a faint ring), a phenotype that was not observed when phage c2 was plated on the wild-type strain (up to 4 mm clear-centred plaques without a halo). Resistance against the P335 phage group was limited to five systems and a single phage (P335), except for Rhea which was unique in providing high resistance against phage TP901-1. It should be noted however that many systems were not tested against this phage group, as many strains could not be constructed due to various issues (Figure 1). None of the tested systems was effective against the *Teubervirus* P087.

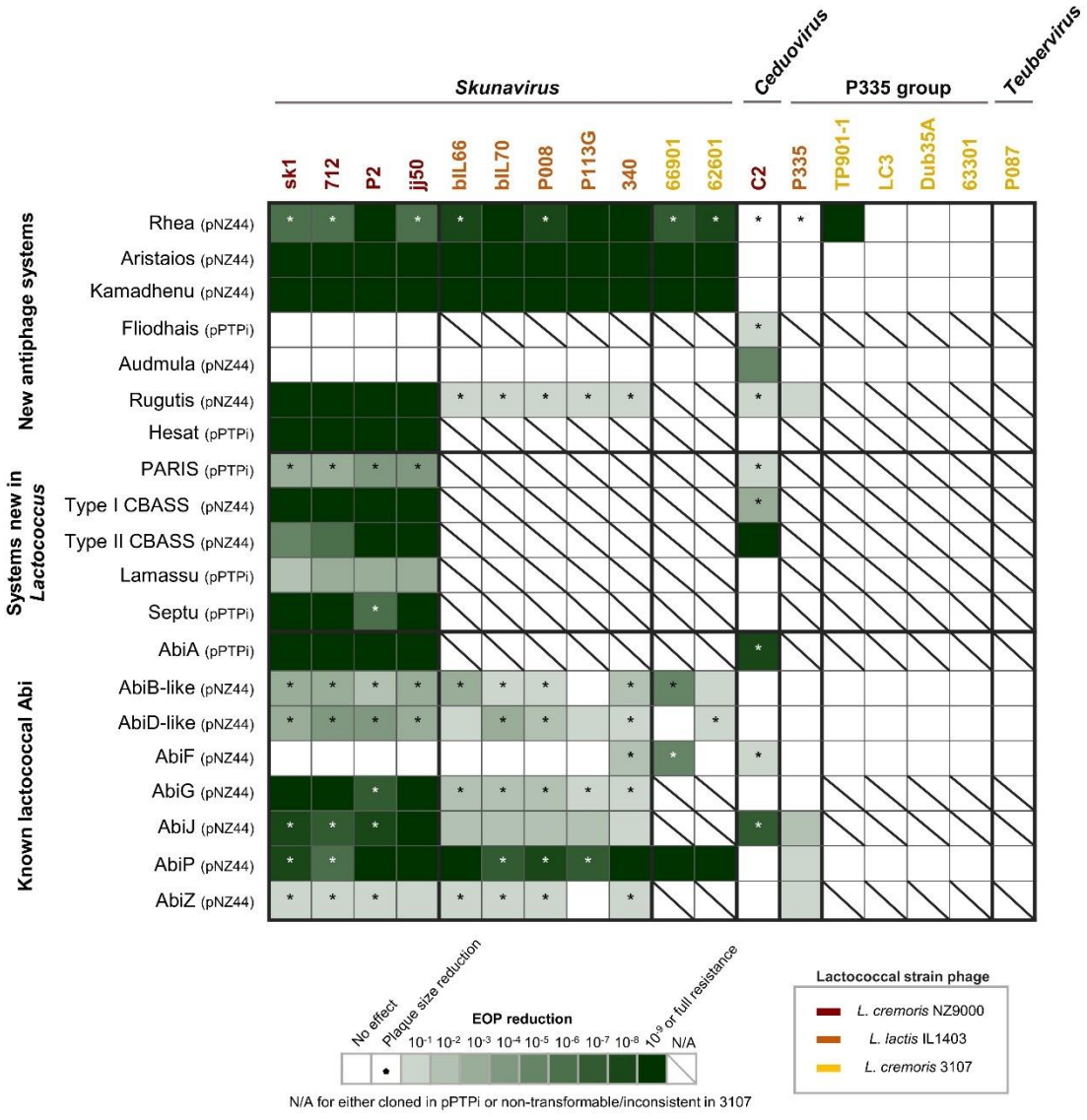


Figure 1. Efficiency of plaquing (EOP) of different phage genus representatives (*Skunavirus*, *Ceduovirus*, P335 group & *Teubervirus*) on different lactococcal host strains (NZ9000, 3107, IL1403) carrying an antiphage system. Detailed EOP information summarised in this figure is presented in Table S3. Data represent the mean of three biological replicates. As the nisin-inducible P*nisA* promoter requires the NisRK two-component system, which is not present in *L. cremoris* 3107 or *L. lactis* IL1403, the pPTPi-derived constructs were only tested in *L. cremoris* NZ9000. Additionally, some of the generated expression constructs, although introduced and tested in *L. cremoris* NZ9000, could not be introduced or were not stable in *L. cremoris* 3107 or *L. lactis* IL1403 (Rugutis, type I and II CBASS, AbiG, AbiJ and AbiZ), and therefore the corresponding EOP values could not be defined.

2.4.4 The newly identified lactococcal antiphage systems are active post phage DNA injection

To examine if the newly identified antiphage systems are active extracellularly or intracellularly, before or after phage adsorption/DNA injection, phage adsorption and transduction assays were performed. Fliodhais, whose antiphage effect was limited to plaque size reduction, and previously characterised lactococcal Abi systems were excluded from these characterisation assays.

Adsorption assays (Figure 2A-C) revealed that the assessed systems do not interfere with phage sk1 or c2 adsorption. Similarly, DNA injection does not appear to be noticeably affected by the tested antiphage systems as the observed transduction frequencies are comparable to those obtained for the control strains (Figure 2D-F). Transduction frequencies observed in the presence of the Audmula and PARIS systems were slightly higher (from $[1.69 \pm 0.37] \times 10^{-4}$ to $[2.71 \pm 0.23] \times 10^{-4}$ and from $[2.90 \pm 0.44] \times 10^{-5}$ to

[3.70 ± 0.71] x 10^{-5} respectively) than the control strains. Conversely, Rhea, Aristaios and Rugutis antiphage systems were associated with an observed transduction frequency reduction of up to one log. A possible explanation for these differences can be the overexpression of the systems interfering with other cellular activities and thus slightly with phage DNA injection. However, this effect is negligible when compared to the observed transduction frequency reduction of a strain expressing the known DNA injection blocking protein Sie_{2009} (57), whose transduction frequencies were below the detection limit (<1.05 x 10^{-7}). Therefore, these antiphage systems do not appear to specifically target phage adsorption or DNA injection steps, indicating that they are active intracellularly at a stage beyond phage DNA injection.

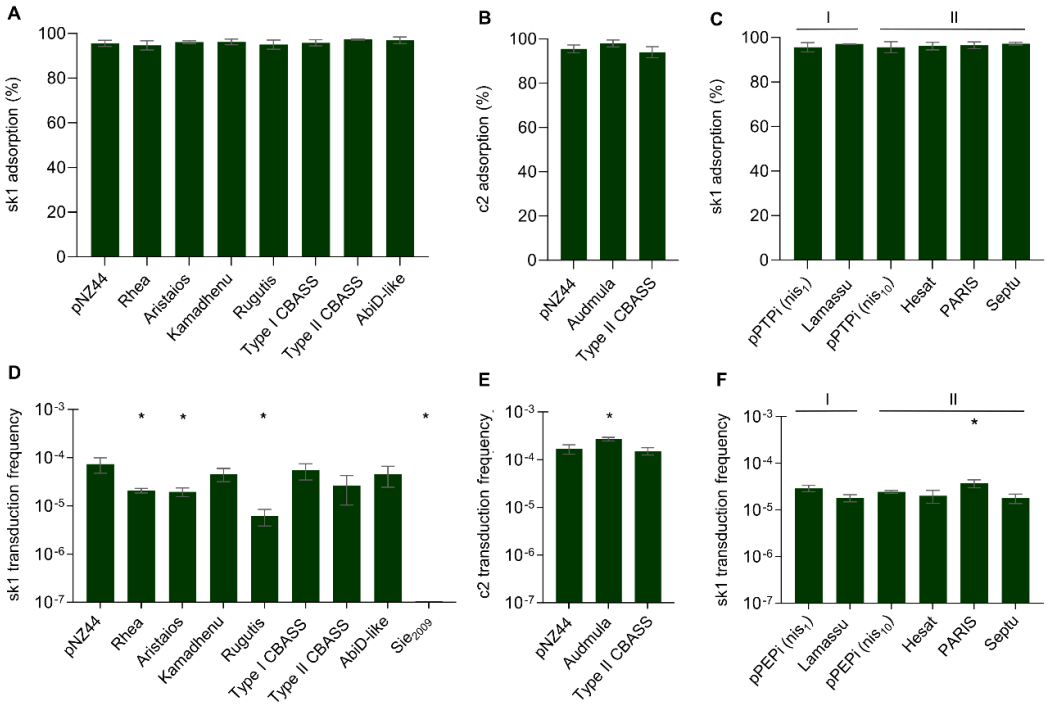


Figure 2. Adsorption and transduction assays. Adsorption percentages of phages sk1 (panel A) and c2 (panel B) to *L. cremoris* NZ9000 strains carrying antiphage systems compared to control strains *L. cremoris* NZ9000::pNZ44 and *L. cremoris* NZ9000::pPTPi

induced at 1 ng/mL (panel C I) or 10 ng/mL nisin (panel C II). Transduction frequencies for *L. cremoris* NZ9000::pNZ44 derivatives transduced with pPTPL-sk1*cos* (panel D) or pPTPi-c2*cos* (panel E) or *L. cremoris* NZ9000::pPEPi derivatives induced at 1 ng/mL (panel F I) or 10 ng/mL (panel F II) transduced with pPTPL-sk1*cos*. Experiments were performed in biological triplicate and data are presented as means ± standard deviation (SD). Asterisks mark statistically significant differences in transduction frequencies between the samples and the relevant controls (unpaired t-test, P-value < 0.05).

2.4.5 Most of the newly identified antiphage systems present Abi phenotypes

To assess if the newly identified antiphage systems exhibit phenotypic characteristics typical of abortive infection systems, lysis profiles of *L. cremoris* NZ9000 and its derivative strains expressing antiphage systems were determined at a low (0.05) and a high (5) multiplicity of infection (MOI) of phage sk1 or c2 (Figure 3). A phenotype of providing phage resistance at a low MOI, yet entering a bactericidal/bacteriostatic state at a high MOI is consistent with an antiphage system acting through abortive infection (15, 58). All tested systems provided phage resistance under low MOI conditions, growing at rates comparable to those of uninfected strains, while corresponding cultures carrying the empty vectors were shown to collapse. The exception to this was Audmula, where delayed lysis occurred 120 min later than the phage-sensitive control, *L. cremoris* NZ9000::pNZ44. However, at a high MOI, all cultures, including those expressing the newly identified antiphage systems demised or entered a state of bacteriostasis. Therefore, all assessed systems, except Audmula, phenotypically behave like an Abi system, though further experimentation is required to validate

this (59). It should be noted that the uninfected control for several strains expressing antiphage systems (notably PARIS and Lamassu) exhibited a decreased growth rate, indicating that expression of certain systems comes at a fitness cost, consistent with previous observations (60, 61).

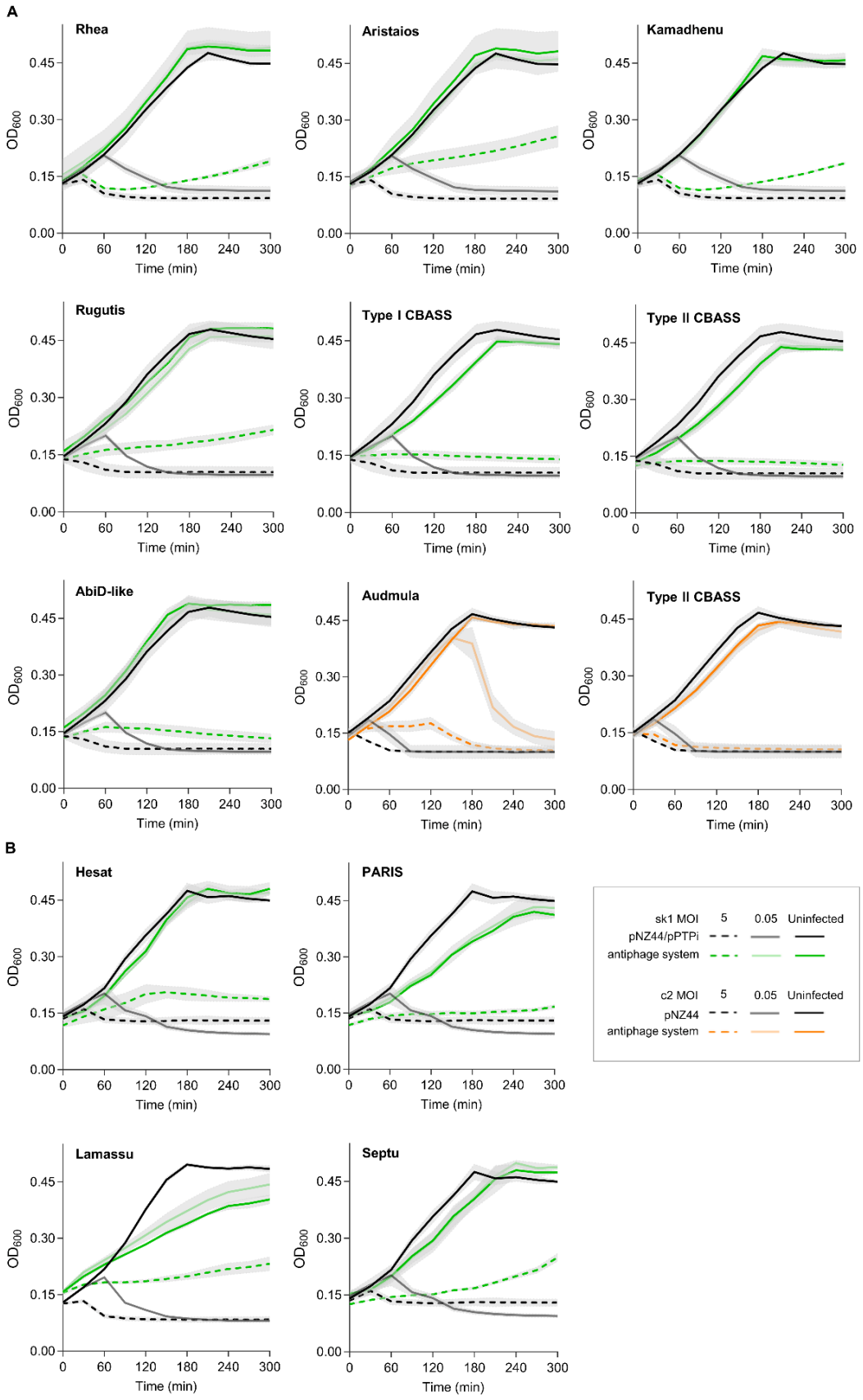


Figure 3. Lysis-in-broth assays. Lysis-in-broth graphs for *L. cremoris* NZ9000::pNZ44 and various antiphage system-expressing derivatives infected by phages sk1 and c2

(panel A), as well as for *L. cremoris*::pPTPi and various antiphage-expressing derivatives infected by phage sk1 (panel B). Each growth curve represents the mean of three biological replicates and the shaded area corresponds to the standard deviation.

2.4.6 Domain and structure prediction of the novel antiphage systems

To obtain information on the possible mode of action for each of the identified antiphage systems, the individual protein(s) of each system were structurally analysed with AlphaFold2 and Dali, and the obtained results were then compared with functional domain predictions obtained by HHpred and InterPro (Table 1). The associated HHpred prediction represents the highest probability hit (> 97 %) with an E-value below 0.001. The Alphafold2 pLDDT values and predicted aligned errors (PAE) plots are present in Figure S5. Z-scores and rmsd values are presented in the figure legends.

Employing HHpred and InterPro, no significant protein domain predictions were found for Rhea. However, based on AlphaFold2 analysis, the C-terminus of Rhea resembles the DNA-binding protein TnsE (Protein Data Bank [PDB] ID 5D17; Figure 4A, B), which is part of a multicomponent transposon complex (62). TnsE is believed to recognise replication intermediates that are typical of conjugative plasmids or of replicating phages, suggesting that this domain is responsible for sensing phage infection with subsequent activation of the antiphage system. Such a mechanism would be reminiscent of type II Lamassu, which protects *Vibrio cholerae* from both phage infection and plasmid invasion by recognising (phage) DNA replication intermediates (63).

The Aristaios antiphage system contains a domain of unknown function (DUF2971) as based on HHpred and InterPro searches. AlphaFold2 analysis revealed that an ADP-ribosyltransferase domain covers most of Aristaios (PDB ID 5ZJ5; Figure 4C, D, E). Such a protein fold is present in a family of bacterial toxins (ADP-ribosylating toxins) that inhibit essential host protein (and potentially phage) functions (64), likely underpinning the abortive infection phenotype elicited by Aristaios. Recently, a DarTG toxin-antitoxin system has been described that depends on the DNA ADP-ribosyltransferase activity of its DarT toxin to modify invading phage DNA and prevent phage replication (65).

AlphaFold2 revealed that Kamadhenu contains a YfbU (PDB ID 1WPB; Figure 4F, G), a two-domain helical protein, which has been reported to be involved in cell death when triggered by DNA damage (66). This would therefore indicate a mechanistic phenomenon in accordance with the observed abortive infection phenotype of Kamadhenu.

The N-terminus of Audmula contains four transmembrane helices, followed by a large C-terminal helical domain that resembles a transglycosylase (PDB ID 3VMR; Figure 4H, I). This suggests that Audmula, the expression of which results in delayed lysis, is involved in cell wall modification, possibly affecting the lysis stage of the phage infection cycle. Cell wall modification resulting in delayed lysis of phage infected cells has previously been demonstrated in *B. subtilis*, where dynamin-like protein DynA forms complexes, thereby possibly stabilising the cell membrane (67).

Based on HHpred outputs, the first component of Fliodhais resembles a lipoprotein that is associated with virulence in *Streptococcus pneumoniae*. AlphaFold2 prediction reveals that Fliodhais is a mono-topic membrane protein (Figure 4K, L). Given the observed mild antiphage activity of this system, it is possible that the antiphage activity is an artefact of this protein complex, perhaps causing premature lysis at a stage when phage particles are not fully matured (due to incomplete assembly or DNA packaging). However, a lipoprotein-containing antiphage system (PD-Lambda-6) has been reported previously (20).

The N-terminus of Rugutis contains a SEC-C motif based on HHpred analysis (PDB ID 2I9W). AlphaFold2 comparison of Rugutis and SEC-C motif containing protein 2I9W reveals that approximately 20 residues at the N-terminus of Rugutis superimpose well with the C-terminus of the SEC-C motif containing protein, while the remainder of the proteins do not share any similarity. Despite the high-quality AlphaFold2 score of the Rugutis structure prediction (Figure 4M), Dali analysis did not yield significant hits. Rugutis, therefore, possesses a structure that is distinct from those present in the Protein Data Bank.

For Hesat, no domain was predicted by HHpred and InterPro. Dali analysis reports significant hits with the colicin-D toxic domain (PDB ID 1V74; Figure 4N, O), which specifically cleaves the anticodon loop of all four tRNA(Arg) iso-acceptors, thereby resulting in cell death (68). Therefore, it is proposed that Hesat upon phage infection is activated to block translation, which is in accordance with its abortive infection phenotype.

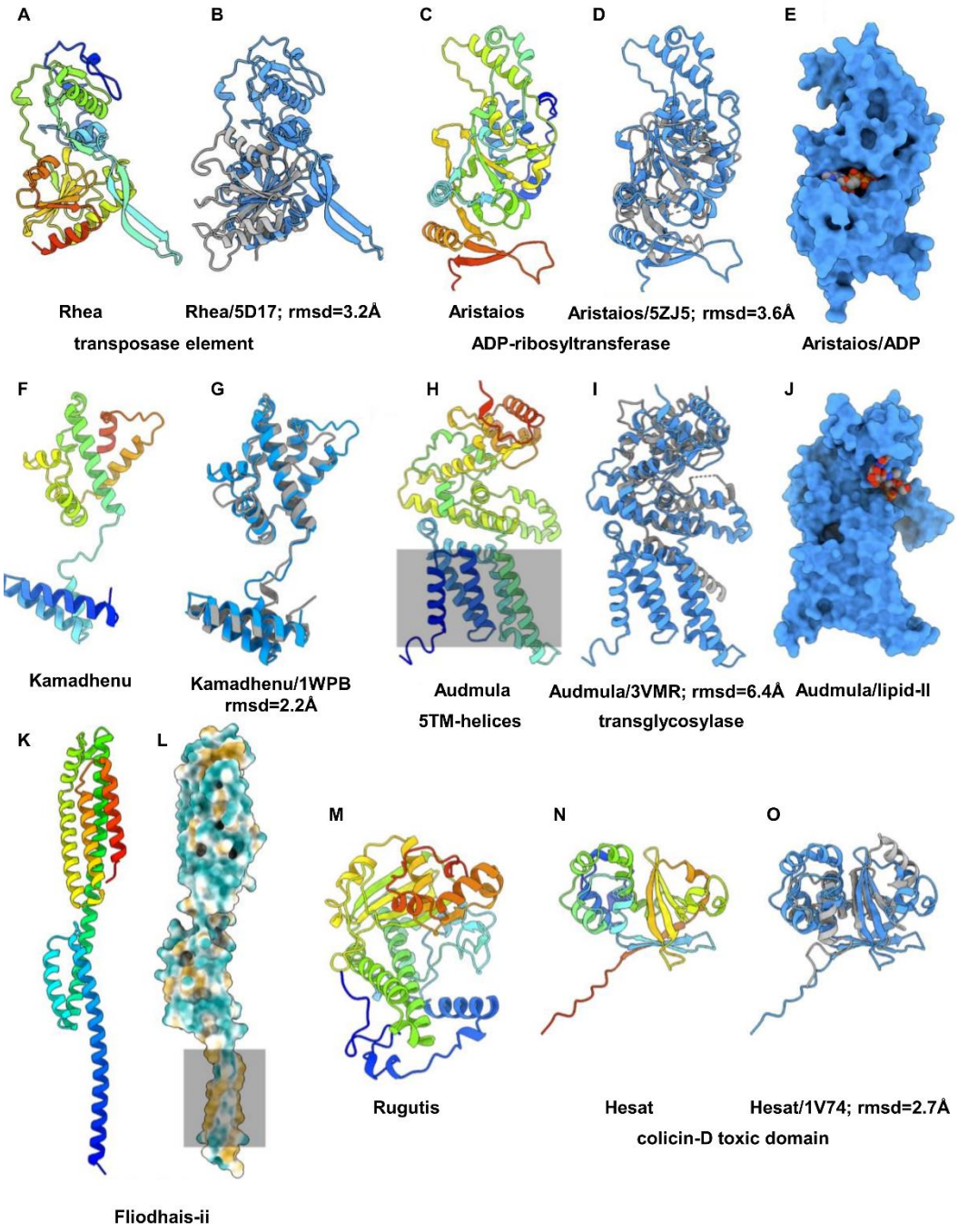


Figure 4. Predicted structures of novel antiphage systems. **A)** Ribbon structure of Rhea (rainbow coloured). **B)** Superimposition of Rhea (blue) with PDB 5D17 (grey), a transposase element (Z=6.2, rmsd=3.2Å). **C)** Ribbon structure of Aristaios (rainbow coloured). **D**) Superimposition of Aristaios (blue) with PDB 5ZJ5 (grey), an ADP-ribosyltransferase (Z=5.6, rmsd=3.6Å). **E)** Aristaios molecular surface (blue) with ADP from 5ZJ5 in the catalytic crevice. **F)** Ribbon structure of the two-domain protein Kamadhenu

(rainbow coloured; no significant hit in the PDB). **G)** Superimposition of Kamadhenu (blue) with PDB 1WPB (grey), the YfbU protein forming a large complex of unknown function (Z=16.4, rmsd=2.2Å). **H)** Ribbon structure of Audmula (rainbow coloured; the 5 trans-membrane helices are boxed in grey). **I)** Superimposition of Audmula (blue) with PDB 3VMR (grey), a transglycosylase; (Z=9.8, rmsd=6.4Å. 3VMR possesses a unique transmembrane helix). **J)** Audmula molecular surface (blue) with Lipid II from 3VMR in the catalytic crevice. **K)** Ribbon structure of Fliodhais-ii (rainbow coloured). Several hits with membrane proteins (boxed in grey) do not point to a defined function. Fliodhais-i (not presented here) was not reliably predicted by AF2, including Fliodhais-i/ii complexes. **L)** Fliodhais-ii molecular surface (coloured according to hydrophobicity, brown). Residues 10-32 form a trans-membrane helix. **M)** Ribbon structure of Rugutis (rainbow coloured; no significant hit in the PDB). **N)** Ribbon structure of Hesat (rainbow coloured). **O)** Superimposition of Hesat (blue) with PDB 1V74 (grey), a colicin-D toxic domain (Z=4.9, rmsd=2.7Å). pLDDT values and predicted aligned errors (PAE) plots present in Figure S5.

2.4.7 Domain and structure prediction of previously described systems

InterPro predictions confirmed that the lactococcal PARIS system consists of an ATPase Associated with a variety of cellular Activities (AAA_21) and a DUF4435 domain, in accordance with the previously described PARIS system (19). Here, based on Alphafold2 prediction we provide the relevant structures (Figure S1) and we report in more detail that the first component of the PARIS system (PARIS-i) resembles a DNA double-strand break repair Rad50 ATPase (PDB ID 3QKU: Figure S1 A,B), supporting the notion that this protein is responsible for sensing invading phage and then activating an associated nuclease by its ATPase activity via a mechanism similar to the Mre11-Rad50 homolog SbcCD (69), as has previously been suggested for the Lamassu system (22). Based on HHpred,

Rousset et al., identified a weak but statistically significant match of part of the second component of PARIS (PARIS-ii) with a Topoisomerase-primase (TOPRIM) domain of OLD family nucleases (19), consistent with our Alphafold2 prediction (PDB ID 6P74; Figure S1B, C). The lactococcal PARIS system was found to exhibit low amino acid similarity with the two distinct *E. coli* PARIS-1 and -2 systems (≤32 %) (19). Nevertheless, AlphaFold2 predictions showed that the two systems superimpose well (Figure S1C, F).

HHpred, InterPro and AlphaFold2/Dali revealed the presence of CBASS-related domains (70–72) in type I and II CBASS (Table 1, Figure S2, (73)). A lactococcal type II CBASS system has previously been described, although its antiphage activity was not explored (71). No lactococcal equivalent of type I CBASS has been reported in literature to our knowledge.

Based on InterPro the lactococcal Lamassu was found to adhere to the described type II Lamassu of the recently described Lamassu family consisting of a Cap4 endonuclease, an unknown protein and a structural maintenance of the chromosomes (SMC) protein (22, 39, 63). Here, AlphaFold2 was able to predict the structures of Lamassu-i, -ii and -iii (Figure S3A, C, E) as well as a hexameric complex by a dimer of the Lamassu-i, -ii, -iii trimer (Figure S3G, H). The second component of Lamassu (Lamassu-ii or LmuC) is believed to be necessary for defensive activity, though its function was not defined (22, 39). AlphaFold2/Dali predict that the D1 domain of this protein resembles a contact-dependent growth inhibition (CDI) DNAse (PDB ID 4G6U; Figure S3D). Bacterial CDI proteins are known to act as toxins or immunity systems (74), suggesting that this is

(part of) the effector of the system. Additionally, the predicted Lamassu-iii (LmuB) globular domain (D1, Figure S3E) is similar to a dsDNA break repair Rad50 ATPase (PDB 7YZO; Figure S3F), supporting previous reports indicating that this protein is responsible for sensing invading phage DNA upon which it activates an associated nuclease (LmuA) by its ATPase activity (22, 63).

InterPro yielded hits with previously reported Septu-related proteins (ATPase and HNH endonuclease (17) for the lactococcal Septu antiphage system. AlphaFold2 was able to predict the structures of the two Septu components (Figure S4A, B), providing novel domain information. Septu-i and -ii contain three domains and AlphaFold2 suggests that domain D3 from Septu-i binds D2 from Septu-ii (Figure S4C, D). Dali analysis reported hits between Septu-i second domain (D2) and an excinuclease ABC (PDB ID 2VF7; Figure S4E). The first Nt domain (D1) of Septu-ii is similar to an HNH endonuclease (PDB ID 5H0O) according to Alphafold2 and Dali, while its third domain (D3) C-terminal domain resembles a GAF domain (PDB ID 8DGD; Figure S4F).

AbiD, AbiD1 and AbiF share modest amino acid similarity (51–53). Here, we predicted the structures of AbiD, AbiD-like, AbiD1 and AbiF (Figure 5A-D). The core of the four proteins superimpose well (Figure 5E). Furthermore, AbiD superimposes well with a Cas13d RNA-guided ribonuclease (PDB ID 6IV8; Figure 5F), including its catalytic site (residue His 293 with AbiD His520) previously associated with abortive infection (75). These results allow amalgamation of these previously distinct systems into a single Abi

group and suggest a similar mode of antiphage action. We propose this antiphage Abi group henceforth to be named AbiD/F.

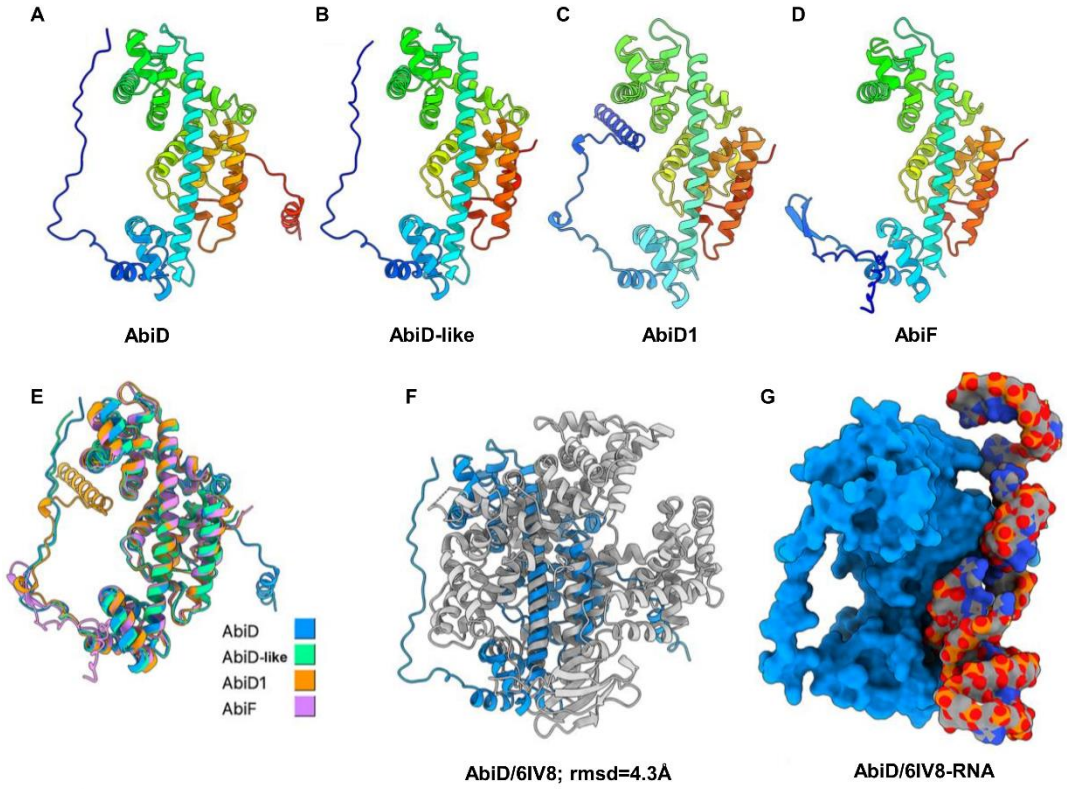


Figure 5: Predicted structures of AbiD-like and related Abi systems. A-D) Ribbon structure of AbiD (A), AbiD-like (B), AbiD1 (C) and AbiF (D) (rainbow coloured). **E)** Superimposition of AbiD (blue), AbiD-like (green), AbiD1 (orange) and AbiF (pink). **F)** Superimposition of AbiD (blue) and PDB 6IV8 (grey), a Cas13d RNA-guided ribonuclease (Z=8.0; rmsd=4.3Å). **G)** Molecular surface of AbiD in contact with that of the RNA from the Cas13d complex. Note the complementarity of the surfaces indicating conservation of the catalytic site. Cas13d catalytic residue His 293 superimposes with AbiD His 520. pLDDT values and predicted aligned errors (PAE) explanation present in Figure S5.

2.4.8 Distribution and conjugation potential of the identified systems

Interestingly, the majority of the confirmed antiphage systems (15 out of 20) are rather rare among the gene families within our collection with only a

few homologues present (≤3 gene family members/homologues, Table S2). In contrast, Rugutis (n=four), Hesat (n=seven), AbiF (n=five), AbiD-like (n=nine), and AbiB-like (n=31) are more abundant gene families. It is worth noting that despite the high occurrence of AbiB homologues, only one out of the 11 (randomly picked from the 31) tested candidates (94 % sequence identity to AbiB) was shown to possess antiviral activity in this study. The remaining homologues share a sequence identity level ranging from 90% to 100% compared to AbiB, with nearly half of them being full-length while the remainder apparently representing truncated versions (with coverage ranging from 16% to 88%). To determine the prevalence of the identified antiphage systems a BLASTP and HMMER search was conducted; obtained hits are listed in Table 2. BLASTP primarily reveals the presence of highly homologous proteins in other lactic acid bacteria (LAB). However, by using a more sensitive search (HMMER), homologues of these systems can be found in many different microorganisms, although their association with antiphage activity still requires verification.

Finally, most of the antiphage systems (13 out of 20) described in this work are encoded either on a pMRC01- or pNP40-like conjugative plasmid (Table S3, Figure S6) or they are carried by a strain possessing either of these plasmid-encoded conjugation mechanisms, implying that they could either be conjugated or co-mobilised to other strains (76, 77) (Table S3). With the exception of Kamadhenu, PARIS, type II CBASS, AbiA, AbiD/F and AbiJ, the identified systems do not appear to be co-localised with other known or herein described systems (when a window of five genes upstream/downstream was employed).

Table 2. Prevalence of the discovered antiphage systems. The BLASTP hits represent the total of distinct hits with a cut-off value of >70 % amino acid identity with an associated E-value of <0.001. The HMMER organisms represent the top two obtained HMMER hits as an example.

	System name	BLASTP hits	BLASTP organisms	HMMER hits	HMMER organisms
New antiphage systems	Rhea	8	*Lactobacillales*	611	*Kosmotoga* sp., *Trichocoleus* sp.
	Aristaios	4	*L. lactis, L. cremoris*	≥10000	*Burkholderia cepacia*, *Geobacillus* sp.
	Kamadhenu	8	*L. lactis, L. cremoris*	914	*Klebsiella pneumoniae*, *Undibacterium rivi*
	Fliodhais-i	5	*L. lactis, L. cremoris, Lactococcus* sp.	5	*L. cremoris*, *L. petauri*
	Fliodhais-ii	47	*L. lactis, L. cremoris, L. garvieae, L. petauri*	415	*Lactobacillus* sp., *Lactiplantibacillus fabifermentans*
	Audmula	2	*L. lactis* & *Enterococcus* sp.	91	*Enterococcus* sp., *Enterococcus lemanii*
	Rugutis	3	*L. lactis, Lactococcus* sp.	6156	*Clostridiales bacterium*, *Pontibacillus litoralis*
	Hesat	18	*Lactococcus* spp., *Streptococcus thermophilus, Lysinibacillus* sp.	111	*Streptococcus varani*, *L. lactis*
Systems new in *Lactococcus*l - like systems	PARIS-i	4	*L. piscium, L. lactis*	9988	*Pseudomonas qingdaonensis*, *Endozoicomonas acroporae*
	PARIS-ii	2	*L. piscium, L. lactis*	955	*Morganella morganii*, *Reichenbachiella agariperforans*

Type I CBASS-i	2	*L. petauri, Lactococcus* sp.	985	*Bacteroidales*, *Lewinella* sp.
Type I CBASS-ii	5	*L. lactis, L. petauri, Streptococcus infantarius, Culicoidibacter larvae*	6684	*Campylobacter concisus*, *Endozoicomonas gorgoniicola*
Type II CBASS-i	2	*L. lactis*	115	*Clostridium liquoris*, *Lachnospira eligens*
Type II CBASS-ii	2	*L. lactis*	4300	*Saprospirales*, Deltaproteobacteria
Type II CBASS-iii	7	*Streptococcaceae*	916	*Bacillus* sp., *Desulfosporosinus metallidurans*
Lamassu-i	1	*L. lactis*	540	*Streptococcus* sp., *Pseudomonas* spp.
Lamassu-ii	2	*L. lactis*	461	*Staphylococcus schleiferi*, *Paenibacillus* sp.
Lamassu-iii	4	*L. lactis, Streptococcus salivarius, Streptococcus sanguinis*	9997	*Bacillus* sp., *Terribacillus saccharophilus*
Septu-i	1	*Lactococcus* sp.	9990	*Candidatus* Brocadiales, *Marinilabiliaceae*
Septu-ii	1	*Lactococcus* sp.	5797	*Streptococcus suis*, *Lactococcus* spp.
AbiD-like	7	*L. cremoris*, *Lactococcus* sp.	8585	*Caloramator proteoclasticus*, *Paenibacillus thermoaerophillus*

2.5 Discussion

The current study constitutes a considerable contribution to knowledge of bacterial immunity against phages by identifying seven novel antiphage systems and five known systems whose functionality had not previously been demonstrated in *Lactococcus*. Although recent studies have focused on genomic co-localisation with known systems for the discovery of new antiphage systems (18, 19, 22), it now appears evident that many antiphage systems remain undetected as they are not necessarily located close to other such systems (Table S3). This is also supported by a recent study (20), where the authors reported that only three out of the 21 discovered systems are situated within a defence island. Additionally, previous studies have not systematically and deliberately searched for plasmid-encoded antiphage systems. Lactococcal plasmid-associated genes are mostly of unknown function and, as phages represent the main bacterial enemy, it was hypothesised that some of these genes of unknown function are associated with antiphage activity. Indeed, one in ten of the candidate systems in this study was ultimately confirmed as an antiphage system, while this may be an underestimation of the actual number of antiphage systems present in this lactococcal strain collection as outlined below.

DefenseFinder and PADLOC were used for the detection of antiphage systems that had previously been found in bacterial species beyond *L. lactis/cremoris*. Even though five systems detected by these two bioinformatic tools were validated, four others did not show any antiphage effect. This lack of activity may be explained by the employment of an insufficiently diverse panel of phages or because the current phages have

evolved to overcome these systems or due to incorrect testing conditions or by the possibility that these systems have been inactivated in *Lactococcus* over time. This could be explained by the elimination of the relevant phage(s) that these systems target or due to the cell fitness cost of maintaining an antiphage system (60, 61). Furthermore, members of the AbiD/F family were shown to require an upstream sequence in addition to the genes for their antiphage activity. Therefore, candidates that did not display antiphage activity could have been false negatives since cloning with native regulatory factors was required or the system is composed of multiple genes (the current study was primarily focused on single or two component candidate antiphage systems). In addition, the many apparently inactive AbiB-like candidates demonstrate that not all members of the same family display the same activity. Therefore, given that a single representative gene was assessed and potentially selected as candidate for most of the gene families, antiphage systems may have been overlooked if an inactive or inappropriate (in terms of activity against the tested phage) member had been selected.

Interestingly, the majority of the systems tested in this study are highly effective against phages of the genus *Skunavirus*, which are the most commonly isolated and problematic phages in the dairy environment, suggesting that the lactococcal phage resistance have expectedly evolved to target the most prevalent and abundant lactococcal phages. The resistance phenotype was not only observed on solid, but also in liquid medium, underlining the biotechnological relevance as *Lactococcus* spp. are applied in the fermentation of milk. All studied systems were found to be

active post-phage DNA injection and with the exception of the newly identified Audmula all tested systems appear to be consistent with an Abi phenotype. CBASS (72), Lamassu (63) and PARIS systems (19) are known to present Abi phenotypes; however, to our knowledge, this has not previously been determined for Septu. Although lysis-in-broth is still widely used to assign systems as Abi (20, 22), it has been reported that the mechanistic characterization of a system as Abi-should not be deduced by the associated (phenotypic) lysis profile (59). Therefore, additional research is required to unravel the mode of action of the systems and thus assignment of the system as a validated Abi.

The newly discovered antiphage systems in this study were shown to be rare among our lactococcal plasmid collection, yet (homologues) seem to be widespread among different bacterial phyla. In addition, almost half of the 23 known lactococcal Abi systems were not found within the 1,020 analysed gene families derived from 53 different strains. This indicates that either some systems are less prevalent than others and/or denoting that our strain collection and derived plasmid library does not represent the full diversity of lactococcal strains that exists. Indeed, it has been reported that most of the known antiphage systems (>50 %) are found in a small proportion of bacterial genomes (<3 %) with rare systems being the main diversity contributors in antiviral arsenals (21). Additionally, there are possible applications for the discovered systems regardless of their distribution considering that bacterial antiphage systems are now used as biotechnological/biomedical tools (78, 79).

Despite the identification of several and diverse domains by HHpred and InterPro for most of the new systems, none of the hits were with proteins known to be involved in phage resistance. AlphaFold2 (AF2) has revolutionised structural biology by allowing accurate protein structure and complexation prediction (80, 81). Indeed, further functional and structural analysis of the antiphage systems using AlphaFold2 and the fold comparator Dali revealed possible modes of action for several of the newly identified systems (46), linking Aristaios, Kamadhenu and Hesat to proteins related to programmed cell death, which is consistent with an abortive infection mode of action. Therefore, AF2 along with Dali could be applied to elucidate the possible functions, structures and complexes of emerging antiphage systems, as the mode of action of more than half of the reported systems (58 %) remains unknown (37). However, these hypothetical modes of action require experimental verification to classify the systems based on their molecular mechanism (21).

The findings of this study led to the grouping of the previously distinct AbiD, AbiD1, AbiF (and AbiD-like), which share sequence similarity and superimpose structurally into a single AbiD/F group of antiphage systems. Members of this group structurally resemble the endonuclease domain of the RNA-guided RNase Cas13, which has previously been associated with abortive infection (75). Therefore, we foresee that structural analysis followed by structural alignments of the existing bacterial antiphage systems may assist in their function- and similarity-based classification. Meanwhile, structural searches of proteins of unknown function against an antiphage

system database may indeed facilitate discovery of previously unknown antiphage systems.

The discovery of plasmid-encoded antiphage systems has clear implications for the dairy industry. The constant increase of the global dairy (https://www.statista.com/statistics/502280/global-dairy-market-value/) and fermented (functional) food products (https://www.statista.com/statistics/1033912/market-value-of-fermented-food-ingredients/) market and the persistent threat of phages in the production of fermented foods (82), necessitates the direct selection of strains encoding a range of antiphage systems or the improvement of phage robustness of existing strains through conjugation or other natural DNA transfer approaches.

The recent surge in discovery of previously unknown antiphage systems and their varying modes of action has greatly improved our understanding of phage-host interactions and phage resistance. Our study provides further evidence on the diversity of the antiphage systems and the significance of plasmids in acquisition of bacterial immunity against phage attack. We expect that future studies will use plasmids as a rich resource for the discovery of unknown antiphage systems in other species and will further confirm the significance of plasmids on the immune system of bacteria. While there is still much to be learnt and understood, we envision continued discovery of the complex bacterial immunity landscape, with the generated knowledge being applied in the food, biotechnology, biomedical industry and beyond.

2.6 Data availability

Sequences were deposited in GenBank under the accession number PRJNA1086404.

Coordinates of predicted structures are accessible on Zenodo (https://doi.org/10.5281/zenodo.11221414). The structures are also presented in the main and supplementary figures.

Any additional information required to reanalyse the data reported in this study is available from the corresponding author upon request.

2.7 References

1. Li,T.T., Tian,W.L. and Gu,C.T. (2019) Elevation *of Lactococcus lactis* subsp. *cremoris* to the species level as *Lactococcus cremoris* sp. nov. and transfer of *Lactococcus lactis* subsp. *tructae* to *Lactococcus cremoris* as *Lactococcus cremoris* subsp. *tructae* comb. nov. *Int J Syst Evol Microbiol*, 71.

2. Mills,S., McAuliffe,O.E., Coffey,A., Fitzgerald,G.F. and Ross,R.P. (2006) Plasmids of lactococci – genetic accessories or genetic necessities? *FEMS Microbiol Rev*, 30, 243–273.

3. Cavanagh,D., Fitzgerald,G.F. and McAuliffe,O. (2015) From field to fermentation: The origins of *Lactococcus lactis* and its domestication to the dairy environment. *Food Microbiol*, 47, 45–61.

4. Aryana,K.J. and Olson,D.W. (2017) A 100-Year Review: Yogurt and other cultured dairy products. *J Dairy Sci*, 100, 9987–10013.

5. Makarova,K., Slesarev,A., Wolf,Y., Sorokin,A., Mirkin,B., Koonin,E., Pavlov,A., Pavlova,N., Karamychev,V., Polouchine,N., *et al.* (2006) Comparative genomics of the lactic acid bacteria. *Proc Natl Acad Sc U S A*, 103, 15611–15616.

6. Hayes,S., Murphy,J., Mahony,J., Lugli,G.A., Ventura,M., Noben,J.-P., Franz,C.M.A.P., Neve,H., Nauta,A. and van Sinderen,D. (2017) Biocidal inactivation of *Lactococcus lactis* bacteriophages: Efficacy and targets of commonly used sanitizers. *Front Microbiol*, 8, 107.

7. Garneau,J.E. and Moineau,S. (2011) Bacteriophages of lactic acid bacteria and their impact on milk fermentations. *Microb Cell Fact*, 10, S20.

8. Zrelovs,N., Dislers,A. and Kazaks,A. (2021) Genome Characterization of Nocturne116, novel *Lactococcus lactis*-infecting phage isolated from moth. *Microorganisms*, 9, 1540.

9. Deveau,H., Labrie,S.J., Chopin,M.-C. and Moineau,S. (2006) Biodiversity and classification of lactococcal phages. *Appl Environ Microbiol*, 72, 4338–4346.

10. Mahony,J., McDonnell,B., Casey,E. and van Sinderen,D. (2016) Phage-host interactions of cheese-making lactic acid bacteria. *Annu Rev Food Sci Technol*, 7, 267–285.

11. Labrie,S.J., Samson,J.E. and Moineau,S. (2010) Bacteriophage resistance mechanisms. *Nat Rev Microbiol*, 8, 317–327.

12. Chopin,M.C., Chopin,A. and Bidnenko,E. (2005) Phage abortive infection in lactococci: variations on a theme. *Curr Opin Microbiol*, 8, 473–479.

13. Millen,A.M., Horvath,P., Boyaval,P. and Romero,D.A. (2012) Mobile CRISPR/Cas-mediated bacteriophage resistance in *Lactococcus lactis*. *PLoS One*, 7, e51663.

14. Kelleher,P., Mahony,J., Bottacini,F., Lugli,G.A., Ventura,M. and van Sinderen,D. (2019) The *Lactococcus lactis* pan-plasmidome. *Front Microbiol*, 10, 707.

15. Lopatina,A., Tal,N. and Sorek,R. (2020) Abortive infection: Bacterial suicide as an antiviral immune strategy. *Annu Rev Virol*, 7, 371–384.

16. Ainsworth,S., Stockdale,S., Bottacini,F., Mahony,J. and van Sinderen,D. (2014) The *Lactococcus lactis* plasmidome: much learnt, yet still lots to discover. *FEMS Microbiol Rev*, 38, 1066–1088.

17. Doron,S., Melamed,S., Ofir,G., Leavitt,A., Lopatina,A., Keren,M., Amitai,G. and Sorek,R. (2018) Systematic discovery of antiphage defense systems in the microbial pangenome. *Science*, 359, eaar4120.

18. Gao,L., Altae-Tran,H., Böhning,F., Makarova,K.S., Segel,M., Schmid-Burgk,J.L., Koob,J., Wolf,Y.I., Koonin,E. V. and Zhang,F. (2020) Diverse enzymatic activities mediate antiviral immunity in prokaryotes. *Science (1979)*, 369, 1077–1084.

19. Rousset,F., Depardieu,F., Miele,S., Dowding,J., Laval,A.L., Lieberman,E., Garry,D., Rocha,E.P.C., Bernheim,A. and Bikard,D. (2022) Phages and their satellites encode hotspots of antiviral systems. *Cell Host Microbe*, 30, 740-753.

20. Vassallo,C.N., Doering,C.R., Littlehale,M.L., Teodoro,G.I.C. and Laub,M.T. (2022) A functional selection reveals previously undetected anti-phage defence systems in the *E. coli* pangenome. *Nat Microbiol*, 7, 1568–1579.

21. Georjon,H. and Bernheim,A. (2023) The highly diverse antiphage defence systems of bacteria. *Nat Rev Microbiol*, 21, 686–700.

22. Millman,A., Melamed,S., Leavitt,A., Doron,S., Bernheim,A., Hör,J., Garb,J., Bechon,N., Brandis,A., Lopatina,A., *et al.* (2022) An expanded arsenal of immune systems that protect bacteria from phages. *Cell Host Microbe*, 30, 1556-1569.

23. Hochhauser,D., Millman,A. and Sorek,R. (2023) The defense island repertoire of the *Escherichia coli* pan-genome. *PLoS Genet*, 19, e1010694.

24. Benler,S., Faure,G., Altae-Tran,H., Shmakov,S., Zheng,F. and Koonin,E. (2021) Ccargo genes of Tn7-like transposons comprise an enormous diversity of defense systems, mobile genetic elements, and antibiotic resistance genes. *mBio*, 12, e0293821.

25. van Mastrigt,O., Di Stefano,E., Hartono,S., Abee,T. and Smid,E.J. (2018) Large plasmidome of dairy *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* FM03P encodes technological functions and appears highly unstable. *BMC Genomics*, 19, 620.

26. Kelly,W.J., Ward,L.J.H. and Leahy,S.C. (2010) Chromosomal diversity in *Lactococcus lactis* and the origin of dairy starter cultures. *Genome Biol Evol*, 2, 729–744

27. Mahony,J., Oliveira,J., Collins,B., Hanemaaijer,L., Lugli,G.A., Neve,H., Ventura,M., Kouwen,T.R., Cambillau,C. and van Sinderen,D. (2017) Genetic and functional characterisation of the lactococcal P335 phage-host interactions. *BMC Genomics*, 18, 146.

28. Wick,R.R., Judd,L.M., Gorrie,C.L. and Holt,K.E. (2017) Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol*, 13, e1005595.

29. Hyatt,D., Chen,G.-L., Locascio,P.F., Land,M.L., Larimer,F.W. and Hauser,L.J. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. 11, 119.

30. Altschul,S.F., Gish,W., Miller,W., Myers,E.W. and Lipman,D.J. (1990) Basic local alignment search tool. *J Mol Biol*, 215, 403–410.

31. Carver,T., Harris,S.R., Berriman,M., Parkhill,J. and McQuillan,J.A. (2012) Artemis: An integrated platform for visualization and analysis of high-throughput sequence-based experimental data. *Bioinformatics*, 28, 464–469.

32. Bateman,A., Coin,L., Durbin,R., Finn,R.D., Hollich,V., Griffiths-Jones,S., Khanna,A., Marshall,M., Moxon,S., Sonnhammer,E.L.L., *et al.* (2004) The Pfam protein families database. *Nucleic Acids Res*, 32*,* D138–D141.

33. Söding,J., Biegert,A. and Lupas,A.N. (2005) The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res*, 33, W244–W248.

34. Sayers,E.W., Beck,J., Bolton,E.E., Bourexis,D., Brister,J.R., Canese,K., Comeau,D.C., Funk,K., Kim,S., Klimke,W., *et al.* (2021) Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res*, 49, D10–D17.

35. Paysan-Lafosse,T., Blum,M., Chuguransky,S., Grego,T., Pinto,B.L., Salazar,G.A., Bileschi,M.L., Bork,P., Bridge,A., Colwell,L., *et al.* (2023) InterPro in 2022. *Nucleic Acids Res*, 51, D418–D427.

36. Krogh,A., Larsson,B., Von Heijne,G. and Sonnhammer,E.L.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J Mol Biol*, 305, 567–580.

37. Tesson,F., Hervé,A., Mordret,E., Touchon,M., d'Humières,C., Cury,J. and Bernheim,A. (2022) Systematic and quantitative view of the antiviral arsenal of prokaryotes. *Nat Commun*, 13, 2561.

38. Abby,S.S., Néron,B., Ménager,H., Touchon,M. and Rocha,E.P.C. (2014) MacSyFinder: A program to mine genomes for molecular systems with an application to CRISPR-Cas systems. *PLoS One*, 9, e110726.

39. Payne,L.J., Todeschini,T.C., Wu,Y., Perry,B.J., Ronson,C.W., Fineran,P.C., Nobrega,F.L. and Jackson,S.A. (2021) Identification and classification of antiviral defence systems in bacteria and archaea with PADLOC reveals new system types. *Nucleic Acids Res*, 49, 10868–10878.

40. Lillehaug,D. (1997) An improved plaque assay for poor plaque-producing temperate lactococcal bacteriophages. *J Appl Microbiol*, 83, 85–90.

41. Mosterd,C. and Moineau,S. (2020) Characterization of a type II-A CRISPR-Cas system in *Streptococcus mutans*. *mSphere*, 5, e00235-20.

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

43. Jumper,J., Evans,R., Pritzel,A., Green,T., Figurnov,M., Ronneberger,O., Tunyasuvunakool,K., Bates,R., Žídek,A., Potapenko,A., *et al.* (2021) Applying and improving AlphaFold at CASP14. *Proteins Struct Funct Bioinf*, 89, 1711–1721.

44. Jumper,J., Evans,R., Pritzel,A., Green,T., Figurnov,M., Ronneberger,O., Tunyasuvunakool,K., Bates,R., Žídek,A., Potapenko,A., *et al.* (2021) Highly accurate protein structure prediction with AlphaFold. *Nature*, 596, 583–589.

45. Mirdita,M., Schütze,K., Moriwaki,Y., Heo,L., Ovchinnikov,S. and Steinegger,M. (2022) ColabFold: making protein folding accessible to all. *Nat Methods*, 19, 679–682.

46. Holm,L. (2022) Dali server: structural unification of protein families. *Nucleic Acids Res*, 50, W210–W215.

47. Pettersen,E.F., Goddard,T.D., Huang,C.C., Meng,E.C., Couch,G.S., Croll,T.I., Morris,J.H. and Ferrin,T.E. (2021) UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Sci*, 30, 70–82.

48. Altschul,S., Madden TL, Schäffer,A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*, 25, 3389–3402.

49. Eddy,S.R. (2011) Accelerated Profile HMM Searches. *PLoS Comput Biol*, 7, e1002195.

50. Emond,E., Dion,E., Walker,S.A., Vedamuthu,E.R., Kondo,J.K. and Moineau,S. (1998) AbiQ, an abortive infection mechanism from *Lactococcus lactis*. *Appl Environ Microbiol*, 64, 4748– 4756.

51. McLandsborough,L.A., Kolaetis,K.M., Requena,T. and McKay,L.L. (1995) Cloning and characterization of the abortive infection genetic determinant *abiD* isolated from pBF61 of *Lactococcus lactis* subsp. lactis KR5. *Appl Environ Microbiol*, 61, 2023–2026.

52. Anba,J., Bidnenko,E., Hillier,A., Ehrlich,D. and Chopin,M.C. (1995) Characterization of the lactococcal *abiD1* gene coding for phage abortive infection. *J Bacteriol*, 177, 3818–3823.

53. Garvey,P., Fitzgerald,G.F. and Hill,C. (1995) Cloning and DNA sequence analysis of two abortive infection phage resistance determinants from the lactococcal plasmid pNP40. *Appl Environ Microbiol*, 61, 4321–4328.

54. Tangney,M. and Fitzgerald,G.F. (2002) Effectiveness of the lactococcal abortive infection systems AbiA, AbiE, AbiF and AbiG against P335 type phages. *FEMS Microbiol Lett*, 210, 67–72.

55. Mahony,J., Murphy,J. and Sinderen,D. van (2012) Lactococcal 936-type phages and dairy fermentation problems: from detection to evolution and prevention. *Front Microbiol*, 3, 1–9.

56. Oliveira,J., Mahony,J., Hanemaaijer,L., Kouwen,T.R.H.M. and van Sinderen,D. (2018) Biodiversity of bacteriophages infecting *Lactococcus lactis* starter cultures. *J Dairy Sci*, 101, 96–105.

57. Mahony,J., McGrath,S., Fitzgerald,G.F. and van Sinderen,D. (2008) Identification and characterization of lactococcal-prophage-carried superinfection exclusion genes. *Appl Environ Microbiol*, 74, 6206– 6215.

58. Garb,J., Lopatina,A., Bernheim,A., Zaremba,M., Siksnys,V., Melamed,S., Leavitt,A., Millman,A., Amitai,G. and Sorek,R. (2022) Multiple phage resistance systems inhibit infection via SIR2-dependent NAD+ depletion. *Nat Microbiol*, 7, 1849–1856.

59. Aframian,N. and Eldar,A. (2023) Abortive infection antiphage defense systems: separating mechanism and phenotype. *Trends Microbiol*, 31, 1003–1012.

60. Westra,E.R., van Houte,S., Oyesiku-Blakemore,S., Makin,B., Broniewski,J.M., Best,A., Bondy-Denomy,J., Davidson,A., Boots,M. and Buckling,A. (2015) Parasite exposure drives selective evolution of constitutive versus inducible defense. *Current Biology*, 25, 1043–1049.

61. Bernheim,A. and Sorek,R. (2020) The pan-immune system of bacteria: antiviral defence as a community resource. *Nat Rev Microbiol*, 18, 113–119.

62. Peters,J.E. and Craig,N.L. (2001) Tn7: smarter than we thought. *Nat Rev Mol Cell Biol*, 2, 806–814.

63. Jaskólska,M., Adams,D.W. and Blokesch,M. (2022) Two defence systems eliminate plasmids from seventh pandemic *Vibrio cholerae*. *Nature*, 604, 323–329.

64. Simon,N.C., Aktories,K. and Barbieri,J.T. (2014) Novel bacterial ADP-ribosylating toxins: structure and function. *Nat Rev Microbiol*, 12, 599–611.

65. LeRoux,M., Srikant,S., Teodoro,G.I.C., Zhang,T., Littlehale,M.L., Doron,S., Badiee,M., Leung,A.K.L., Sorek,R. and Laub,M.T. (2022) The DarTG toxin-antitoxin system provides phage defence by ADP-ribosylating viral DNA. *Nat Microbiol*, 7, 1028–1040.

66. Amitai,S., Kolodkin-Gal,I., Hananya-Meltabashi,M., Sacher,A. and Engelberg-Kulka,H. (2009) *Escherichia coli* MazF leads to the simultaneous selective synthesis of both “death proteins” and “survival proteins”. *PLoS Genet*, 5, e1000390.

67. Guo,L., Sattler,L., Shafqat,S., Graumann,P.L. and Bramkamp,M. (2022) A bacterial dynamin-like protein confers a novel phage resistance strategy on the population level in *Bacillus subtilis*. *mBio*, 13, e03753-21.

68. Graille,M., Mora,L., Buckingham,R.H., van Tilbeurgh,H. and de Zamaroczy,M. (2004) Structural inhibition of the colicin D tRNase by the tRNA-mimicking immunity protein. *EMBO J*, 23, 1474–1482.

69. Käshammer,L., Saathoff,J.-H., Lammens,K., Gut,F., Bartho,J., Alt,A., Kessler,B. and Hopfner,K.-P. (2019) Mechanism of DNA end sensing and processing by the Mre11-Rad50 complex. *Mol Cell*, 76, 382-394.e6.

70. Richmond-Buccola,D., Hobbs,S.J., Garcia,J.M., Toyoda,H., Gao,J., Shao,S., Lee,A.S.Y. and Kranzusch,P.J. (2024) A large-scale type I CBASS antiphage screen identifies the phage prohead protease as a key determinant of immune activation and evasion. *Cell Host Microbe, 32, 1074–1088.*

71. Fatma,S., Chakravarti,A., Zeng,X. and Huang,R.H. (2021) Molecular mechanisms of the CdnG-Cap5 antiphage defense system employing 3′,2′-cGAMP as the second messenger. *Nat Commun*, 12, 6381.

72. Cohen,D., Melamed,S., Millman,A., Shulman,G., Oppenheimer-Shaanan,Y., Kacen,A., Doron,S., Amitai,G. and Sorek,R. (2019) Cyclic GMP–AMP signalling protects bacteria against viral infection. *Nature*, 574, 691–695.

73. Millman,A., Melamed,S., Amitai,G. and Sorek,R. (2020) Diversity and classification of cyclic-oligonucleotide-based anti-phage signalling systems. *Nat Microbiol*, 5, 1608–1615.

74. Morse,R.P., Nikolakakis,K.C., Willett,J.L.E., Gerrick,E., Low,D.A., Hayes,C.S. and Goulding,C.W. (2012) Structural basis of toxicity and immunity in contact-dependent growth inhibition (CDI) systems. *Proc Natl Acad Sci U S A*, 109, 21480–21485.

75. Meeske,A.J., Nakandakari-Higa,S. and Marraffini,L.A. (2019) Cas13-induced cellular dormancy prevents the rise of CRISPR-resistant bacteriophage. *Nature*, 570, 241–245.

76. Ortiz Charneco,G., Kelleher,P., Buivydas,A., Streekstra,H., van Themaat,E.V.L., de Waal,P.P., Mahony,J. and van Sinderen,D. (2021)

Genetic dissection of a prevalent plasmid-encoded conjugation system in *Lactococcus lactis*. *Front Microbiol*, 12, 680920.

77. Ortiz Charneco,G., Kelleher,P., Buivydas,A., Dashko,S., de Waal,P.P., van Peij,N.N.M.E., Roberts,R.J., Mahony,J. and van Sinderen,D. (2023) Delineation of a lactococcal conjugation system reveals a restriction-modification evasion system. *Microb Biotechnol*, 16, 1250–1263.

78. Adli,M. (2018) The CRISPR tool kit for genome editing and beyond. *Nat Commun*, 9, 1911.

79. Singh,M., Bindal,G., Misra,C.S. and Rath,D. (2022) The era of Cas12 and Cas13 CRISPR-based disease diagnosis. *Crit Rev Microbiol*, 48, 714–729.

80. Tunyasuvunakool,K., Adler,J., Wu,Z., Green,T., Zielinski,M., Žídek,A., Bridgland,A., Cowie,A., Meyer,C., Laydon,A., *et al.* (2021) Highly accurate protein structure prediction for the human proteome. *Nature*, 596, 590–596.

81. Evans,R., O'Neill,M., Pritzel,A., Antropova,N., Senior,A., Green,T., Žídek,A., Bates,R., Blackwell,S., Yim,J., *et al.* (2022) Protein complex prediction with AlphaFold-Multimer. *bioRxiv*, 10.1101/2021.10.04.463034, preprint: not peer reviewed.

82. Briggiler Marcó,M. and Mercanti,D.J. (2021) Bacteriophages in dairy plants. *Adv Food Nutr Res*, 97, 1–54.

2.8 Supplementary data

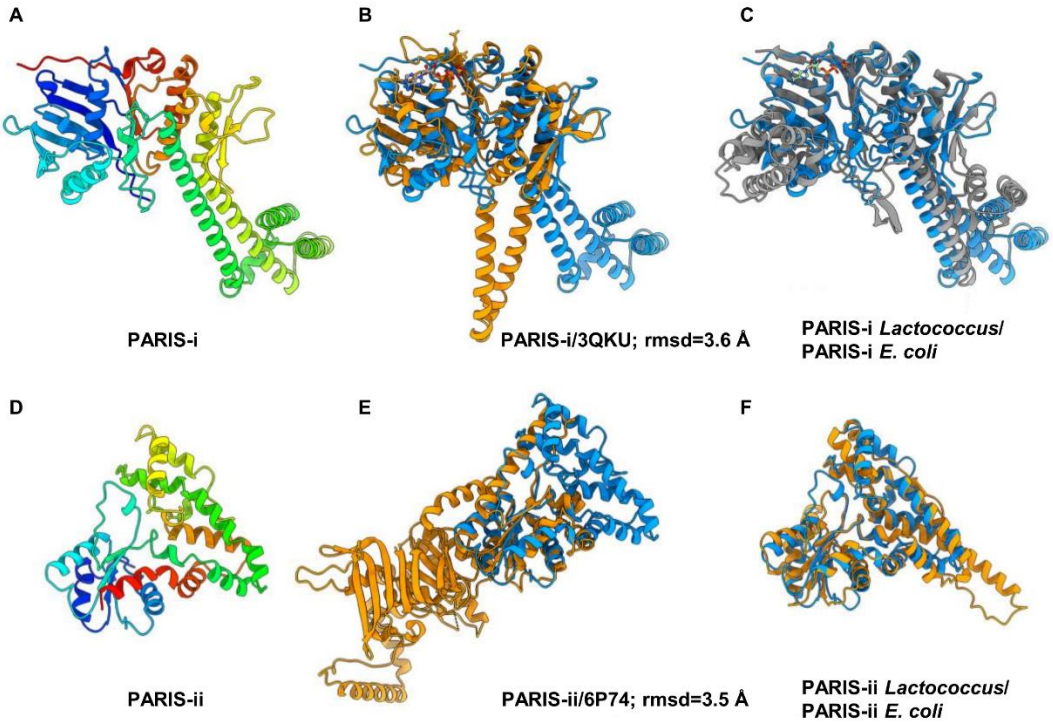


Figure S1: Predicted structures of PARIS. A) Ribbon structure of PARIS-i (rainbow coloured). B) Superimposition of PARIS-i (blue) with PDB 3QKU (orange), a DNA double-strand break repair Rad50 ATPase; (z=18.4 rmsd=3.6 Å). C) Superimposition of *Lactococcus* PARIS-i (blue) with *E. coli* PARIS-i (orange). D) Ribbon structure of PARIS-ii (rainbow coloured). E) Superimposition of PARIS-ii (blue) with PDB 6P74 (orange), an ATP dependent endonuclease; (z=5.2 rmsd=3.5 Å). F) Superimposition of *Lactococcus* PARIS-ii (blue) with *E. coli* PARIS-ii (orange). pLDDT values and predicted aligned errors (PAE) plots are present in Figure S5.

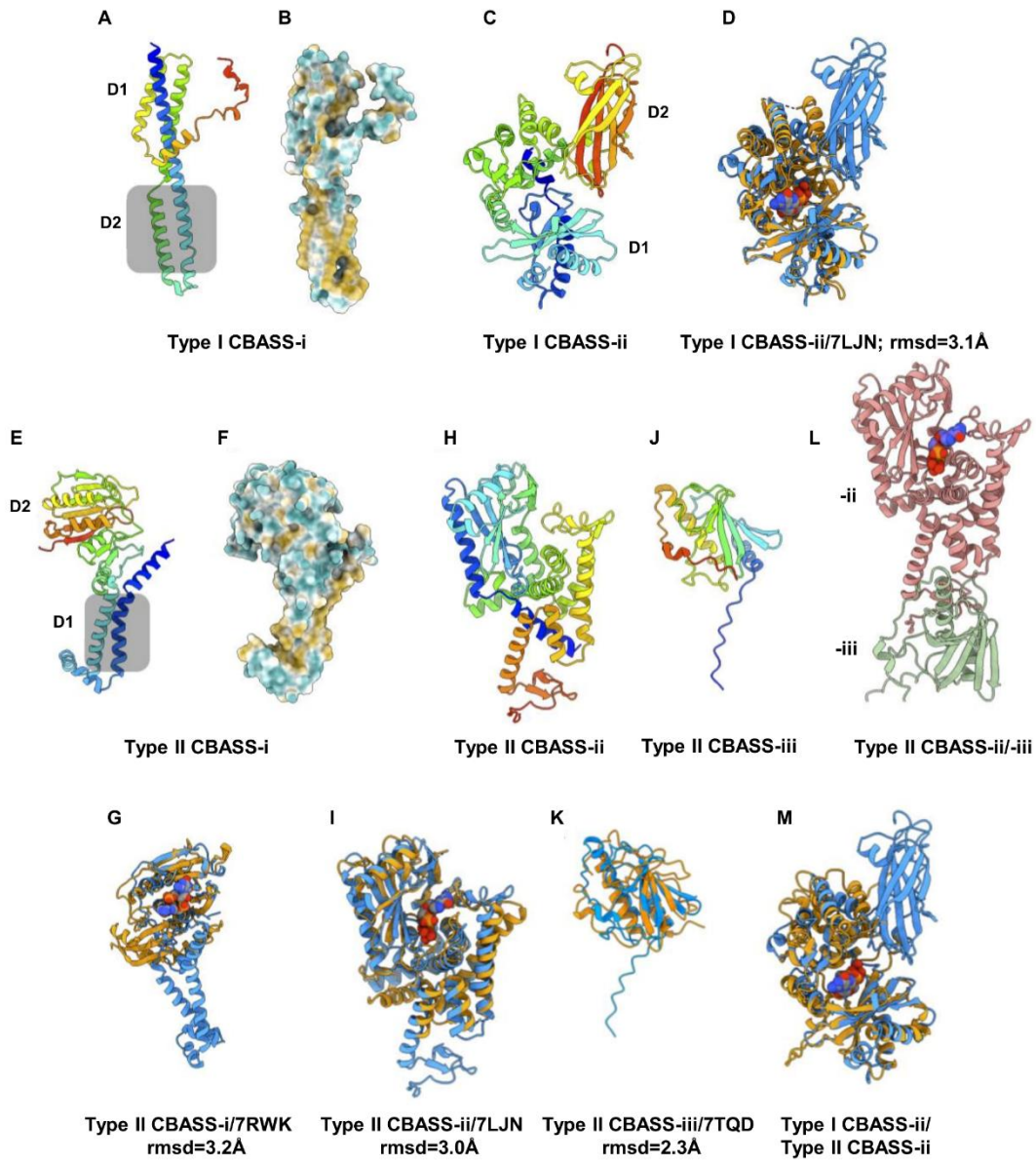


Figure S2: Predicted structures of type I and II CBASS. A) Ribbon structure of type I CBASS-i (rainbow coloured; the two trans-membrane helices are boxed grey). B) Type I CBASS-i molecular surface (hydrophobic surfaces are coloured in beige). C) Ribbon structure of type I CBASS-ii (rainbow coloured) two domains. D) Superimposition of type I CBASS-ii N-terminal domain D1 (blue) with PDB 7LJN (orange), a nucleotidyltransferase (CD-NTase) (Z=20.9, rmsd=3.1Å). Note that a complex of type I CBASS-i/type I CBASS-ii could not be confidently predicted by AF2. E) Ribbon structure of type II CBASS-i (rainbow coloured; the two trans-membrane helices of domain D1 are boxed grey). F) type II CBASS-i molecular surface (hydrophobic surfaces are coloured in beige). G) Superimposition of type II CBASS-i (blue) with PDB 7RWS (orange, with cGAMP inside), a cGAMP signalling

protein that activates HNH nuclease domain for DNA degradation (Z=13.4, rmsd=3.2Å). H) Ribbon structure of type II CBASS-ii (rainbow coloured). I) Superimposition of type II CBASS-i (blue) with PDB 7LNJ (orange), a nucleotidyltransferase (CD-NTase) with GTP inside (Z=29.9, rmsd=3.0Å). J) Ribbon structure of type II CBASS-iii (rainbow coloured; no significant PDB hit). K) Superimposition of type II CBASS-iii (blue) with PDB 7TQD (orange), a Cap2 domain (Z=8.2, rmsd=2.3Å). L) Ribbon structure of a complex between type II CBASS-ii (salmon coloured) and type II CBASS-iii (pale green). A GTP from 7LNJ is located inside the type II CBASS-ii structure. To note, no complex could be confidently predicted with type II CBASS-i. M) Superimposition of type I CBASS-ii (blue) and type II CBASS-ii (orange) with GTP inside. pLDDT values and predicted aligned errors (PAE) are present in Figure S5.

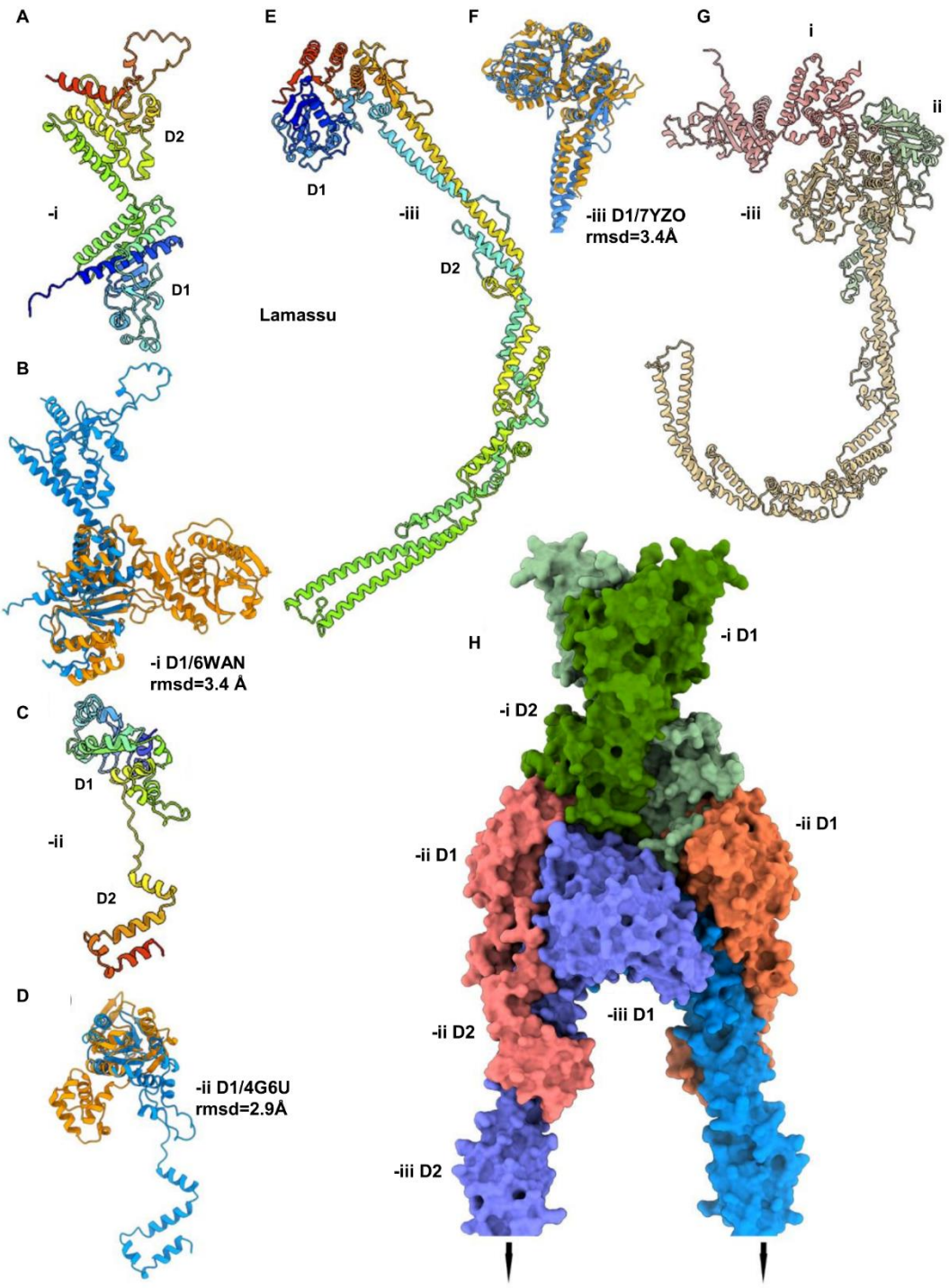


Figure S3: Predicted structures of Lamassu. A) Ribbon structure of the two-domain protein Lamassu-i (rainbow coloured). B) Superimposition of Lamassu-i D1 domain (blue) with PDB 6WAN (orange), a CARF domain (Z=11.2 rmsd=3.4 Å). C) Ribbon structure of the two-domain protein Lamassu-ii (rainbow coloured). D) Superimposition of Lamassu-ii D1 domain (blue) with PDB 4G6U (orange), a contact-dependent growth inhibition (CDI) DNAse (Z=7.5, rmsd=2.9Å). E) Ribbon structure of Lamassu-iii (rainbow coloured). F)

Superimposition of Lamassu-iii D1 domain (blue) with PDB 7YZO (orange), an endonuclease (Z=27.8, rmsd=3.4Å). G) Complex of the three Lamassu components (i, ii, iii). H) Molecular surface of the Lamassu three components (i, ii, iii) forming a hexamer (the Lamassu-iii domain D2 is not displayed completely). pLDDT values and predicted aligned errors (PAE) plots are present in Figure S5.

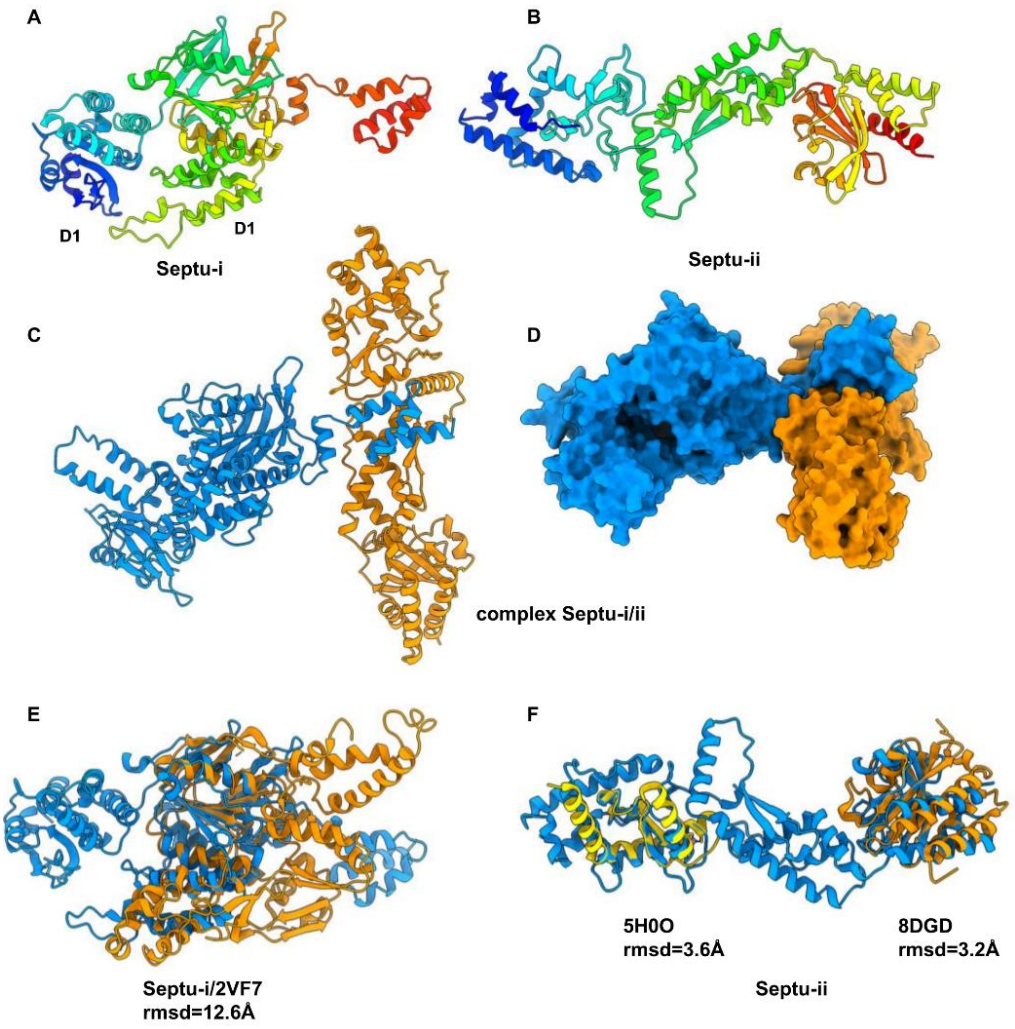


Figure S4: Predicted structures of Septu. A) Ribbon structure of the three-domain protein Septu-i (rainbow coloured). B) Ribbon structure of the three-domain protein Septu-ii (rainbow coloured). C) Complex of the two Septu components (i, blue; ii, orange). D) Molecular surface of the two Septu components (i, blue; ii, orange). E) Superimposition of Septu-i D2 domain (blue) with PDB 2VF7 (orange), an excinuclease ABC (Z=12.9, rmsd=12.6Å). F) Superimposition of Septu-ii (blue) D1 domain with PDB 5H0O (yellow) a

HNH endonuclease (Z=6.9, rmsd=3.6Å) and D3 domain with PDB 8DGD (orange), a GAF domain (Z=9.0, rmsd=3.2Å). pLDDT values and predicted aligned errors (PAE) plots are present in Figure S5.

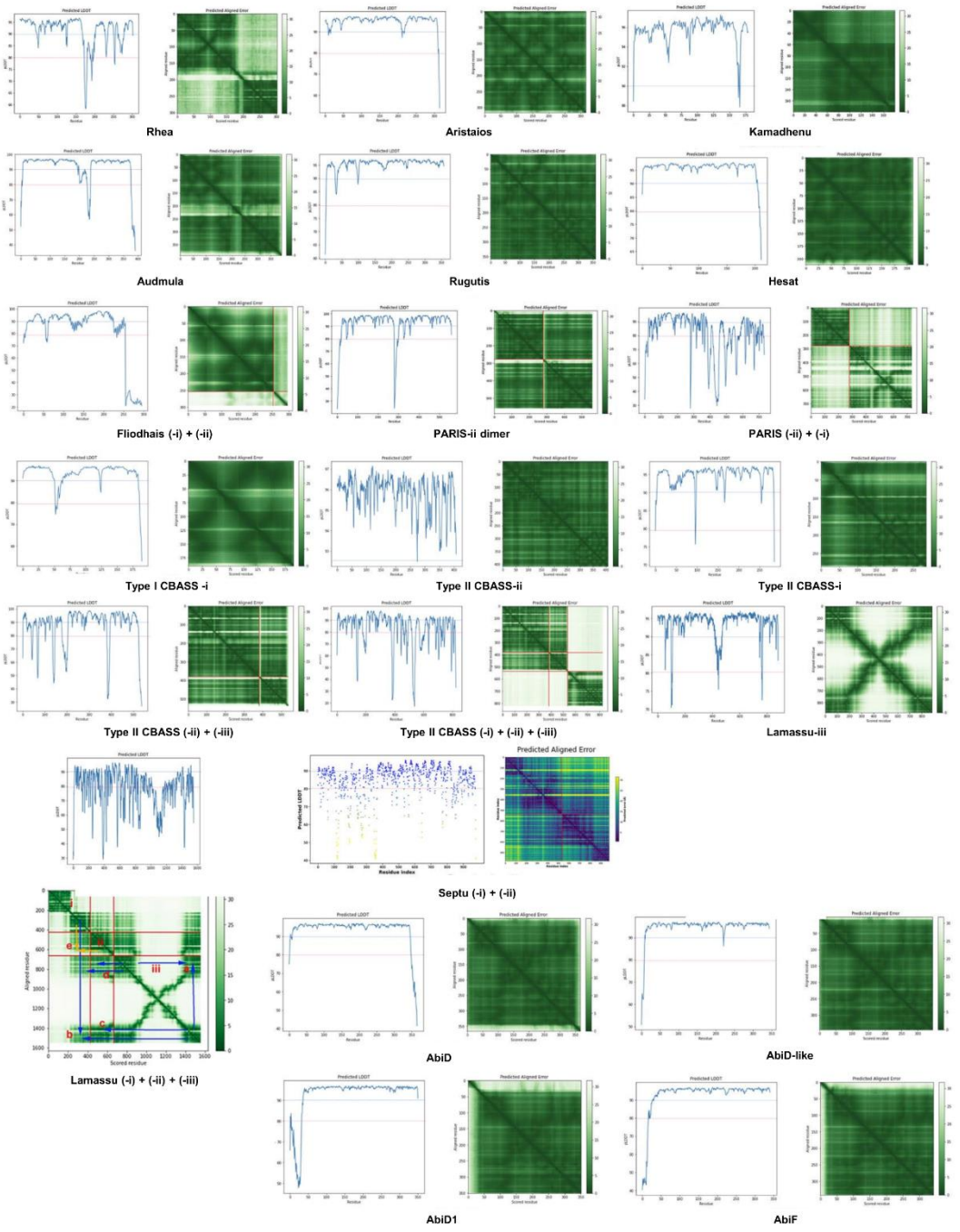


Figure S5. pLDDT values and predicted aligned errors (PAE) plots of predicted structures (Related to Figure 4, Figure 5 and Figure S1-4). The pLDDT plots (left) and PAE plots (right) correspond to unique proteins or complexes. The pLDDT values (Y axis) are plotted for each residue (X axis). The pLDDT values of 80 % and 90 % are identified

by a red and blue line, respectively. In PAE plots (residue number *vs* residue number) a green dot indicates that two residues are close in space. In complexes, the folded proteins or domains form squares along the diagonal while interactions between proteins or domains appear as squares at the vertical/horizontal intersection of the domains/proteins. For example, in Lamassu ternary complex ([-i]+[-ii]+[-iii]), (a) indicated an interaction between -iii Nt and -iii Ct; (b) between -iii Ct and -i D2; (c) between -iii Ct and -ii; (d) between -iii Nt, -ii and -i D2; (e) between -i D2 and -ii; (f) -i D1 does not interact with other proteins/complexes.

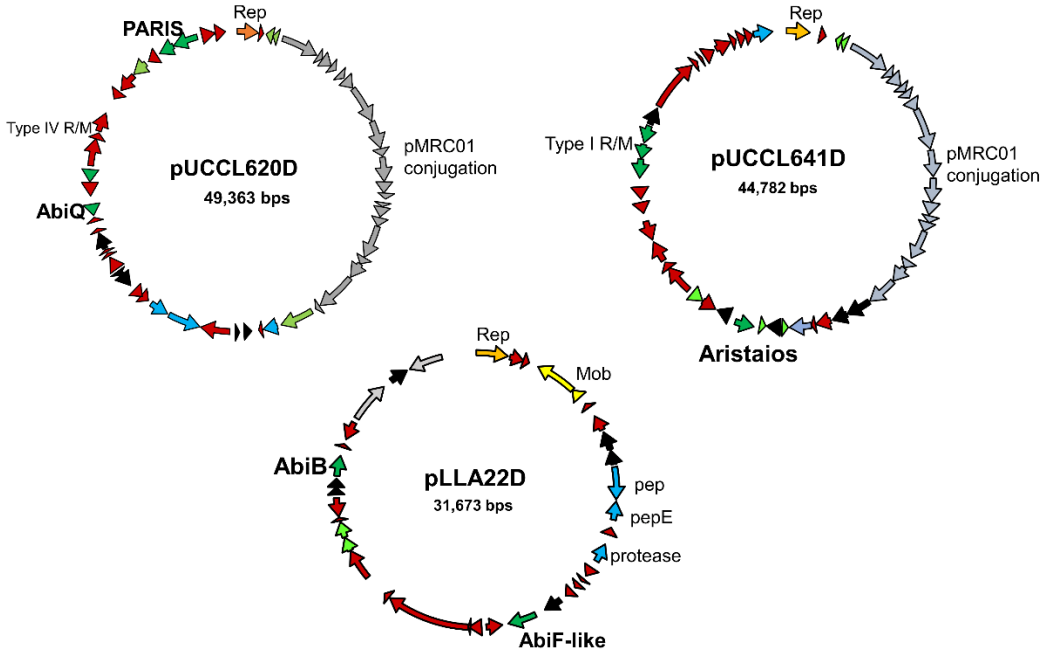


Figure S6. Example of lactococcal plasmids carrying novel and previously described antiphage systems.

Table S1. Bacterial strains, bacteriophages and plasmids used in this study.

Bacterial strain, bacteriophage or plasmid	Source	Identifier
L. cremoris NZ9000	(1)	RefSeq: NC_017949.1
L. cremoris 3107	(2)	Refseq: NZ_CP031538.1
L. lactis IL1403	(3)	RefSeq: NC_002662.1
L. cremoris MG1363	(4)	RefSeq: NC_009004.1
E. coli EC101	(5)	RefSeq: NZ_VIBV00000000.1
sk1	(6)	RefSeq: NC_001835.1
712	(7)	RefSeq: NC_008370.1
jj50	(8)	RefSeq: NC_008371.1
p2	(9)	RefSeq: NC_042024.1
c2	(10)	RefSeq: NC_001706.1
66901	(11)	RefSeq: NC_049410.1
62601	(11)	RefSeq: NC_049407.1
TP901-1	(12)	RefSeq: NC_002747.1
LC3	(13)	RefSeq: NC_005822.1
Dub35A	(14)	GenBank: KX160220.1
63301	(15)	RefSeq: NC_031017.1
P087	(16)	RefSeq: NC_012663.1
P008	(17)	RefSeq: NC_008363.1
bIL170	(18)	RefSeq: NC_001909.1
bIL66	(19)	GenBank: L35175.1 and AF539445.1
P113G	(20)	GenBank: KC182548.1
340	(21)	RefSeq: NC_021853.1
P335	Felix d'Hérelle Reference Center for Bacterial Viruses	GenBank: DQ838728.1
pNZ44	(22)	N/A
pPTPi	(23)	N/A
pPEPi	This study (Andrius Buivydas personal communication)	N/A
pPTPL-ski*cos*	(24)	N/A
pPTPi-c2*cos*	This study	N/A
All plasmids are listed and described in Table S2 and methods details	This study	N/A

Table S2. Gene families, selected candidate plasmid-encoded antiphage systems and cloning information. All gene families and their members are illustrated in Sheet 1. Genes selected for cloning are in bold. All selected genes encoding antiphage candidates and their details related to cloning can be found in Sheet 2.

Available at: https://academic.oup.com/nar/advance-article/doi/10.1093/nar/gkae671/7730533#supplementary-data:~:text=gkae671_Supplemental_Files

Table S3. Detailed efficiency of plaquing (EOP) data, conjugation potential and co-localisation information. Sheet 1: Detailed EOP data of different phage genus representatives (*Skunavirus*, *Ceduovirus*, P335 group & *Teubervirus*) on different lactococcal hosts (NZ9000, 3107, IL1403) carrying the antiphage systems related to antiphage spectrum of the identified antiphage systems. Sheet 2: Example of EOP calculation and plaque size reduction of phage sk1 in the presence or Rhea. Sheet 3: Conjugation potential of the antiphage systems, co-localisation with known systems and number of other family members in the same family related to the distribution and conjugation potential of the identified systems.

Available at: https://academic.oup.com/nar/advance-article/doi/10.1093/nar/gkae671/7730533#supplementary-data:~:text=gkae671_Supplemental_Files

2.9 Supplementary data references

1. Linares,D.M., Kok,J. and Poolman,B. (2010) Genome sequences of *Lactococcus lactis* MG1363 (revised) and NZ9000 and comparative physiological studies. *J Bacteriol*, 192, 5806–5812.

2. Erazo Garzon,A., Mahony,J., Bottacini,F., Kelleher,P. and van Sinderen,D. (2019) Complete genome sequence of *Lactococcus lactis* subsp. *cremoris* 3107, host for the model lactococcal p335 bacteriophage TP901-1. *Microbiol Resour Announc*, 8, e01635-18.

3. Bolotin,A., Wincker,P., Mauger,S., Jaillon,O., Malarme,K., Weissenbach,J., Ehrlich,S.D. and Sorokin,A. (2001) The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res*, 11, 731–753.

4. Wegmann,U., O'Connell-Motherway,M., Zomer,A., Buist,G., Shearman,C., Canchaya,C., Ventura,M., Goesmann,A., Gasson,M.J., Kuipers,O.P., *et al.* (2007) Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. *J Bacteriol*, 189, 3256–3270.

5. Law,J., Buist,G., Haandrikman,A., Kok,J., Venema,G. and Leenhouts,K. (1995) A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. *J Bacteriol*, 177, 7011–7018.

6. Chandry,P.S., Moore,S.C., Boyce,J.D., Davidson,B.E. and Hillier,A.J. (1997) Analysis of the DNA sequence, gene expression, origin of replication and modular structure of the *Lactococcus lactis* lytic bacteriophage sk1. *Mol Microbiol*, 26, 49–64.

7. Rincé,A., Tangney,M. and Fitzgerald,G.F. (2000) Identification of a DNA region from lactococcal phage sk1 protecting phage 712 from the abortive infection mechanism AbiF. *FEMS Microbiol Lett*, 182, 185–191.

8. Josephsen,J. and Vogensen,F.K. (1989) Identification of three different plasmid-encoded restriction/modification systems in *Streptococcus lactis* subsp. *cremoris* W56. *FEMS Microbiol Lett*, 59, 161–166.

9. Bebeacua,C., Tremblay,D., Farenc,C., Chapot-Chartier,M.P., Sadovskaya,I., van Heel,M., Veesler,D., Moineau,S. and Cambillau,C. (2013) structure, adsorption to host, and infection mechanism of virulent lactococcal phage p2. *J Virol*, 87, 12302–12312.

10. Higgins,D.L., Sanozky-Dawes,R.B. and Klaenhammer,T.R. (1988) Restriction and modification activities from *Streptococcus lactis* ME2 are encoded by a self-transmissible plasmid, pTN20, that forms cointegrates during mobilization of lactose-fermenting ability. *J Bacteriol*, 170, 3435–3442.

11. Oliveira,J., Mahony,J., Hanemaaijer,L., Kouwen,T.R.H.M. and van Sinderen,D. (2018) Biodiversity of bacteriophages infecting *Lactococcus lactis* starter cultures. *J Dairy Sci*, 101, 96–105.

12. Christiansen,B., Johnsen,M.G., Stenby,E., Vogensen,F.K. and Hammer,K. (1994) Characterization of the lactococcal temperate phage TP901-1 and its site- specific integration. *J Bacteriol*, 176, 1069–1076.

13. Lillehaug,D., Lindqvist,B.H. and Birkeland,N.K. (1991) Characterization of φLC3, a *Lactococcus lactis* subsp. *cremoris* temperate bacteriophage

with cohesive single-stranded DNA ends. *Appl Environ Microbiol*, 57, 3206–3211.

14. Mahony,J., Oliveira,J., Collins,B., Hanemaaijer,L., Lugli,G.A., Neve,H., Ventura,M., Kouwen,T.R., Cambillau,C. and van Sinderen,D. (2017) Genetic and functional characterisation of the lactococcal P335 phage-host interactions. *BMC Genomics*, 18, 146.

15. Oliveira,J., Mahony,J., Lugli,G.A., Hanemaaijer,L., Kouwen,T., Ventura,M. and van Sinderen,D. (2016) Genome sequences of eight prophages isolated from *Lactococcus lactis* dairy strains. *Genome Announc*, 4, 4–5.

16. Villion,M., Chopin,M.C., Deveau,H., Ehrlich,S.D., Moineau,S. and Chopin,A. (2009) P087, a lactococcal phage with a morphogenesis module similar to an *Enterococcus faecalis* prophage. *Virology*, 388, 49–56

17. Loof,M., Lembke,J. and Teuber,M. (1983) Characterization of the Genome of the *Streptococcus lactis* “subsp. *diacetylactis*” Bacteriophage P008 Wide-spread in German Cheese Factories. *Syst Appl Microbiol*, 4, 413–423.

18. Crutz-Le Coq,A.M., Cesselin,B., Commissaire,J. and Anba,J. (2002) Sequence analysis of the lactococcal bacteriophage bIL170: Insights into structural proteins and HNH endonucleases in dairy phages. *Microbiology (N Y)*, 148, 985–1001.

19. Bidnenko,E., Ehrlich,D. and Chopin,M.C. (1995) Phage operon involved in sensitivity to the *Lactococcus lactis* abortive infection mechanism AbiD1. *J Bacteriol*, 177, 3824–3829.

20. Loof,M. and Teuber,M. (1986) Heteroduplex analysis of the genomes of *Streptococcus lactis* “subsp. *diacetylactis*” bacteriophages of the P008-type isolated from German cheese factories. *Syst Appl Microbiol*, 8, 226–229.

21. Mahony,J., Kot,W., Murphy,J., Ainsworth,S., Neve,H., Hansen,L.H., Heller,K.J., Sørensen,S.J., Hammer,K., Cambillau,C., *et al.* (2013) Investigation of the relationship between lactococcal host cell wall polysaccharide genotype and 936 phage receptor binding protein phylogeny. *Appl Environ Microbiol*, 79, 4385–4392.

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

23. O’Driscoll,J., Glynn,F., Cahalane,O., O’Connell-Motherway,M., Fitzgerald,G.F. and van Sinderen,D. (2004) Lactococcal plasmid pNP40 encodes a novel, temperature-sensitive restriction-modification system. *Appl Environ Microbiol*, 70, 5546–5556

24. Mahony,J., McGrath,S., Fitzgerald,G.F. and van Sinderen,D. (2008) Identification and characterization of lactococcal-prophage-carried superinfection exclusion genes. *Appl Environ Microbiol*, 74, 6206–6215.

Chapter III

Functional and practical insights into three lactococcal antiphage systems

This chapter was published in:

Grafakou A., Mosterd C., de Waal P.P., van Rijswijck I.M.H., van Peij N.N.M.E., Mahony J. and van Sinderen D. (2024) Functional and practical insights into three lactococcal antiphage systems. *Appl Environ Microbiol*, 0:e01120-24, 10.1128/aem.01120-24.

A.G, J.M. and D.v.S. led the study. A.G. performed the experiments and the analyses. C.M. performed and analysed the milk acidification assay. P.d.W., I.v.R. and N.v.P. contributed with meaningful discussion of the results. J.M. and D.v.S. supervised and examined the results of the experiments. The initial concept manuscript was written by A.G. All authors contributed to editing the manuscript.

3.1 Abstract

The persistent challenge of phages in dairy fermentations requires the development of starter cultures with enhanced phage-resistance. Recently, three plasmid-encoded lactococcal antiphage systems, named Rhea, Aristaios and Kamadhenu, were discovered. These systems were found to confer high levels of resistance against various *Skunavirus* members. In the present study, their effectiveness against phage infection was confirmed in milk-based medium, thus validating their potential to ensure reliable dairy fermentations. We furthermore demonstrated that Rhea and Kamadhenu do not directly hinder phage genome replication, transcription or associated translation. Conversely, Aristaios was found to interfere with phage transcription. Two of the antiphage systems are encoded on pMRC01-like conjugative plasmids, and the Kamadhenu-encoding plasmid was successfully transferred by conjugation to three lactococcal strains, each of which acquired substantially enhanced phage-resistance against *Skunavirus* members. Such advances in our knowledge of the lactococcal phage resistome and the possibility of mobilising these protective functions to bolster phage protection in sensitive strains provides practical solutions to the ongoing phage problem in industrial food fermentations.

3.2 Introduction

The production of a plethora of fermented dairy foods, such as cheese, buttermilk and sour cream, has long depended on the employment of *Lactococcus lactis* and *Lactococcus cremoris* (1, 2). These economically and industrially important starter bacteria are members of the lactic acid bacteria (LAB), which is a group of Gram-positive, non-spore forming, micro-aerophilic bacteria (3). Many LAB, including *L. lactis* and *L. cremoris,* enjoy a generally recognized as safe (GRAS) status according to the Food and Drug Administration (FDA) due to their historic safe application in food products. As dominant components of many dairy starter cultures, their primary functions in milk fermentations are to decrease milk pH by producing lactic acid, metabolise milk proteins and, in certain cases, produce antimicrobial substances such as bacteriocins, thus contributing to the organoleptic properties and safety of the final product (1, 4).

However, the intensive application of strains of these two lactococcal species is accompanied by the persistent challenge of infecting (bacterio)phages (5). A phage-infected starter culture may partially or completely lyse, leading to delayed or failed fermentations with major economic and environmental consequences (6). Among the described lactococcal phages (7, 8), members of the *Skunavirus* genus are the most frequently encountered and problematic for the dairy industry (9). The continuous threat posed by phages in the dairy fermentation environment has inspired major research efforts to discover and implement lactococcal antiphage mechanisms. Until recently, described antiphage systems of *Lactococcus* spp. encompassed various Type I, II and III Restriction-

Modification (RM) systems (10–12), a type III-A Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein (CRISPR-Cas) system (13), five Superinfection exclusion (Sie) proteins (Sie_{2009}, Sie_{IL409} or Sie_{309}, $Sie_{F7/2AB}$ or Sie_{285}, $Sie_{mg2/312}$, Sie_{T712} (14, 15)) and 23 abortive infection (Abi) systems (4).

Abi systems are among the most prevalent lactococcal antiphage strategies. Upon activation the infected cell prevents a crucial step in the phage propagation process which typically affects its own viability yet preventing completion of the phage life cycle and consequently averting infection of neighbouring cells (16, 17). Most of the currently known lactococcal Abi systems are encoded by one or two plasmid-borne genes and have been shown to affect different points of the phage reproduction cycle, including DNA replication (AbiA, AbiF, AbiK, AbiP, AbiR, AbiT), transcription (AbiB, AbiG, AbiU, AbiV) and translation (AbiC) (3, 4). Recently, seven additional lactococcal antiphage systems have been described, of which five behave as Abi systems (Rhea, Aristaios, Kamadhenu, Rugutis, Hesat), while the classification/mode of action for the remaining two (Fliodhais, Audmula) is not yet known. Furthermore, the antiphage activity of five additional systems (PARIS, type II and II CBASS, Lamassu, Septu), whose activity had not been previously established in lactococci, was recently reported (18).

There is a technological need for phage-robust starter cultures which simultaneously satisfy consumer demands for products with specific organoleptic features (1, 19). Although genetically modified organisms (GMOs) may address such requirements (20), their use by the food industry

faces considerable consumer opposition and is subject to strict and varying regulatory conditions in various jurisdictions, such as the European Union regulatory framework for deliberate release of GMOs (21). Nonetheless, many desired traits are encoded by conjugative or mobilizable plasmids that by natural processes can be transferred or co-transferred between strains (22). Conjugation is a natural process that involves transfer of genetic material from a donor to a recipient cell via a conjugative apparatus through direct cell-to-cell contact (23). Since conjugation is not classified as genetic modification under Directive 2001/18/EC and is exempt from labelling requirements in the United States, it can be employed to generate phage robust dairy starter cultures which are not considered GMOs (24).

The aim of the current study was to characterize and evaluate mechanistic details of the recently described plasmid-encoded Rhea, Aristaios and Kamadhenu lactococcal antiphage systems which in particular confer high resistance against many members of the most prevalent lactococcal phage genus *Skunavirus* (18) rendering these systems of particular interest to the dairy industry. In the current study, we also apply this resource of transferable phage resistance for the development of phage robust strains.

3.3 Materials and methods

3.3.1 Strains, bacteriophages, cultivation media and growth conditions

Bacterial strains, relevant plasmids and bacteriophages used in this study are listed in Table 1. Lactococcal strains were grown at 30 °C in M17 broth (Oxoid, United Kingdom) supplemented with 0.5 % (w/v) glucose (GM17) for 16-20 h. GM17 was supplemented with either chloramphenicol (5 μg/ml, to select for strains carrying pNZ44 or pJP005 and their derivatives, Sigma-Aldrich, Ireland) or streptomycin (500 μg/ml, to select for *L. cremoris* MG1614, Sigma Aldrich). Propagation of bacteriophages, spot and plaque assays, and phage titration were performed as previously described by Grafakou et al. (18). Efficiency of plaquing (EOP) was estimated from three biological replicates by spotting appropriate dilutions of the phage lysate on a relevant sensitive host.

Table 1. Bacterial strains and bacteriophages used in this study.

Bacterial strains and bacteriophages	Characteristics[a]	Source or Reference
L. cremoris NZ9000::pNZ44	Laboratory strain, host of the antiphage systems and for phage sk1	(25, 26)
L. cremoris 3107::pNZ44	Dairy starter strain, host of the antiphage systems and for phage 62601	(26, 27)
L. cremoris NZ9000/3107::pNZ44 + Rhea	Antiphage system constitutively expressed in *L. cremoris* NZ9000/3107::pNZ44	(18)
L. cremoris NZ9000/3107::pNZ44 + Aristaios	Antiphage system constitutively expressed in *L. cremoris* NZ9000/3107::pNZ44	(18)
L. cremoris NZ9000/3107::pNZ44 + Kamadhenu	Antiphage system constitutively expressed in *L. cremoris* NZ9000/3107::pNZ44	(18)
L. cremoris NZ9000::pNZ44 + AbiP	Antiphage system constitutively expressed in *L. cremoris* NZ9000/3107::pNZ44	(18, 28)
L. cremoris UCCL624	Donor for conjugation experiments carrying among other plasmids pUCCL624B (antiphage systems-encoding conjugative plasmid)	NCBI accession numbers SAMN40630563 and PP556517
L. cremoris NZ9000::pJP005	Laboratory strain, recipient for conjugation experiments, Cm^r	(25, 29)
L. cremoris MG1614	Plasmid free laboratory strain, used as recipient for conjugation experiments, Str^r	(30)
L. lactis UCCL643	Industrial strain, recipient for conjugation experiments	Strain previously isolated from dairy environment

L. cremoris NZ9000::pJP005::pUCCL624B	Transconjugant	This study
L. cremoris MG1614::pUCCL624B	Transconjugant	This study
L. lactis UCCL643::pUCCL624B	Transconjugant	This study
sk1	*Skunavirus* propagated on *L. cremoris* NZ9000::pNZ44	(31)
62601	*Skunavirus* propagated on *L. cremoris* 3107::pNZ44	(32)
p2	*Skunavirus* propagated on *L. cremoris* NZ9000	(33)
712	*Skunavirus* propagated on *L. cremoris* NZ9000::pNZ44	(34)
jj50	*Skunavirus* propagated on *L. cremoris* NZ9000	(35)
phUCCL643.2	*Skunavirus* propagated on *L. lactis* UCCL643	Phage previously isolated from dairy environment
phUCCL643.3	*Skunavirus* propagated on *L. lactis* UCCL643	Phage previously isolated from the dairy environment
phUCCL643.4	*Skunavirus* propagated on *L. lactis* UCCL643	Phage previously isolated from the dairy environment
phUCCL643.5	*Skunavirus* propagated on *L. lactis* UCCL643	Phage previously isolated from the dairy environment

[a]Cm^r, chloramphenicol resistant; Str^r, streptomycin resistant

3.3.2 One step phage growth curves for ECOI and burst size determination

One step phage growth curves were conducted as described by Moineau et al. (36) with some modifications. Two milliliters of bacterial culture (optical density at 600 nm, OD_{600nm} of ~0.8) were harvested by centrifugation (7,000 × *g* for 2 min) and resuspended in 900 µl of GM17 supplemented with 10 mM $CaCl_2$ (GM17+$CaCl_2$). Phage sk1 lysate was added at a multiplicity of infection (MOI) of 0.1. After 5 min of phage adsorption at 30 ˚C, bacteria were harvested as previously described and washed twice in 1 ml of GM17+$CaCl_2$ to remove free phages. The suspension was serially diluted 10,000-fold in GM17+$CaCl_2$ to reduce the number of bacteria available for residual free phage adsorption and incubated at 30°C. Samples were withdrawn immediately (time zero, t_0) and every 15 min for 75 min and plated on the phage-sensitive host (*L. cremoris* NZ9000::pNZ44) after appropriate dilution. The initial titer (t_0) defined the number of centres of infection (COI) formed. The efficiency of COI formation (ECOI) for each sample was calculated as the COI from the test strain divided by the COI obtained from the reference strain *L. cremoris* NZ9000::pNZ44. The burst size of sk1 on a test strain was determined as (phage titer at the end of the single step growth curve [60 min] - initial titer [15 min])/initial titer [15 min]. Data are presented as mean ± standard deviation (SD) from three biological replicates.

3.3.3 DNA replication assay

Quantification of intracellular sk1 DNA in phage-infected cells of *L. cremoris* NZ9000::pNZ44 and its phage-resistant derivatives expressing the three antiphage systems (Table 1) was evaluated using quantitative PCR (qPCR) as previously described with some modifications (37). Ten milliliters of GM17 broth inoculated with 2 % of fresh overnight culture of relevant strains was incubated at 30 °C until an OD_{600nm} of 0.2 was reached. The culture was subsequently supplemented with a final concentration of 10 mM $CaCl_2$. Phage lysate was added at an MOI of 0.03. The infected cultures were incubated at 30 ˚C and 1 ml samples of the culture were taken after 5, 15 and 25 min, and centrifuged in pre-chilled tubes at 14,000 × *g* for 2 min at 4 ˚C. The supernatant was discarded to avoid quantifying residual phages. The pellet was resuspended in 1 ml of ice-cold ¼ strength Ringer's solution (Merck, Ireland), flash-frozen in ethanol (70 %, -80 ˚C) and stored at -80 ˚C prior to DNA extraction. Subsequently, the frozen samples were thawed on ice and the pellets were washed once in ¼ strength Ringer's solution. Total DNA was extracted using the Purelink™ Genomic DNA extraction kit (Invitrogen) according to the manufacturer's instructions. Three biological replicates were performed for each strain.

LightCycler® 480 SYBR Green I Master (Roche, Ireland) and a LightCycler® 480 Instrument (Roche) were used to perform the qPCR assays. Previously optimized qPCR primers targeting the sk1 small terminase-encoding gene (ORF1, NCBI Taxonomy ID 2905675) were used (38). Three technical replicates (15 µl reaction mixture) containing 5 µl of sample or sk1 DNA (for the construction of the standard curve, see below)

and 500 nM of each primer were prepared for each sample. The initial denaturation step was performed at 95 °C for 5 min, followed by 40 cycles of: (i) denaturation at 95 °C for 15 s; (ii) annealing at 55 °C for 30 s; and (iii) extension at 72 °C for 30 s. Negative controls without template DNA were included in the analysis.

To quantify sk1 DNA, a standard curve was constructed by extracting DNA from 1 ml pure phage lysate (Norgen Biotek, Canada) according to the manufacturer's instructions with slight modifications. Prior to DNA extraction, a phage lysate was treated with 2 µl of DNAse I (Roche) for 30 min followed by ethylenediaminetetraacetic acid (EDTA; 5 mM) inactivation for 20 min at room temperature. The DNA was quantified using a Qubit™ 1X dsDNA High Sensitivity (HS; quantitation range 0.1 to 120 ng) (Thermo Fischer Scientific, United Kingdom), then serially diluted and analysed by qPCR as described above. To generate the best-fit line, the obtained cycle threshold (C_T) values were plotted against the range of known quantities of DNA that were converted to DNA copies (per ml of standard sample) considering the molecular weight of phage sk1 as 17,654.67 kDa (39). Data are presented as mean ± standard deviation (SD) from three biological replicates and an unpaired t-test was performed to determine statistically significant differences (P-value < 0.01).

3.3.4 Transcription assay

Quantification of intracellular sk1 RNA was evaluated in sk1 infected cells of *L. cremoris* NZ9000::pNZ44 and its phage-resistant derivatives

expressing the three antiphage systems (Table 1) by RT-qPCR. Bacterial strains were cultivated and infected as described above for the replication assays apart from adding sk1 phage lysate at MOI of 0.1. Infected cultures were initially incubated at 30 °C and after 10 and 25 min, 1 ml sample were centrifuged in pre-chilled tubes at 14,000 × *g* for 2 min at 4 ˚C as an initial screening. To obtain a more detailed profile of *L. cremoris* NZ9000::pNZ44 carrying the Aristaios antiphage system, another round of infection was performed along with the reference strain and samples were collected at 5, 10, 15, 25, 35 and 60 min time points. Pellets were resuspended and stored in 500 µl of RNA*later* (Invitrogen). Prior to RNA extraction 500 µl of ¼ strength Ringer's solution was added to the samples, which were then centrifuged at 10,000 × *g* for 5 min to remove RNAlater. Total RNA was extracted using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions with the modification of adding 12 µl of DNAse mix (DNAse and incubation buffer included in the kit) to each sample and incubating at 30 °C. Three biological replicates were performed for each strain. cDNA was synthesized using SuperScript™ III reverse transcriptase (RT; Invitrogen) according to the manufacturer's instructions. For each 20 µl reaction 250 ng of random oligomeric primers (Invitrogen) and 5 µl of total RNA were used. Reactions containing the same mix but replacing the RT enzyme with water were included as controls to verify appropriate DNA digestion.

LightCycler® 480 SYBR Green I Master (Roche) and a LightCycler® 480 Instrument (Roche) were also employed for the RT-qPCR experiments. According to the time of their appearance, phage transcripts have been

divided into three categories, corresponding to early, middle or late-expressed regions (31). Primers targeting the middle and late transcripts were used in this study. Primers annealing to the predicted Holliday junction endonuclease-encoding gene of sk1 (NCBI Taxonomy ID 2905675; open reading frame 53 [ORF53]; sk1_hol_F ACGTTCAAGTCTAAGGGAAAC and sk1_hol_R TGGTACATCAACATAGCCAATG) were designed to quantify the middle transcripts. Primers were validated and optimized by determining the primer efficiency (89.8 %) and the R^2 (0.99) during a standard curve construction as described in the previous paragraph. Optimized qPCR primers targeting the sk1 small terminase-encoding gene were applied (38) to quantify the late transcripts. Three technical replicates were prepared and analysed as described for the qPCR experiments with slight modifications as follows: 5 µl of cDNA added for each reaction and the extension step was performed at 72 °C for 20 s. Phage sk1 cDNA in infected lactococcal cells was quantified and interpreted as described for the replication assay.

3.3.5 Protein overexpression and antibody generation

Phage sk1 capsid-, tail- and receptor binding protein (RBP)-encoding genes (ORF6, ORF11 and ORF18, respectively; NCBI Taxonomy ID 2905675) were amplified from a fresh lysate using Phusion Green High-Fidelity DNA Polymerase (Thermo Fisher Scientific) according to the manufacturer's instructions. The NZYEasy Cloning & Expression kit I (NZYTech, Portugal) and *E. coli* vector pHTP1 was selected, since it contains two poly-histidine (6xHis) sequences allowing subsequent

recombinant protein purification by affinity chromatography. Therefore, primers (Table S1) were designed by omitting the start codon for the forward primer and including an in-frame stop codon to the reverse primer to exclude the C-terminal His-tag according to the manufacturer's instructions. The purified PCR-generated fragments were cloned into the linearized pHTP1 *E. coli* vector. Ligation mixtures were introduced into competent *E. coli* BL21 (DE3) (40) according to the manufacturer's instructions. The integrity of recombinant plasmid sequences was verified by Sanger sequencing (Eurofins Genomics, Germany) using vector-specific primers (Table S1). *E. coli* BL21 (DE3) harbouring a plasmid of interest was inoculated (1 %) to 100 ml of LB media (Thermo Fisher Scientific) supplemented with 50 µg/ml kanamycin (Sigma) and cultures were incubated with agitation (200 rpm) at 37 ˚C until an OD_{600nm} of 0.4-0.5 was reached. Induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; PanReac, United States) followed and cultures were incubated at 30 ˚C for 4-6 hours. Cells were harvested at 4,600 × *g* for 30 min and the pellet was resuspended in 10 ml lysis buffer (10 mM Tris-HCl pH 7.5, 300 mM NaCl, 50 mM $CaCl_2$, 10 mM imidazole, 25 mg/ml lysozyme). The cell suspensions were lysed by sonication and recombinant proteins were purified as previously described by Lavelle et al. (41). Antibodies targeting the purified and dialysed phage sk1 capsid, tail and RBP were generated with a standard rabbit immunization protocol (63 days) executed by Davids Biotechnologie GmbH (Germany, https://www.davids-bio.com/pages/polyclonal-rabbit-antisera.html).

3.3.6 Detection of intracellular phage proteins during infection

Fresh overnight cultures of *L. cremoris* NZ9000::pNZ44 and its derivatives (Table 1) were added (1 %) to 100 ml of GM17 and incubated at 30 °C until an OD_{600nm} of ~0.2 was reached. Cells were harvested by centrifugation (at 2,600 × *g* for 5 min) and the obtained cell pellet was resuspended in 10 ml of GM17 supplemented with 10 mM $CaCl_2$ and subsequently infected with phage sk1 at an MOI of 1. Two-ml samples were taken at 0, 15, 25, 30 and 60 min post-infection and centrifuged at 14,000 × *g* for 2 min at 4 °C. The obtained cell pellets were flash-frozen (−80°C) and analyzed for intracellular phage proteins production by Western blotting. Cell pellets were resuspended in 80 µl of sodium dodecyl sulfate (SDS) loading buffer (10 % glycerol, 3 % SDS, 0.0625 M Tris pH 6.8, 0.01 % bromophenol blue) and were incubated at 100 °C for 5 min prior to SDS-PAGE gel loading. The samples were centrifuged (at 14,000 × *g* for 1 min) and 20 µl protein solution was separated at 160 V for 90 min on a 12 % SDS-PAGE gel. The proteins were electroblotted (100 V for 30 min) onto a nitrocellulose membrane (0.45 µm, Thermo Fisher Scientific) using a 1 X CAPS solution (pH 10.5; Sigma) as transfer buffer in a Mini-PROTEAN® 3 Cell apparatus (Bio-Rad, Ireland). The membranes were subsequently soaked in PBS (Sigma) for several minutes and then treated with Intercept® (PBS) blocking buffer (LI-COR, United Kingdom) for 1 h on a platform shaker STR6 (Stuart Scientific). Membranes were then incubated (1 h, gentle shaking) with primary antibody, having been diluted 1:5,000 (in the case of antibodies directed against the major capsid or tail protein) or 1:2,500 (for antibodies against the RBP) in blocking buffer supplemented

with 0.25 % Tween-20 (Sigma). After four washes (5 min each with gentle shaking) with PBS supplemented with 0.1 % Tween-20, the membrane was incubated (1 h, shaking) with secondary antibody (IRDye® 680RD Goat anti-Rabbit IgG; LI-COR) diluted 1:7,500 in blocking buffer. This was followed by four washes with PBS supplemented with 0.1 % Tween-20 (5 min each with gentle shaking) and a final rinse in PBS to remove residual Tween-20. The protein bands were visualized with IRDye 680RD application and an optimal auto-exposure time using ChemiDoc MP imager (Bio-Rad).

3.3.7 Milk acidification assay

L. cremoris 3107::pNZ44 and derivative strains expressing either of the three antiphage systems (Table 1) were grown overnight at 30 °C in M17 supplemented with 0.5 % lactose (LM17). Fresh overnight cultures (2 %) were added directly to 10 % $^{w}/_{v}$ Reconstituted Skimmed Milk (RSM, Carbery Milk Products, Ireland) supplemented with 5 µg/ml chloramphenicol and the cultures were grown overnight at 30 °C. Subsequently, a 2 % inoculum was added to 10 ml of fresh 10 % RSM and cultures were then incubated at 30 °C for three hours at which point they were infected with 100 µl of *Skunavirus* 62601 ($1*10^8$ PFU/ml). Uninfected cultures were included as controls and were supplemented with 100 µl of sterile-filtered overnight culture (*L. cremoris* 3107::pNZ44, LM17) instead of phage lysate. The pH was recorded every 3 hours for a total of 9 hours, with a final reading after 24 hours. Data are presented as mean ± SD from three biological replicates.

3.3.8 Conjugation experiments

Conjugation was performed using the spread solid mating approach as described by Ortiz Charneco et al. (42). Agar plates were supplemented with streptomycin, chloramphenicol or nisin to select for recipient cells, i.e. *L. cremoris* MG1614, *L. cremoris* NZ9000::pJP005 and *L. lactis* UCCL643, respectively.

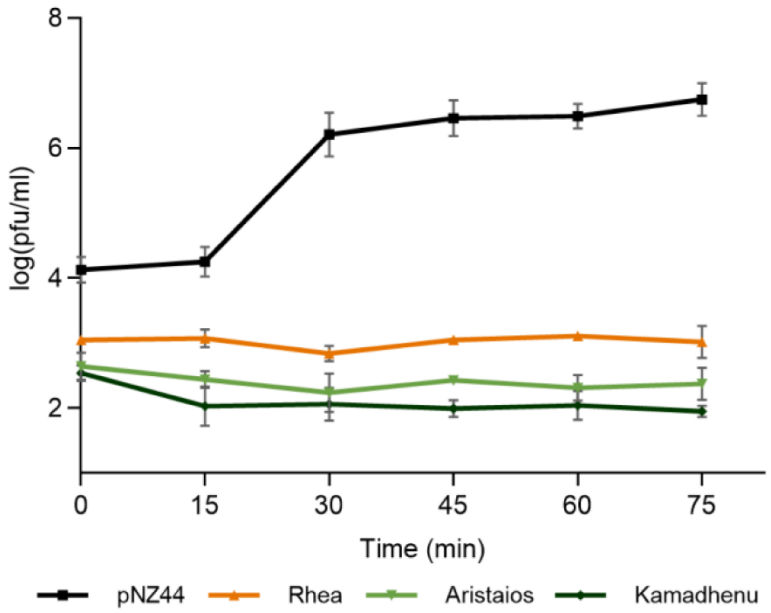
The presence of pUCCL624B in *L. cremoris* MG1614, *L. cremoris* NZ9000::pJP005 and *L. lactis* UCCL643 was verified by colony PCR using Phusion Green High-Fidelity DNA Polymerase (Thermo Fisher Scientific) according to the manufacturer's instructions and pUCCL624B- and NZ9000/MG1614/UCCL643-specific primers (Table S2), confirming conjugative transfer of pUCCL624B to the recipient strain.

3.4 Results

3.4.1 Rhea, Aristaios and Kamadhenu antiphage systems dramatically reduce the burst size and ECOI of phage sk1

To date, in addition to the previously established 23 lactococcal abortive infection systems (AbiA to AbiZ (4)), ten additional antiphage systems that behave as Abi systems (Rhea, Aristaios, Kamadhenu, Hesat, Rugutis, PARIS, type I and II CBASS, Lamassu, Septu) have been described to be present in representatives of this genus (18). The objective of the current study was to further characterize the plasmid-encoded Rhea, Aristaios and Kamadhenu lactococcal antiphage systems. The effectiveness of Rhea, Aristaios and Kamadhenu antiphage systems against many members of the most prevalent lactococcal phage genus *Skunavirus* and specifically against *Skunavirus* sk1 in solid and liquid growth medium had previously been demonstrated (18). To further evaluate the impact of these systems on phage development, the burst size and the ECOI of phage sk1 on *L. cremoris* NZ9000::pNZ44 and its derivatives expressing each of the three antiphage mechanisms (Table 1) was determined using one step phage growth curves (Figure 1A). Infected cells of *L. cremoris* NZ9000::pNZ44 release approximately 180 progeny sk1 virions per infected cell (Figure 1B). However, the strains containing the three antiphage systems produce no detectable phage progeny (burst size close to zero) (Figure 1B). Similarly, a 11-fold (for Rhea) and approximately 30-fold (for Aristaios and Kamadhenu) ECOI reduction was observed in the presence of these antiphage systems relative to the control (Figure 1B).

A



B

Strain	ECOI	Burst size
NZ9000::pNZ44	1	180 ± 65
NZ9000::pNZ44+Rhea	0.088 ± 0.035	0.16 ± 0.40
NZ9000::pNZ44+Aristaios	0.032 ± 0.002	-0.22 ± 0.29
NZ9000::pNZ44+Kamadhenu	0.029 ± 0.018	0.16 ± 0.57

Figure 1. A) One step phage growth curve of phage sk1 and B) Efficiency of centre of infection (ECOI) and burst size of phage sk1 on *L. cremoris* NZ9000::pNZ44 and its phage resistant derivatives harbouring Rhea, Aristaios and Kamadhenu antiphage systems. Experiments were performed in biological triplicate and data are presented as means ± SD.

3.4.2 Phage DNA replication is not impaired in the presence of Rhea, Aristaios and Kamadhenu

Previously it was shown that the three antiphage systems do not interfere with phage adsorption or DNA injection and exhibit an Abi-like phenotype (18). To further assess which step of the phage life cycle is targeted by the Rhea, Aristaios and Kamadhenu antiphage systems, we investigated the replication, transcription and translation process of phage sk1 in sensitive host cells (*L. cremoris* NZ9000::pNZ44) and cells harbouring the antiphage systems (*L. cremoris* NZ9000::pNZ44 expressing Rhea, Aristaios, Kamadhenu or AbiP antiphage systems; Table 1). AbiP was previously described to interfere with phage DNA replication and was therefore used as a positive control (28). DNA replication of phage sk1 was studied by quantifying the sk1 *terS* (small terminase-encoding) gene using qPCR 5, 15 and 25 min post phage infection. Phage sk1 DNA was already detected in all samples 5 min post phage infection (Figure 2). After 15 min, a more than a ten-fold increase of *terS* was observed for all samples except for the culture expressing AbiP (positive control). After 25 min, similar levels of DNA copies/ml were recorded for all samples except for the culture expressing AbiP. Additionally, AbiP was shown to prevent *terS* increase after 25 min compared to the 15 min time point. Therefore, in contrast to AbiP, lactococcal cultures expressing Rhea, Aristaios and Kamadhenu antiphage systems do not interfere with sk1 phage replication and allow DNA multiplication at a level similar to the reference strain.

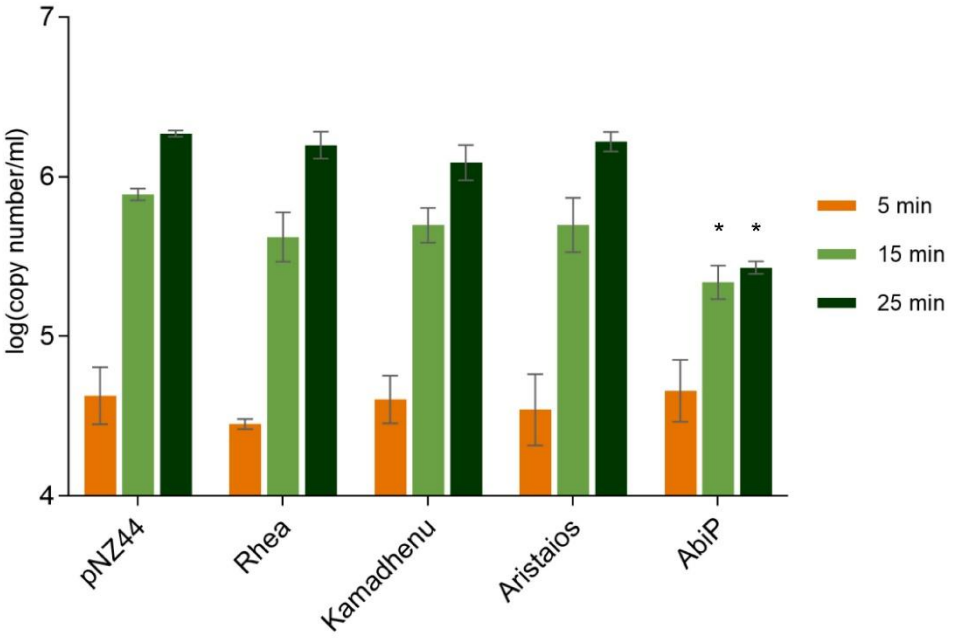


Figure 2. Quantification of phage sk1 DNA on different time intervals by qPCR in *L. cremoris* NZ9000::pNZ44 and its phage resistant derivatives harbouring Rhea, Aristaios, Kamadhenu and AbiP antiphage systems. Experiments were performed in biological triplicate and data are presented as means ± SD. Asterisks mark statistically significant differences between the phage resistant derivatives and the reference strain at respective time points (unpaired t-test, P-value < 0.01).

3.4.3 Aristaios interferes with phage transcription

Transcription of phage sk1 in sensitive cells (*L. cremoris* NZ9000::pNZ44) and cells harbouring phage-resistance systems (*L. cremoris* NZ9000::pNZ44 carrying Rhea, Aristaios and Kamadhenu antiphage systems; Table 1) was examined by quantifying the sk1 *hol* (encoding a predicted Holliday junction endonuclease; part of the middle transcript) and *terS* (encoding the presumed small terminase; part of the late transcript) cDNA with RT-qPCR 10 and 25 min post phage infection

(Figure 3A). Phage sk1 middle and late transcript cDNA was detected for all assessed samples 10 min post phage infection followed by an increase at 25 min in strains harbouring Rhea and Kamadhenu, but not for the Aristaios antiphage system. A detailed profile for Aristaios antiphage system (Figure 3B), confirmed that at 25 min post-infection there were approximately 10 times fewer copies of cDNA (for both middle and late transcripts) when this system was expressed compared to the reference strain. Interestingly, the additional time-point at 60 min showed an increase in the cDNA copies for the reference strain, indicating a second round of infections, compared to the strain carrying Aristaios where at 60 min a decrease is observed that could be explained by the activation of the antiphage system and cell lysis without release of phage progeny, confirming the previous one step phage growth curve result. Therefore, the Aristaios antiphage system appears to interfere with sk1 transcription, in contrast to Rhea and Kamadhenu antiphage systems for which no impact was observed.

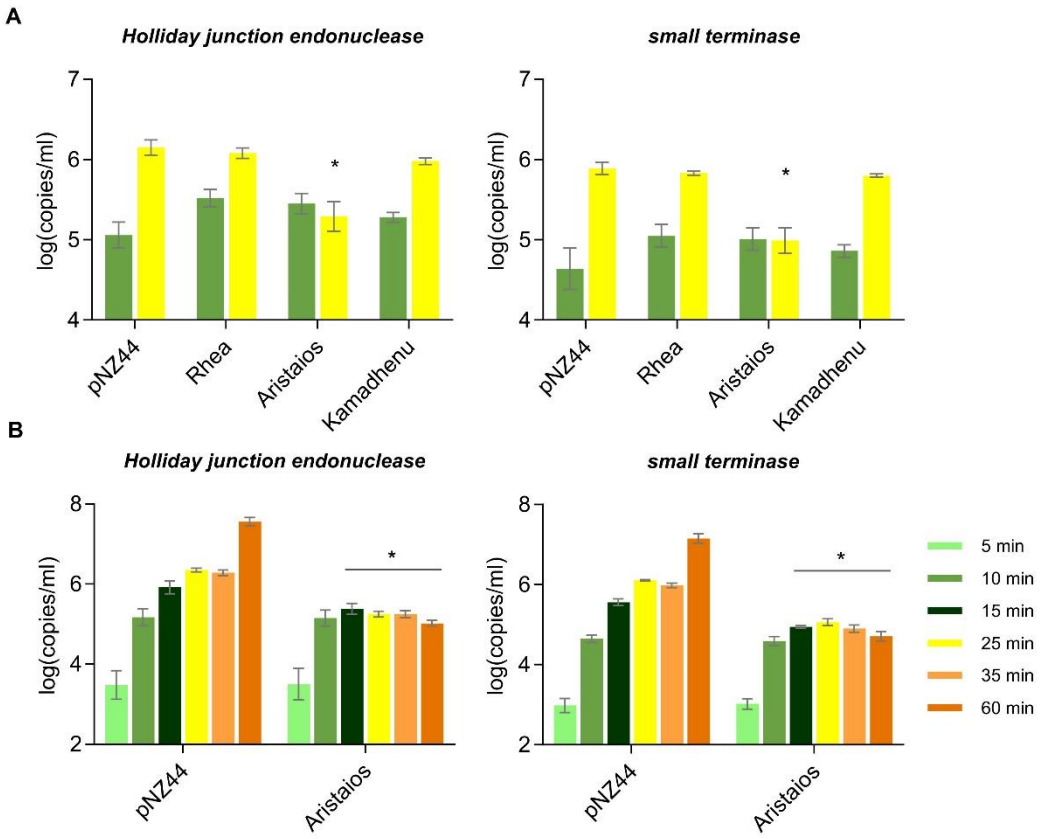


Figure 3. Quantification of phage sk1 cDNA on different time intervals by qPCR in *L. cremoris* NZ9000::pNZ44 and its phage resistant derivatives harbouring Rhea, Aristaios and Kamadhenu antiphage systems. Experiments were performed in biological triplicate and data are presented as means ± SD. Asterisks mark statistically significant differences between the phage resistant derivatives and the reference strain at respective timepoints (unpaired t-test, P-value < 0.01).

3.4.4 Aristaios downstream impact on phage translation

The effect of Rhea, Aristaios and Kamadhenu antiphage systems (Table 1) on the production of phage structural proteins was investigated at different time intervals by using polyclonal antibodies specific to sk1 capsid, tail and RBP proteins (Figure 4). In cell extracts of the reference strain (*L. cremoris* NZ9000::pNZ44), sk1 phage proteins were detected 15 (capsid

and tail) or 30 min (RBP) post-phage infection, followed by an increased signal at the next time point (Figure 4). A similar profile was recorded for the cell extracts of Rhea and Kamadhenu expressing strains. Consistent with the finding that Aristaios interferes with phage sk1 transcription, sk1 capsid, tail and RBP proteins were either not detected at any time point (capsid; Figure 4A) or there was a substantially reduced signal detection (tail and RBP; Figure 4B and 4C) in the presence of Aristaios. These results indicate that Rhea and Kamadhenu do not hinder the production of sk1 capsid, RBP and tail protein, and likely do not interfere with phage mRNA translation. In contrast, Aristaios seems to directly or indirectly interfere with transcription of *Skunavirus* sk1 with a consequent impact on the produced protein levels.

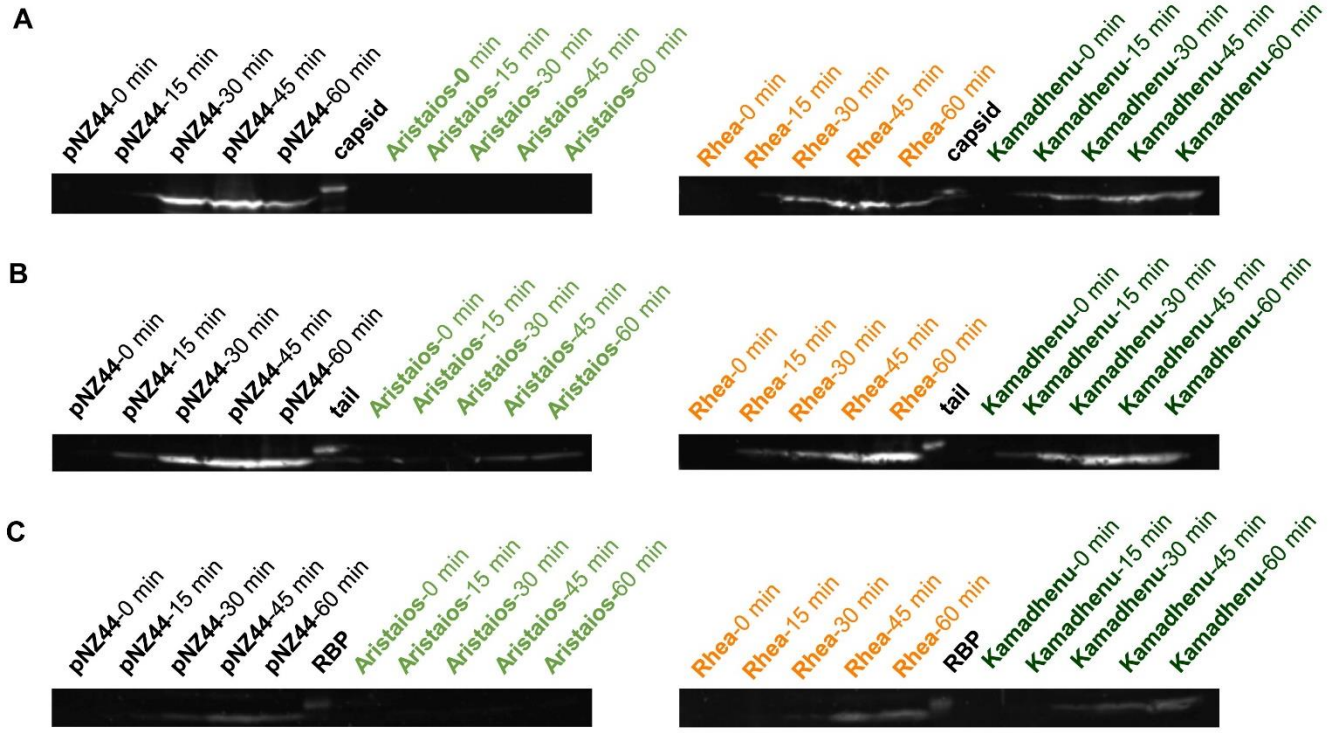


Figure 4. Detection of sk1 phage capsid (panel A), tail (B) and RBP (C) in *L. cremoris* NZ9000::pNZ44 and its phage resistant derivatives harbouring Rhea, Aristaios and Kamadhenu antiphage systems with western blot in different time points.

3.4.5 Rhea, Aristaios and Kamadhenu antiphage systems are active in milk-based growth medium

Previously, it was shown that the three antiphage systems are effective against multiple *Skunavirus* members in three different lactococcal strains (*L. cremoris* NZ9000, *L cremoris* 3107 and *L. lactis* IL1403) on solid semisynthetic medium (18). To assess the effectiveness of these systems in a milk-based medium, which is more relevant for dairy applications, *L. cremoris* 3107::pNZ44 and its phage-resistant derivatives expressing Rhea, Aristaios and Kamadhenu (Table 1) were grown and infected with *Skunavirus* 62601 in reconstituted skimmed milk (Figure 5). *L. cremoris* 3107 is a dairy starter strain with plasmids encoding the ability to utilize lactose (27), while *Skunavirus* 62601 can infect *L. cremoris* 3107, unlike *Skunavirus* sk1. Therefore, it allows growth in milk-based medium in contrast to the laboratory strain *L. cremoris* NZ9000 that was used for the remainder of this study. The pH of the milk was measured at different time points starting at the point of inoculation. After 6 hours, all infected cultures exhibited similar acidification profiles, except for the infected reference strain, where acidification was notably affected. Phage resistance of strains expressing either of the three antiphage systems was maintained overnight, as shown by a further pH decrease. Therefore, under the tested conditions, the Rhea, Aristaios and Kamadhenu antiphage systems were effective against this member of the *Skunavirus* genus in a milk-based medium at a level that was equivalent to that observed in synthetic liquid laboratory medium (18).

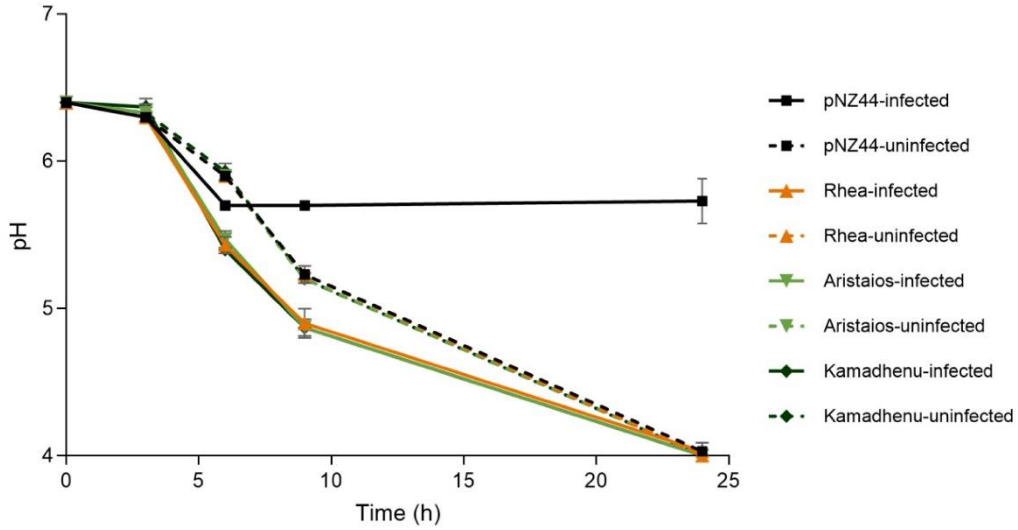


Figure 5. Milk acidification curves of *L. cremoris* NZ9000::pNZ44 and its phage resistant derivatives harbouring Rhea, Aristaios and Kamadhenu antiphage systems. Experiments were performed in biological triplicate and data are presented as means ± SD.

3.4.6 Phage-resistant strains generated by natural transfer of Kamadhenu antiphage system encoding plasmid

Previously, it was reported that Aristaios and Kamadhenu antiphage systems are encoded on pMRC01-like conjugative plasmids (18), among other antiphage systems, based on BLASTP alignment (43) against the two prevalent lactococcal conjugation systems on pNP40 and pMRC01 (42). Therefore, these antiphage systems could potentially be transferred to starter strains of interest by conjugation, to generate natural derivatives with enhanced antiphage characteristics that could be used in the food industry.

To evaluate this possibility, plasmid pUCCL624B (Kamadhenu-encoding plasmid, Table 1) was selected as a representative for conjugation

experiments, as it encodes several additional (predicted) antiphage systems including AbiA, AbiZ and Kamadhenu, as well as an RM system (Figure 6). Indeed, pUCCL624B was successfully transferred by conjugation to three strains (*L. cremoris* NZ9000::pJP005, *L. cremoris* MG1614 and *L. lactis* UCCL643*; Table* 1). The presence of pUCCL624B in *L. cremoris* MG1614, *L. cremoris* NZ9000::pJP005 and *L. lactis* UCCL643 was further verified by PCR using pUCCL624B/NZ9000/MG1614/UCCL643-specific primers (Table S1).

The three transconjugants (Table 1) were subsequently challenged against skunaviruses that infect the recipient strain (i.e. sk1, p2,712 and jj50 for *L. cremoris* NZ9000::pJP005 & MG1614 and phages phUCCL643.2-5 for *L. lactis* UCCL643). Indeed, the transconjugants provided either over five log efficiency of plaquing (EOP) reduction (*L. cremoris* NZ9000::pJP005::p60975B *&* *L. cremoris* MG1614::pUCCL624B) or three to five log EOP reduction (UCCL643::pUCCL624B) against the above-mentioned phages of the genus *Skunavirus* that infect the respective strains (Table S2). Therefore, it was confirmed that pUCCL624B can be transferred by conjugation delivering associated antiphage traits.

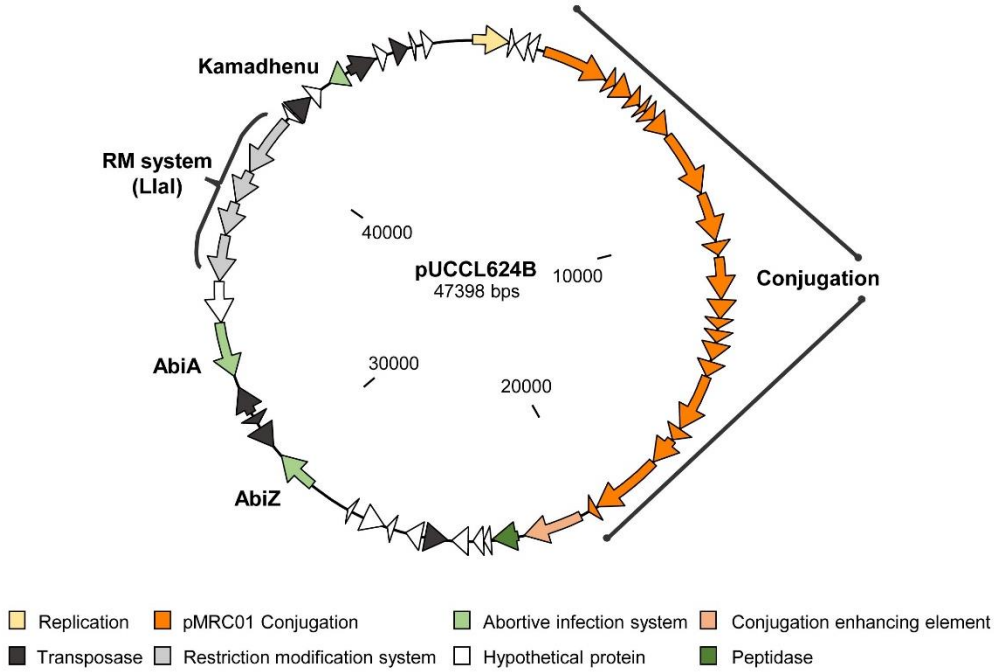


Figure 6. Plasmid map of the pMRC01-like conjugative plasmid pUCCL624B carrying Kamadhenu antiphage system among other antiphage systems.

3.5 Discussion

Three recently discovered lactococcal antiphage systems, namely Rhea, Aristaios and Kamadhenu, were observed to behave as Abi systems and to be highly effective against all tested phages of the genus *Skunavirus* (18). Members of the *Skunavirus* genus are the most problematic lactococcal phages (44), rendering the Rhea, Aristaios and Kamadhenu antiphage systems particularly interesting to the dairy fermentation industry.

In the current study, the burst size and the ECOI of phage sk1 was observed to drop dramatically in the presence of these systems, resembling similar observations for AbiG, AbiQ, AbiU, AbiT (45–48). Severe ECOI and burst size reductions are typical of Abi systems, confirming the Abi phenotype for the Rhea, Aristaios and Kamadhenu antiphage systems. Furthermore, it was previously shown that the three systems allow sk1 phage adsorption/transduction and are therefore active post phage DNA injection (18). Here, phage DNA replication was observed to proceed unimpaired in the presence of the described antiphage systems. In contrast, AbiP was observed to affect DNA replication, consistent with previous literature reports (28).

Rhea and Kamadhenu do not evidently block any of the investigated steps of the phage lytic cycle (adsorption, DNA injection, DNA replication, transcription, translation). However, these systems could interfere with other steps, such as DNA packaging, virion assembly, or host lysis, which were not investigated in the current study. Aristaios was found to (directly or indirectly) inhibit sk1 transcription with a consequent impact on phage protein production. This finding supports the previously proposed

hypothesis that the Aristaios protein hinders an essential host or phage function as it resembles an ADP-ribosyltransferase (18), a domain also found in bacterial toxins (49) including a toxin-antitoxin antiphage system (50). Therefore, these results confirm that these antiphage systems are active post-phage DNA injection. Notably, several (i.e. six) characterized lactococcal Abi systems have been demonstrated to interfere with phage DNA replication, while four and one of them have been shown to interfere with transcription and translation, respectively (4).

Furthermore, it is known that phages may bypass antiphage systems through phage-specific mutations. Future isolation of phage mutants that escape these antiphage systems may provide further mechanistic details (i.e. activation) as demonstrated for antiphage systems in other bacterial species (51). Therefore, thoroughly characterized antiphage systems with diverse mechanisms could be combined to develop starter cultures with stronger phage-resistance that are harder to be bypassed by phages through single point mutations. Interestingly, the synergistic protection of combined systems, such as Zorya II with Druantia III or ietAS and tmn with Gabija, Septu I or PrrC, has recently been demonstrated in *E. coli* (52).

Additionally, along with the high effectiveness of the three antiphage systems against members of the genus *Skunavirus* in synthetic medium, the three antiphage systems were found to retain their protective action against a member of the *Skunavirus* genus in a milk-based medium, highlighting their industrial significance. This knowledge can be applied to improve dairy fermentations by either employing strains carrying (any of the) antiphage systems as starters or by transferring the plasmids encoding

these mechanisms to strains of interest. Interestingly, two of the three systems of this study are encoded on a pMRC01-like plasmid. Plasmid pMRC01 has previously been successfully transferred to over 30 different lactococcal strains, including commercial starter strains (53). Here, the Kamadhenu-encoding plasmid (which also encodes AbiA, AbiZ and an RM system) was successfully transferred to three lactococcal stains, including laboratory and industrial strains, offering protection against all tested *Skunavirus* members. This finding demonstrates that genetically transferable antiphage activities can be harnessed for the development of phage robust strains.

Although more than 30 genetically distinct lactococcal Abi systems have been described, their mechanisms of action appear to be diverse, affecting different critical stages of the phage multiplication cycle, such as DNA replication, transcription and translation. However, antiphage systems can be overcome through phage-specific mutations, resulting in delayed fermentations and downstream economic sequences. This highlights the necessity of expanding the arsenal of antiphage systems by discovering previously unknown antiphage systems and understanding their diverse mechanisms, that may be combined for the generation of more robust starter cultures.

3.6 Data availability

This study did not generate new unique reagents or sequencing data. Any additional information required to reanalyse the data reported in this paper is available from the corresponding author upon request.

3.7 References

1. Cavanagh,D., Fitzgerald,G.F. and McAuliffe,O. (2015) From field to fermentation: The origins of *Lactococcus lactis* and its domestication to the dairy environment. *Food Microbiol*, 47, 45–61.

2. Aryana,K.J. and Olson,D.W. (2017) A 100-Year Review: Yogurt and other cultured dairy products. *J Dairy Sci*, 100, 9987–10013.

3. Makarova,K., Slesarev,A., Wolf,Y., Sorokin,A., Mirkin,B., Koonin,E., Pavlov,A., Pavlova,N., Karamychev,V., Polouchine,N., *et al.* (2006) Comparative genomics of the lactic acid bacteria. *Proc Natl Acad Sc U S A*, 103, 15611–15616.

4. Ainsworth,S., Stockdale,S., Bottacini,F., Mahony,J. and van Sinderen,D. (2014) The *Lactococcus lactis* plasmidome: much learnt, yet still lots to discover. *FEMS Microbiol Rev*, 38, 1066–1088.

5. Hayes,S., Murphy,J., Mahony,J., Lugli,G.A., Ventura,M., Noben,J.P., Franz,C.M.A.P., Neve,H., Nauta,A. and Van Sinderen,D. (2017) Biocidal inactivation of *Lactococcus lactis* bacteriophages: Efficacy and targets of commonly used sanitizers. *Front Microbiol*, 8, 107.

6. Garneau,J.E. and Moineau,S. (2011) Bacteriophages of lactic acid bacteria and their impact on milk fermentations. *Microb Cell Fact*, 10, 1–10.

7. Deveau,H., Labrie,S.J., Chopin,M.C. and Moineau,S. (2006) Biodiversity and classification of lactococcal phages. *Appl Environ Microbiol*, 72, 4338–4346.

8. Zrelovs,N., Dislers,A. and Kazaks,A. (2021) Genome characterization of nocturne116, novel *Lactococcus lactis*-infecting phage isolated from moth. *Microorganisms*, 9, 1540.

9. Mahony,J., Murphy,J. and van Sinderen,D. (2012) Lactococcal 936-type phages and dairy fermentation problems: From detection to evolution and prevention. *Front Microbiol*, 3, 1–9.

10. Nyengaard,N., Vogensen,F.K. and Josephsen,J. (1995) Restriction-modification systems in *Lactococcus lactis*. *Gene*, 157, 13–18.

11. Schouler,C., Clier,F., Lerayer,A.L., Ehrlich,S.D. and Chopin,M.C. (1998) A type IC restriction-modification system in *Lactococcus lactis*. *J Bacteriol*, 180, 407–11.

12. Su,P., Im,H., Hsieh,H., Kang'A,S. and Dunn,N.W. (1999) LlaFI, a type III restriction and modification system in *Lactococcus lactis*. *Appl Environ Microbiol*, 65, 686–93.

13. Millen,A.M., Samson,J.E., Tremblay,D.M., Magadán,A.H., Rousseau,G.M., Moineau,S. and Romero,D.A. (2019) *Lactococcus lactis* type III-A CRISPR-Cas system cleaves bacteriophage RNA. *RNA Biol*, 16, 461–468.

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

15. Mahony,J., McGrath,S., Fitzgerald,G.F. and van Sinderen,D. (2008) Identification and characterization of lactococcal-prophage-carried superinfection exclusion genes. *Appl Environ Microbiol*, 74, 6206–6215.

16. Chopin,M.C., Chopin,A. and Bidnenko,E. (2005) Phage abortive infection in lactococci: Variations on a theme. *Curr Opin Microbiol*, 8, 473–479.

17. Lopatina,A., Tal,N. and Sorek,R. (2020) Abortive Infection: Bacterial suicide as an antiviral immune strategy. *Annu Rev Virol*, 7, 371–384.

18. Grafakou,A., Mosterd,C., Beck,M.H., Kelleher,P., McDonnell,B., de Waal,P.P., van Rijswijck,I.M.H., van Peij,N.N.M.E., Cambillau,C., Mahony,J., *et al.* (2024) Discovery of antiphage systems in the lactococcal plasmidome. *Nucleic Acids Res*, 52, 9760–9776.

19. Mills,S., O'Sullivan,O., Hill,C., Fitzgerald,G. and Ross,R.P. (2010) The changing face of dairy starter culture research: From genomics to economics. *Int J Dairy Technol*, 63, 149–170.

20. Dal Bello,F., Bocquet,L., Bru,A., Laulund,S., Machielsen,R., Raneri,M., Sewalt,V., van Peij,N., Ville,P., Volonté,F., *et al.* (2024) New Genomic Techniques applied to food cultures: a powerful contribution to innovative, safe, and sustainable food products. *FEMS Microbiol Lett*, 371, fnae010.

21. Pedersen,M.B., Iversen,S.L., Sørensen,K.I. and Johansen,E. (2005) The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. *FEMS Microbiol Rev*, 29, 611–624.

22. Mills,S., McAuliffe,O.E., Coffey,A., Fitzgerald,G.F. and Ross,R.P. (2006) Plasmids of lactococci-genetic accessories or genetic necessities? *FEMS Microbiol Rev*, 30, 243–273.

23. Kohler,V., Keller,W. and Grohmann,E. (2019) Regulation of gram-positive conjugation. *Front Microbiol*, 10, 1134.

24. Derkx,P.M., Janzen,T., Sørensen,K.I., Christensen,J.E., Stuer-Lauridsen,B. and Johansen,E. (2014) The art of strain improvement of industrial lactic acid bacteria without the use of recombinant DNA technology. *Microb Cell Fact*, 13, S5.

25. Linares,D.M., Kok,J. and Poolman,B. (2010) Genome sequences of *Lactococcus lactis* MG1363 (revised) and NZ9000 and comparative physiological studies. *J Bacteriol*, 192, 5806–5812.

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

27. Erazo Garzon,A., Mahony,J., Bottacini,F., Kelleher,P. and van Sinderen,D. (2019) Complete genome sequence of *Lactococcus lactis* subsp. *cremoris* 3107, host for the model lactococcal p335 bacteriophage TP901-1. *Microbiol Resour Announc*, 8, e01635-18.

28. Domingues,S., Chopin,A., Ehrlich,S.D. and Chopin,M.C. (2004) The lactococcal abortive phage infection system AbiP prevents both phage DNA replication and temporal transcription switch. *J Bacteriol*, 186, 713–721.

29. Van Pijkeren,J.P. and Britton,R.A. (2012) High efficiency recombineering in lactic acid bacteria. *Nucleic Acids Res*, 40, 1–13.

30. Gasson,M.J. (1983) Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol*, 154, 1–9.

31. Chandry,P.S., Moore,S.C., Boyce,J.D., Davidson,B.E. and Hillier,A.J. (1997) Analysis of the DNA sequence, gene expression, origin of replication and modular structure of the *Lactococcus lactis* lytic bacteriophage sk1. *Mol Microbiol*, 26, 49–64.

32. Oliveira,J., Mahony,J., Hanemaaijer,L., Kouwen,T.R.H.M. and van Sinderen,D. (2018) Biodiversity of bacteriophages infecting *Lactococcus lactis* starter cultures. *J Dairy Sci*, 101, 96–105.

33. Bebeacua,C., Tremblay,D., Farenc,C., Chapot-Chartier,M.P., Sadovskaya,I., van Heel,M., Veesler,D., Moineau,S. and Cambillau,C. (2013) Structure, adsorption to host, and infection mechanism of virulent lactococcal phage p2. *J Virol*, 87, 12302–12312.

34. Rincé,A., Tangney,M. and Fitzgerald,G.F. (2000) Identification of a DNA region from lactococcal phage sk1 protecting phage 712 from the abortive infection mechanism AbiF. *FEMS Microbiol Lett*, 182, 185–191.

35. Josephsen,J. and Vogensen,F.K. (1989) Identification of three different plasmid-encoded restriction/modification systems in *Streptococcus lactis* subsp. *cremoris* W56. *FEMS Microbiol Lett*, 59, 161–166.

36. Moineau,S., Durmaz,E., Pandian,S. and Klaenhammer,T.R. (1993) Differentiation of two abortive mechanisms by using monoclonal antibodies directed toward lactococcal bacteriophage capsid proteins. *Appl Environ Microbiol*, 59, 208–212.

37. Ruiz-Cruz,S., Erazo Garzon,A., Kelleher,P., Bottacini,F., Breum,S.Ø., Neve,H., Heller,K.J., Vogensen,F.K., Palussière,S., Courtin,P., *et al.* (2022) Host genetic requirements for DNA release of lactococcal phage TP901-1. *Microb Biotechnol*, 15, 2875–2889.

38. Muhammed,M.K., Krych,L., Nielsen,D.S. and Vogensen,F.K. (2017) A high-throughput qPCR system for simultaneous quantitative detection of dairy *Lactococcus lactis* and *Leuconostoc* bacteriophages. *PLoS One*, 12, e0174223.

39. Stothard,P. (2000) The sequence manipulation suite: JavaScript Programs for analyzing and formatting protein and DNA sequences. *Biotechniques*, 28, 1102–1104.

40. Studier,F.W. and Moffatt,B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol*, 189, 113–130.

41. Lavelle,K., Goulet,A., McDonnell,B., Spinelli,S., van Sinderen,D., Mahony,J. and Cambillau,C. (2020) Revisiting the host adhesion determinants of *Streptococcus thermophilus* siphophages. *Microb Biotechnol*, 13, 1765–1779.

42. Ortiz Charneco,G., Kelleher,P., Buivydas,A., Streekstra,H., van Themaat,E.V.L., de Waal,P.P., Mahony,J. and van Sinderen,D. (2021)

Genetic dissection of a prevalent plasmid-encoded conjugation system in *Lactococcus lactis*. *Front Microbiol*, 12, 680920.

43. Altschul,S., Madden,T.L., Schäffer,A.A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*, 25, 3389–3402.

44. Mahony,J., Murphy,J. and Van Sinderen,D. (2012) Lactococcal 936-type phages and dairy fermentation problems: From detection to evolution and prevention. *Front Microbiol*, 3, 1–9.

45. O'Connor,L., Coffey,A., Daly,C. and Fitzgerald,G.F. (1996) AbiG, a genotypically novel abortive infection mechanism encoded by plasmid pCI750 of *Lactococcus lactis* subsp. *cremoris* UC653. *Appl Environ Microbiol*, 62, 3075–3082.

46. Emond,E., Dion,E., Walker,S.A., Vedamuthu,E.R., Kondo,J.K. and Moineau,S. (1998) AbiQ, an abortive infection mechanism from *Lactococcus lactis*. *Appl Environ Microbiol*, 64, 4748–4756.

47. Dai,G., Su,P., Allison,G.E., Geller,B.L., Zhu,P., Kim,W.S. and Dunn,N.W. (2001) Molecular characterization of a new abortive infection system (AbiU) from *Lactococcus lactis* LL51-1. *Appl Environ Microbiol*, 67, 5225–32.

48. Bouchard,J.D., Dion,E., Bissonnette,F. and Moineau,S. (2002) Characterization of the two-component abortive phage infection mechanism AbiT from *Lactococcus lactis*. *J Bacteriol*, 184, 6325–6332.

49. Simon,N.C., Aktories,K. and Barbieri,J.T. (2014) Novel bacterial ADP-ribosylating toxins: structure and function. *Nat Rev Microbiol*, 12, 599–611.

50. LeRoux,M., Srikant,S., Teodoro,G.I.C., Zhang,T., Littlehale,M.L., Doron,S., Badiee,M., Leung,A.K.L., Sorek,R. and Laub,M.T. (2022) The DarTG toxin-antitoxin system provides phage defence by ADP-ribosylating viral DNA. *Nat Microbiol*, 7, 1028–1040.

51. Stokar-Avihail,A., Fedorenko,T., Hör,J., Garb,J., Leavitt,A., Millman,A., Shulman,G., Wojtania,N., Melamed,S., Amitai,G., *et al.* (2023) Discovery of phage determinants that confer sensitivity to bacterial immune systems. *Cell*, 186, 1863-1876.

52. Wu,Y., Garushyants,S.K., van den Hurk,A., Aparicio-Maldonado,C., Kushwaha,S.K., King,C.M., Ou,Y., Todeschini,T.C., Clokie,M.R.J., Millard,A.D., *et al.* (2024) Bacterial defense systems exhibit synergistic anti-phage activity. *Cell Host Microbe*, 32, 557-572.

53. Hickey,R.M., Twomey,D.P., Ross,R.P. and Hill,C. (2001) Exploitation of plasmid pMRC01 to direct transfer of mobilizable plasmids into commercial lactococcal starter strains. *Appl Environ Microbiol*, 67, 2853–2858.

3.8 Supplementary data

Table S1. Oligonucleotides used in this study for the phage protein cloning for antibody production and for screening/confirming transconjugants in the conjugation experiments.

Oligonucleotide name (targeted phage-gene/plasmid/strain_direction)	**Oligonucleotide sequence** (5'→3')
sk1ORF6_F	TCAGCAAGGGCTGAGGAATAAACCTGATTTAATCGAAAAA
sk1ORF6_R	TCAGCGGAAGCTGAGGTTATGAAACTGTAATTACTGCA
sk1ORF11_F	TCAGCAAGGGCTGAGGAAATTAGATTATAATTCACGTGA
sk1ORF11_R	TCAGCGGAAGCTGAGGTTATGAATGGTCAGTTACTGA
sk1ORF18_F	TCAGCAAGGGCTGAGGACAATTAAAAACTTCACGTTTTT
sk1ORF18_R	TCAGCGGAAGCTGAGGTTATTTAATGAAGTAACTTCCG
pUCCL624B_F	AGCAGCCTGCAGAGGAGGCACTCACCATGGAATTAACCTTTGGTTT
UCCL624B_R	AGCAGCGGTACCTTACTTCAAAATGTATTCAA
NZ9000::pJP005_F*	GTCATCAAACATACTTTCGTC
NZ9000::pJP005_R*	AAGTTTTGCCATTGTTTCTCC
MG1614_F*	GTCATCAAACATACTTTCGTC

MG1614_R*	AAGTTTTGCCATTGTTTCTCC
UCCL643_F	ACGCCTATTGGAACCACTC
UCCL643_R	TTTTGTCTTGTCCCCTTCTC

*These oligonucleotides amplify region of the C1 type cell wall polysaccharide (CWPS) of NZ9000 & MG1614 in contrast to the C2 CWPS type of the donor strain UCCL624 (1, 2)

Table S2. Obtained phage-resistance of the transconjugants (*L. cremoris* NZ9000::pJP005::pUCCL624B, *L. cremoris* MG1614::pUCCL624B & UCCL643::pUCCL624B) compared to the recipient/refence strains (*L. cremoris* NZ9000::pJP005, *L. cremoris* MG1614 & *L. lactis* UCCL643).

	Lactococcal strain			
Phage	NZ9000::pJP005	NZ9000::pJP005::pUCCL624B	MG1614	MG1614::pUCCL624B
sk1	1	≤ 1.32 (± 0.29) × 10^{-6}	1	≤ 2.03 (± 0.53) × 10^{-6}
p2	1	≤ 3.03 (± 1.05) × 10^{-6}	1	≤ 2.57 (± 0.79) × 10^{-6}
712	1	≤ 3.98 (± 1.11) × 10^{-6}	1	≤ 9.70 (± 3.97) × 10^{-6}
jj50	1	≤ 8.07 (± 3.18) × 10^{-5}	1	≤ 7.38 (± 3.90) × 10^{-5}

Table S2. Obtained phage-resistance of the transconjugants (*L. cremoris* NZ9000::pJP005::pUCCL624B, *L. cremoris* MG1614::pUCCL624B & UCCL643::pUCCL624B) compared to the recipient/refence strains (*L. cremoris* NZ9000::pJP005, *L. cremoris* MG1614 & *L. lactis* UCCL643) (continued).

	Lactococcal strain	
Phage	UCCL643	UCCL643::pUCCL624B
phUCCL643.2	1	$1.39 (\pm 0.44) \times 10^{-3}$
phUCCL643.3	1	$1.63 (\pm 0.65) \times 10^{-5}$
phUCCL643.4	1	$2.08 (\pm 1.05) \times 10^{-5}$
phUCCL643.5	1	$1.83 (\pm 1.19) \times 10^{-4}$

3.9 Supplementary data references

1. Ainsworth,S., Sadovskaya,I., Vinogradov,E., Courtin,P., Guerardel,Y., Mahony,J., Grard,T., Cambillau,C., Chapot-Chartier,M.P. and van Sinderen,D. (2014) Differences in lactococcal cell wall polysaccharide structure are major determining factors in bacteriophage sensitivity. *mBio*, 5, 1–11.

2. Mahony,J., Kot,W., Murphy,J., Ainsworth,S., Neve,H., Hansen,L.H., Heller,K.J., Sørensen,S.J., Hammer,K., Cambillau,C., *et al.* (2013) Investigation of the relationship between lactococcal host cell wall polysaccharide genotype and 936 phage receptor binding protein phylogeny. *Appl Environ Microbiol*, 79, 4385–4392.

Chapter IV

The Great Phage Escape: Activating and escaping lactococcal antiphage systems

This chapter will be submitted for publication as:

Mosterd C., Grafakou A., de Waal P.P., van Rijswijck I.M.H., van Peij N.N.M.E., Péchoux C., Kulakauskas S., Cambillau C., Mahony J. and van Sinderen D. The Great Phage Escape: Activating and escaping lactococcal antiphage systems.

C.M., A.G, J.M. and D.v.S. led the study. C.M. and A.G. performed the experiments and the analyses unless otherwise stated. C.P. and S.K. performed the TEM to measure the cell wall thickness. C.C. performed the AlphaFold2 predictions and analysis. P.d.W., I.v.R. and N.v.P. contributed with meaningful discussion of the results. J.M. and D.v.S. supervised and examined the results of the experiments. The initial concept manuscript was written by A.G and C.M. All authors contributed to editing the manuscript.

4.1 Abstract

To obtain mechanistic insights into recently identified antiphage systems, we isolated 66 phage escape mutants which had become insensitive to 13 plasmid-encoded lactococcal phage resistance systems (i.e. Rhea, Kamadhenu, Rugutis, Audmula, PARIS, type II CBASS, Septu, AbiA, AbiB, AbiD/F AbiG, AbiJ, AbiP). Genome analysis of these phage escape mutants identified a total of 15 mutated genes. Six of the encoded proteins appear to activate specific antiphage systems, while two others appear to act as targets. Furthermore, AbiA escape mutants were found to be insensitive to AbiJ, while distinct antiphage systems (AbiG and AbiP) were observed to be activated by a major phage tail protein, indicating mechanistic commonalities. Based on escape mutant sequence analysis and previously predicted domains, we propose that Audmula modifies the cell wall to intracellularly confine phage progeny thus delaying cell lysis. The obtained advances in our understanding of lactococcal antiphage mechanisms may not only benefit the dairy industry but may also be useful for biotechnological or biomedical applications, while simultaneously providing fundamental insights into phage-host interactions.

4.2 Introduction

Milk fermentation for cheese production greatly relies on *Lactococcus lactis* and *Lactococcus cremoris* strains, thus rendering these bacteria economically and industrially important (1). However, the omnipresence of bacteriophages in milk processing environments presents an ongoing challenge for the dairy fermentation industry (2), where infection of starter cultures may cause delayed or even failed fermentations, with serious environmental and economic consequences (3). This constant phage threat (4) has, in recent decades, fuelled significant research efforts to study *Lactococcus lactis*/*cremoris* and their infecting phages (5–8).

Therefore, it is not surprising that *Lactococcus* was the source of some of the earliest discovered antiphage systems, including 23 abortive infection (Abi) systems (9, 10). Antiphage systems employ diverse strategies to protect bacteria against their infecting phages. Among other recently described mechanisms (11), they include systems that prevent phage adsorption or phage DNA injection or restrict the nucleic acids of the incoming phage genome (12). Abi systems are considered the last line of bacterial defence against phages (13) and represent one of the most common lactococcal antiphage systems (9). Their activation upon phage infection and prior to the completion of the phage cycle, is detrimental for the monad but significantly limits phage release, thereby protecting the rest of the bacterial community from infection (13).

Despite the intense search for antiphage systems until the 2000s, predominantly in lactococci, it recently became evident that much of the

bacterially-encoded phage immunity landscape remains unexplored (14). This led to numerous studies and novel approaches to discover previously unknown antiphage systems (15–18), with recent discoveries triggering intensified research aimed at comprehending the diverse mechanisms that govern these systems (11). However, despite various antiphage systems being mechanistically explored (19–21), there is an abundance of, as yet, uncharacterized antiphage systems.

Recently the activity of 20 plasmid-encoded, mainly Abi-like, antiphage systems was described in *Lactococcus,* yet their mode of action remains largely unclear (22). Abi system contains at least one module responsible for sensing the phage infection and one that kills the cell or shuts down metabolism (13). To define phage-derived genes and associated proteins that represent potential triggers or targets of various lactococcal antiphage systems, phage escape mutants have been isolated and analysed (23–28). A similar approach has been applied in other bacteria to confirm phage proteins that activate antiphage systems (29–33).

The aim of the current study was to generate mechanistic insights into triggers or targets of recently described lactococcal antiphage systems (22). By improving our understanding of the mechanism of action of lactococcal antiphage systems, diverse and transferable phage-resistance systems can be employed for the development of phage robust starter cultures. Furthermore, mechanistic insights into antiphage systems may prove valuable in the development of biotechnological and biomedical tools, as has been the case with restriction enzymes, CRISPR-Cas-based

technologies or retron-derived DNA for genome editing (34–37), while simultaneously enhancing our knowledge of phage-host interactions.

4.3 Materials and methods

4.3.1 Strains, bacteriophages, plasmids, media and growth conditions

Bacterial strains, plasmids, bacteriophages and antiphage systems used in this study are listed in Table 1. *L. cremoris* strains were grown at 30 °C for 16-20 hours in M17 broth (Thermo Fisher Scientific, United Kingdom) supplemented with 0.5 % glucose (GM17). For preparation of GM17 agar plates, GM17 was supplemented with 1.5 % (w/v) agar. Strains carrying plasmids pNZ44, pPTPi, pPEPi (or their derivatives, Table 1) were grown in GM17 supplemented with either chloramphenicol (5 µg/mL, Sigma-Aldrich, Ireland), tetracycline (5 µg/mL), erythromycin (10 µg/mL, Sigma-Aldrich), respectively, or a combination of these antibiotics if a strain harboured more than one plasmid. For the induction of the P*nis*A promoter, cultures of strains carrying pPTPi, pPEPi or their derivatives were supplemented with 1 – 10 ng/mL nisin from *L. lactis* (Sigma-Aldrich), when an OD_{600nm} of 0.2 - 0.3 was reached.

Table 1. Lactococcal strains, plasmids, bacteriophages and antiphage systems.

Lactococcal strains, plasmids, bacteriophages and antiphage systems	Characteristics[a]	Source or Reference
L. cremoris NZ9000	Host strain for the antiphage systems and control strain for trigger assay	(38)
L. cremoris 3107	Host strain for the antiphage systems	(39)
L. lactis IL1403	Host strain for the antiphage systems	(40)
pNZ44	High copy number vector carrying the antiphage systems, Cm^r	(41)
pPTPi	Nisin-inducible, low copy number vector carrying the antiphage systems, cloning vector for the phage genes, Tc^r	(42)
pPEPi	Nisin-inducible, low copy number vector carrying the antiphage systems, cloning vector for the phage genes, Em^r	(22)
sk1	*Skunavirus* infecting *L. cremoris* NZ9000::pNZ44, used for phage mutant isolation	(43)
sk713	*Skunavirus* infecting *L. cremoris* NZ9000::pNZ44, used for phage mutant isolation	This study
p2	*Skunavirus* infecting *L. cremoris* NZ9000::pNZ44, used for phage mutant isolation	(44)
c2	*Ceduovirus* infecting *L. cremoris* NZ9000::pNZ44, used for phage mutant isolation	(45)
sv62601	*Skunavirus* infecting *L. cremoris* 3107::pNZ44, used for phage mutant isolation	(6)
sv66901	*Skunavirus* infecting *L. cremoris* 3107::pNZ44, used for phage mutant isolation	(6)
bIL66	*Skunavirus* infecting *L. cremoris* IL1403::pNZ44, used for phage mutant isolation	(23)
P008	*Skunavirus* infecting *L. cremoris* IL1403::pNZ44, used for phage mutant isolation	(46)
50501	*Ceduovirus* infecting *L. cremoris* 3107::pNZ44, used for phage mutant isolation	This study
56006	*Ceduovirus* infecting *L. lactis* IL1403::pNZ44, used for phage mutant isolation	This study
svUCCL624	*Skunavirus* infecting *L. cremoris* NZ9000::pNZ44, used for phage mutant isolation	This study

Rhea	Confirmed antiphage system cloned in pNZ44	(22)
Kamadhenu	Confirmed antiphage system cloned in pNZ44	(22)
Audmula	Confirmed antiphage system cloned in pNZ44	(22)
Rugutis	Confirmed antiphage system cloned in pNZ44	(22)
PARIS	Confirmed antiphage system cloned in pPEPi	(22)
Type I CBASS	Confirmed antiphage system cloned in pNZ44	(22)
Type II CBASS	Confirmed antiphage system cloned in pNZ44	(22)
Lamassu	Confirmed antiphage system cloned in pPTPi	(22)
Septu	Confirmed antiphage system cloned in pPTPi	(22)
AbiA	Confirmed antiphage system cloned in pPTPi	(22)
AbiB	Confirmed antiphage system cloned in pNZ44	(22)
AbiD/F	Confirmed antiphage system cloned in pNZ44	(22)
AbiF	Confirmed antiphage system cloned in pNZ44	(22)
AbiG	Confirmed antiphage system cloned in pNZ44	(22)
AbiJ	Confirmed antiphage system cloned in pNZ44	(22)
AbiP	Confirmed antiphage system cloned in pNZ44	(22)

[a]Cm^r, chloramphenicol resistant; Tc^r, tetracycline resistant; Em^r, erythromycin resistant

4.3.2 Phage escape mutant isolation

To obtain phage mutants that had become insensitive to an imposed antiphage system or so-called “(phage) escape mutants”, bacterial host strains carrying a given antiphage system were challenged with high titre lysates (≥10^8 PFU/ml) of skunaviruses or ceduoviruses sensitive to that antiphage system (Table 1). Plaque assays were performed using the double agar method as previously described (47) to isolate single plaques of potential phage escape mutants. For this purpose, GM17 was supplemented with 10 mM $CaCl_2$ and either 1 % (solid agar) or 0.4 % (semi-solid) bacteriological agar. 300 µL of a fresh overnight bacterial culture was mixed with 10 µL phage lysate (or dilutions thereof) in 4 mL of semi-solid agar and the mixture was poured onto solid agar plates. In cases where plaque visualisation was challenging, agar was either supplemented with an additional 0.4 % glycine or the semi-solid medium was prepared with 0.2 % agarose instead of agar. Phage lysates were diluted using SM buffer (10 mM $CaCl_2$, 100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl at pH 7.5).

4.3.3 Phage escape mutant propagation

Single plaques of potential phage escape mutants were propagated by transferring 2 % (v/v) of a fresh overnight culture of the relevant strain carrying a given antiphage system to GM17 broth, supplemented with $CaCl_2$ (10 mM) and the single plaque. The resulting lysates were filtered (pore size 0.45 µm, Sarstedt AG & Co. KG) and stored at 4 °C. If propagation of the escape mutant on the strain carrying the antiphage system was

unsuccessful, propagation was performed using wild-type/control strain, i.e. *L. cremoris* NZ9000 carrying either 'empty' cloning vector pNZ44 or pPTPi. Prior to phage escape mutant analysis, spot assays were used to confirm the resistance/insensitivity of the obtained escape mutant against the strain carrying the defence system and compared to the wild-type phage (by determining plaque-forming units [PFUs]). Additionally, if the same gene was found to be mutated across escape mutants of multiple antiphage systems, it was tested using spot assays if the escape mutant obtained against one antiphage system was also able to circumvent the activity of other antiphage systems. For these spot assays, 300 µL of an overnight bacterial culture of *L. cremoris* NZ9000 carrying a given antiphage system (or wild-type strain carrying the empty vector as a control), was mixed with 4 mL of semi-solid agar and poured onto plates with solidified medium (bottom layer). Ten-fold serial dilutions were performed in SM buffer for both wild-type phage and a candidate escape mutant. 10 µL of each phage dilution was spotted on the soft agar overlay. Plates were then incubated overnight at 30 °C.

4.3.4 Sequencing and genome analysis of escape mutants

Total phage DNA was extracted using the Phage DNA extraction kit (Norgen Biotek, Canada) as recommended by the manufacturer. Genomes of individual escape mutants were sequenced using Illumina MiSeq technology by GenProbio, Parma, Italy. MIRA (Mimicking Intelligent Read Assembly) version 4.0.2 was used for *de novo* assembly of MiSeq-derived phage genome sequences to generate a consensus sequence. Open

reading frames (ORFs) were predicted using a combination of Prodigal version 2.6 and BLASTX (48, 49), followed by manual assessment, curation, and correction of predicted ORFs. Functional annotations were generated using BLASTP (50) analysis against the non-redundant protein database (nr) provided by the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi), as well as using the MEGAnnotator pipeline (51). Proposed protein functions were validated by querying protein domain database Pfam (52) and the NCBI Conserved Domain Database (53), and by performing structural similarity prediction searches using HHpred (54). The generated sequences were analysed for the presence of single nucleotide polymorphisms (SNPs) when compared to the wild-type genome sequence (SNP analysis).

4.3.5 Cloning of candidate phage-derived trigger genes

Upon identification of a phage gene that was mutated across different escape mutants obtained for a given antiphage system, the original (i.e. non-mutated) gene was cloned into either of the low-copy, nisin-inducible vectors pPTPi or pPEPi as described previously with the following modifications (22). Phage genes to be cloned were amplified using Phusion High-Fidelity Polymerase (Thermo Fisher Scientific) from the relevant phage lysates according to the manufacturer's instructions and with relevant primers (Table S1; Eurofins Genomics). FastDigest restriction enzymes BamHI, SalI and EcoRI (Table S1; Thermo Fisher Scientific) were used according to the manufacturer's instructions. Transformants carrying the

desired recombinant plasmids were screened by colony PCR using pPTPi/pPEPi_F 5'-CTATCTTGAGAAAGTATTGGTAA-3' and pPTPi/pPEPi_R 5'-TGGCGGACAATAAGTCCTC-3' primers. Sequence integrity of obtained recombinant plasmids was verified by Sanger sequencing (Eurofins Genomics, Germany). The recombinant plasmid containing the phage gene (Table S1) was subsequently transformed by electroporation into *L. cremoris* NZ9000 carrying a plasmid encoding the relevant antiphage system (Table S1) or carrying the corresponding empty vector (pNZ44, pPTPi or pPEPi) as previously described (22). Phage gene sequence integrity was verified by Sanger sequencing (Eurofins Genomics) in these latter constructed strains using PCR-generated amplicons of the phage gene as a template. The functionality of the antiphage systems in these latter constructed strains was verified by a spot assay using a phage sensitive to the antiphage system, yet able to infect the wild-type strain.

4.3.6 Antiphage system trigger assay

To examine the impact of transcriptional induction of a cloned phage gene (trigger assay) on bacterial growth, an adapted version of the protocol developed by Stokar-Avihail et al. was used (33). Ten-fold serial dilutions of overnight cultures grown in GM17 supplemented with the relevant antibiotics (chloramphenicol, tetracycline and/or erythromycin) were prepared and spotted in parallel on GM17 agar with and without the inducing agent (nisin). Antiphage systems encoded on pPTPi or pPEPi (Table S1) were induced simultaneously with the phage gene (candidate phage-

derived trigger), while antiphage systems on pNZ44 (Table S1) were constitutively expressed during introduction and induction of the phage gene. Plates were incubated at 30 °C for 48 hours and CFUs (colony forming units) were determined. The relative toxicity of each induced phage gene for each strain was determined by dividing the number of CFUs obtained in the absence of nisin by the number of CFUs obtained in the presence of (an otherwise non-toxic amount of) nisin. A phage protein was considered a trigger if its toxicity to the strain carrying the antiphage system exceeded the toxicity to the control strain by at least one log. When a phage trigger was identified, the mutated version of the gene (originating from a corresponding escape mutant) was also cloned and tested in the same trigger assay. In all cases, a nisin concentration of 10 ng/ml was used for induction.

4.3.7 Transmission electron microscopy (TEM)

Cell wall analysis by TEM was performed by MIMA2, Jouy-en-Josas, France (MIMA2, INRAE, 2018. Microscopy and Imaging Facility for Microbes, Animals and Foods, https://mima2.jouy.hub.inrae.fr/). Cultures of *L. cremoris* NZ9000::pNZ44 and NZ9000::pNZ44-Audmula were grown until an OD_{600} of ~0.4. Samples were fixed with 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 hours at room temperature. Samples were contrasted with 0.2 % Oolong Tea Extract (OTE) in cacodylate buffer, post-fixed with 1 % osmium tetroxide containing 1.5 % potassium cyanoferrate, gradually dehydrated in ethanol (30 % to 100 %) and substituted gradually in mix of ethanol-epon and embedded in Epon

(Delta microscopie, France). Thin sections (70 nm) were collected onto 200 mesh copper grids a Hitachi HT7700 electron microscope operated at 80kV (Milexia, France), and images were acquired with a charge-coupled device camera (AMT).

For TEM imaging, samples were prepared as follows: Two mL of culture 45 minutes post-infection with phage c2 was harvested by centrifugation. The pellet was resuspended in fixative buffer (0.1 M HEPES + 2.5 % glutaric dialdehyde + 3 % formaldehyde) kept at 30 °C and incubated on ice for 45 minutes. Samples were centrifuged at 12,000 × *g* for 5 minutes at 4 °C; the supernatant was removed and the pellet was covered in cold fixative buffer. The fixed samples were subsequently sent to BioEM Lab (University of Basel, Switzerland) for imaging.

4.3.8 Phage protein structure and protein complex prediction

We performed predictions with either a Colab notebook running AlphaFold v2.3.1 (https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb) or HPC resources from GENCI-IDRIS running AlphaFold v2.3.1 (55). The pLDDT values of predicted structures were stored in the PDB files as B-factors and plotted with the Colab or the IDRIS resources. The final predicted protein or domain structures were submitted to the Dali server (56) to identify the closest structural homologues in the PDB. Visual representations of the structures were prepared with ChimeraX (57).

4.3.9 Quantification and statistical analysis

Data are presented as mean ± standard deviation (SD) from three biological replicates and an unpaired t-test was performed to determine statistically significant differences (P-value < 0.05).

4.4 Results

4.4.1 Isolation of phage escape mutants that bypass lactococcal antiphage systems

In a previous study, 20 plasmid-encoded antiphage systems found in 53 lactococcal strains were identified and their activity was experimentally confirmed against phages of the genera *Skunavirus* and *Ceduovirus* (22). The previously described antiphage activity of these systems was exploited to select phages that bypass the antiphage activity conferred by these systems thus generating putative escape mutants. In total, escape mutant isolation efforts were attempted for 16 of these 20 lactococcal antiphage systems (Table 1). Two systems (Aristaios and Hesat) were excluded from this analysis as these were previously shown to provide either full resistance or full sensitivity to all tested phages. Additionally, two antiphage systems (Fliodhais and AbiZ) were excluded from this analysis due to their relatively modest activity against the tested phages and under the conditions employed (EOP ≥ 10^{-1}).

For the remaining 16 antiphage systems, potential phage escape mutants were obtained at low frequencies on *L. cremoris* NZ9000, *L. cremoris* 3107 or *L. lactis* IL1403 expressing these systems in challenges with *Skunavirus* or *Ceduovirus* members (Table 1). However, for three of the 16 tested systems we failed to isolate stable escape mutants, either because plaques could not be propagated (AbiF) or the isolated plaques did not result in increased insensitivity towards the antiphage system (type I CBASS and Lamassu). For the remaining 13 systems, a total of 66 escape mutants (EMs) were isolated (Table S2). The genomes of these 66 escape

mutant phages were sequenced and compared to that of the wild-type reference phage for the detection of mutations that may explain the obtained insensitivity of the escape mutant phages towards the antiphage systems (Table S2).

4.4.2 Analysis of mutations required to circumvent lactococcal antiphage systems

Sequence analysis of the mutated phages showed that a single point mutation appeared to be sufficient in ~70 % of the isolated phages (47 out of 66) to circumvent the antiphage system. Of the remaining 19 isolated phage mutants, twelve, six and one mutant(s) were shown to carry two, three or four point mutations in their respective genome (Table S2).

Further analysis facilitated identification of a total of 15 genes (Table 2) which had been mutated repeatedly across the assessed 66 escape mutant phages (Table S2). For nine of the 13 antiphage systems, the same gene or pair of genes (the latter in the case of AbiA) was shown to be mutated for all sequenced escape mutants of a given antiphage system (the four exceptions being the PARIS, type II CBASS, AbiB, AbiD/F antiphage systems). When genotypically distinct phages were used for escape mutant isolation (i.e. in the case of Rhea, Audmula, AbiJ, AbiP) the obtained mutations were shown to be present in the same homologue among the tested phages. Finally, whereas most phage escape mutants were shown to carry mutations in coding regions, four mutants harbour an additional mutation in an intergenic region (one escape mutant of Rugutis and AbiG

and two of AbiB). Additionally, two (out of three) isolated sk1 escape mutants of type II CBASS and two (out of six) escape mutants of PARIS possess mutations only in (the same) intergenic regions (corresponding to the early expressed region of sk1 for CBASS II and c2 for PARIS escape mutants respectively). In more detail, intergenic mutations were located for CBASS between sk1 ORF48 and 49, and for PARIS preceding c2 ORF 1.

Table 2. Synopsis of the number of mutations per mutant, the functions of all the mutated genes, the shared mutated gene function(s) across the mutants of each antiphage system and the categorization of the shared mutated genes into functional groups.

Antiphage system	Number of mutations per mutant	All mutated genes functions	Shared mutated gene function(s) across mutants of each antiphage system	Categorization of the shared mutated gene into functional groups
Kamadhenu	1	Sak-like SSAP	Sak-like SSAP	DNA replication/recombination
AbiA	2	Erf-like SSAP Holliday junction resolvase	Erf-like SSAP	
AbiJ	1-3	Sak/Erf-like SSAP Hypothetical protein Terminase large subunit AAA ATPase Major capsid protein	Holliday junction resolvase	
Septu	1-4	SSB Tape measure protein Sak-like SSAP Terminase large subunit	Sak/Erf-like SSAP	
AbiG	2-3	Major tail protein Intergenic region	SSB	Structural proteins/ Unknown function
AbiP	1	Major tail protein	Major tail protein	
AbiB	1-3	Hypothetical proteins Intergenic region Major capsid protein	Major tail protein	
Rhea	1-3	Terminase large subunit Terminase small subunit Receptor binding protein	Hypothetical protein (sk1_ORF35)	DNA packaging & lysis

Rugutis	1-2	Terminase large subunit Intergenic region	Major capsid protein	
Audmula	1	Lysin	Terminase large subunit	
PARIS	1-3	Intergenic region/ Hypothetical proteins	Terminase large subunit	Unknown function
AbiD/F	1	Hypothetical proteins	Lysin	
Type II CBASS	1-2	Intergenic region Hypothetical protein (sk1_ORF48)	Hypothetical protein (c2_ORF2)	N/A

4.4.3 Categorisation of the mutated genes based on (predicted) function

Analysis of the genes mutated in phages that circumvent the antiphage systems, allowed their grouping into four categories based on their (predicted) function as follows (Table 2): (i) DNA replication and recombination, (ii) structural proteins, (iii) DNA packaging and lysis, and (iv) unknown function. Each escape mutant category will be discussed below.

4.4.3.1 DNA replication and recombination

Interestingly, the activity of Kamadhenu, Septu, AbiA and AbiJ was circumvented by the challenged phage through the acquisition of mutations either in predicted single-strand DNA binding (SSB) or single-strand DNA annealing protein (SSAP) encoding genes. SSAPs and SSBs are known to be involved in DNA replication and recombination processes in phages (58–60). In a previous study, a lactococcal strain which constitutively expressed *kamadhenu* provided full resistance against all tested *Skunavirus* and *Ceduovirus* members (22). However, Kamadhenu escape mutants were isolated when *L. cremoris* 3107 bearing *kamadhenu* was challenged with phage svUCCL624. The genomes of Kamadhenu escape mutants all contained a mutation in the exact same genomic position of the Sak-like (sensitivity to AbiK) SSAP-encoding gene (Table S2). Both c2 escape mutants of AbiA possessed in addition to a mutation in the Holliday junction resolvase-encoding gene, an identical mutation in the Erf-like (Essential recombination function) protein-encoding gene, another member of the

SSAP family. Erf proteins and Holliday junction resolvases are known to be involved in homologous recombination during the circularization of the linear dsDNA phage genome upon entry into the host cell or the strand resolution of DNA recombination intermediates, respectively (61–64). Finally, all nine AbiJ phage escape mutants obtained following challenges by three different phages were shown to contain genomic mutations in the genes encoding either the Sak-like or Erf-like protein. Interestingly, all mutations in the *sak*-like genes of AbiJ and Kamadhenu escape mutants were found in identical positions of *sak* homologues in different phages.

All Septu-insensitive escape mutants shared the same mutation in a ssDNA binding protein-encoding gene with two out of the three mutants bearing additional mutations in different genes (Table S2).

4.4.3.2 Structural proteins

Escape mutant analysis for three antiphage systems (AbiB, AbiG and AbiP) revealed mutations in structural protein-encoding genes. Specifically, two out of the three phage sv66901-derived escape mutants which were shown to have become insensitive to AbiB contained a genomic mutation in the gene encoding the major capsid protein. Additionally, all assessed AbiG- and AbiP-insensitive escape mutants harboured mutation(s) in the major tail protein-encoding gene.

4.4.3.3 DNA packaging and lysis

The next category of phage genes that were found to be mutated among escape mutants of various antiphage systems is that of specific enzyme-encoding genes. Interestingly, all Rhea-escape mutants were shown to harbour at least one point mutation in the ATPase domain of the terminase large subunit-encoding gene. Similarly, all c2 escape mutants of Rugutis were found to possess a mutation in the ATPase domain of the terminase large subunit-encoding gene. Audmula escape mutants of three different members of the genus *Ceduovirus* were obtained and all were shown to harbour a mutation in the active site of their lysin.

4.4.3.4 Proteins of unknown function

Escape mutants of PARIS, type II CBASS, AbiD/F and AbiB antiphage systems harboured mutations in genes which are located in the early expressed region of the corresponding phage genome, and for which no function could be assigned based on domain searches, structural homology analysis and comparative sequence analysis (InterProScan, HHpred and BLASTN/BLASTP) (54, 65, 66). Most of the PARIS-insensitive escape mutants harbour a mutation in a gene of unknown function, though this gene does not exhibit homology to the Ocr-encoding gene which was described to activate PARIS in *E. coli*. Nonetheless, AF2 structural prediction showed that the corresponding proteins (c2_ORF2 and Ocr) possess a highly negatively charged cluster while the second component of PARIS from both *E. coli* and *Lactococcus* is predominately positively charged in a central area

(Figure S1). Notably, *in silico* structural analysis based on AF2 did not reveal any fold resemblance between the two hypothetical proteins (sk1_ORF24 and sv62601_ORF26), in which AbiD/F-insensitive phage escape mutants of two different phages had acquired a mutation (Figure S2).

4.4.4 Cross-insensitivity of escape mutants

In cases where the same gene was mutated by phages to circumvent genetically distinct antiphage systems, the ability of escape mutants obtained against one antiphage system to circumvent other antiphage systems was evaluated (Table S3). Among the evaluated combinations of escape mutants and antiphage systems, the two AbiA c2 escape mutants (EM41 and EM42, Table S2) were observed to also overcome the activity of AbiJ. Whereas AbiA (EOP $<10^{-6}$) and AbiJ (EOP $\sim10^{-6}$) provide near full resistance against wild-type c2, the EM41 and EM42 escape mutants displayed EOP values of $>10^{-1}$ when challenged with AbiA or AbiJ (Table S4). Compared to wild-type c2, plaque sizes of the escape mutants were reduced. This reduction was more pronounced when plated on strains expressing the antiphage system. In the case of EM41, this phenomenon was only observed when plated on the strain expressing AbiJ. This indicates that overcoming the activity of AbiA and AbiJ comes at a fitness cost. Escape mutants of AbiA and AbiJ all possess mutations in their Erf-like SSAP-encoding gene and the ability of AbiA escape mutants to overcome AbiJ suggests a shared mode of action or activation. This was the only case of escape mutants showing cross-insensitivity towards a distinct antiphage system from the system they had been obtained from (Table S3).

4.4.5 Identification of phage proteins that trigger antiphage systems

It was previously established that 12 out of 13 antiphage systems for which we isolated escape mutants in the current study, exhibit typical characteristics of abortive infection systems, Audmula being the exception (22). As the escape mutants can overcome the activity of the antiphage systems by introducing one or more mutations in certain genes of the phage genome, it is possible that the encoded proteins act as a trigger or target of the antiphage systems. Since abortive infection systems are known to induce cell death (or lead to a bacteriostatic state) upon recognition of phage infection (13), expression of a trigger protein along with the antiphage system is therefore expected to result in increased cell toxicity, as has previously been described for several antiphage systems (33).

We aimed to investigate if any of the products encoded by phage genes, in which mutations had been shown to allow insensitivity against certain antiphage systems, act as the molecular trigger of the corresponding systems. For this reason, the wild-type version of these mutated phage genes was cloned in a plasmid under nisin-inducible transcriptional control and introduced into *L. cremoris* NZ9000 carrying the relevant antiphage system or the control strain. Phage genes which upon expression cause (higher) toxicity to the strain carrying the antiphage system compared to the control strain were considered as abortive infection triggers (trigger assay). The (relative) toxicity of each induced phage gene for each strain was determined by dividing the number of CFUs obtained in the absence of nisin (before induction) by the number of CFUs obtained in the presence of nisin

(after induction). A phage protein was considered a trigger if its toxicity to the strain carrying the antiphage system exceeded the toxicity to the control strain by at least one log.

As candidate triggers, the wild-type versions of 15 genes that had been mutated in (several of) the analysed escape mutants were selected for cloning (Table 2). In addition, sk1p48 (locus tag in the reference sk1 genome) was selected as a candidate trigger for type II CBASS, as one escape mutant harboured a mutation in this gene (therefore a total of 16 phage genes were cloned and tested as candidate triggers, see Table 2). Furthermore, the intergenic mutations found in type II CBASS escape mutants are located adjacent to the promoter regulating sk1p48 (43). Among the 16 evaluated phage genes, the nisin-mediated induction of six (i.e. genes encoding c2 phage terminase large subunit [c2_ter], sv66901 phage major capsid protein [sv66901_mcp], sk1 and sv62601 phage hypothetical proteins [sk1_ORF24 and sv62601_ORF26], p2 and sk713 phage major tail proteins [p2_mtp and sk713_mtp]) was shown to cause reduced viability in the strain expressing the antiphage system (Rugutis, AbiB, AbiD/F [both sk1_ORF24 and sv62601_ORF26], AbiG and AbiP, respectively) compared to the wild-type strain, suggesting that their expression leads to activation of the abortive infection system and subsequent cell death (Figure 1D, Figure 2).

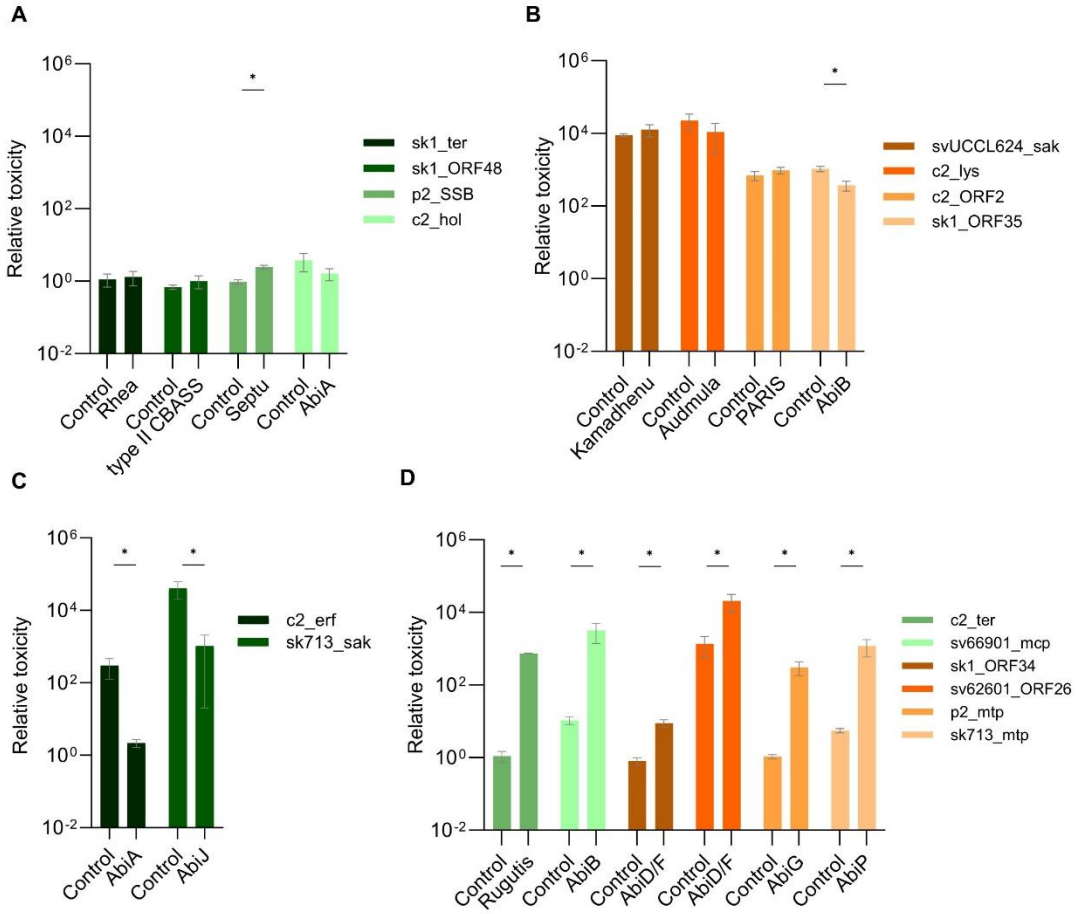


Figure 1. Relative toxicity of putative phage triggers to control strains and strains expressing relevant antiphage systems (CFU/ml before induction divided by CFU/ml after induction). A) Phage proteins not toxic to the wild-type strain or to the strain expressing the antiphage system. B) Phage proteins displaying similar toxicity to the wild-type strain or strain expressing the antiphage system. C) Phage proteins more toxic to the wild-type strain than to the strain expressing the antiphage system. D) Phage proteins more toxic to the strain expressing the antiphage system compared to the wild-type strain (and thus identified as phage triggers). Experiments were performed in biological triplicate and data are presented as means ± standard deviation (SD). Asterisk marks statistically significant differences in relative toxicity of the phage proteins to the relevant antiphage system compared to the control (unpaired t-test, P-value < 0.05). Phage proteins p2_SSB and sk1_ORF35 were not considered more toxic to the strain expressing the relevant antiphage system or to the wild-type strain, respectively, as the toxicity to the strain carrying the antiphage system did not exceed the toxicity to the control strain by at least one log (see

Methods). sk1_ter (sk1 terminase large subunit); sk1_ORF48 (sk1 ORF48 encoded protein); p2_SSB (p2 SSB); c2_hol (c2 Holliday junction resolvase); svUCCL624_sak (svUCCL624 Sak-like SSAP); c2_lys (c2 lysin); c2_ORF2 (c2 ORF2 encoded protein); sk1_ORF35 (sk1 ORF35 encoded protein); c2_erf (c2 Erf-like SSAP); sk713_sak (sk713 sak-like SSAP); c2_ter (c2 terminase large subunit); sv66901_mcp (sv66901 major capsid protein); sk1_ORF24 (sk1 ORF24 encoded protein); sv62601_ORF26 (sv62601 ORF26 encoded protein); p2_mtp (p2 major tail protein); sk713_mtp (sk713 major tail protein)

Interestingly, all three assessed phage SSAPs were found to be toxic to the host cells (Figures 1B and 1C), with two phage SSAPs being more toxic to the wild-type strain than to the strain expressing the antiphage system (AbiA and AbiJ), suggesting that SSAPs represent targets of antiphage systems, as previously hypothesized ((60); Figure 2). This finding further explains why AbiA-insensitive escape mutants were also found in this study to be insensitive to AbiJ (Table S3, Table S4, Figure 2).

The remaining eight evaluated phage proteins were either not toxic to the strain expressing the antiphage system or the wild-type strain (Figure 2A) or equally toxic to both the strain expressing the antiphage system and the wild-type strain (Figure 2B). The latter could be due to either the activation of Abi systems already present in the host genome or the inherent toxicity of the corresponding phage proteins at the specific expression level.

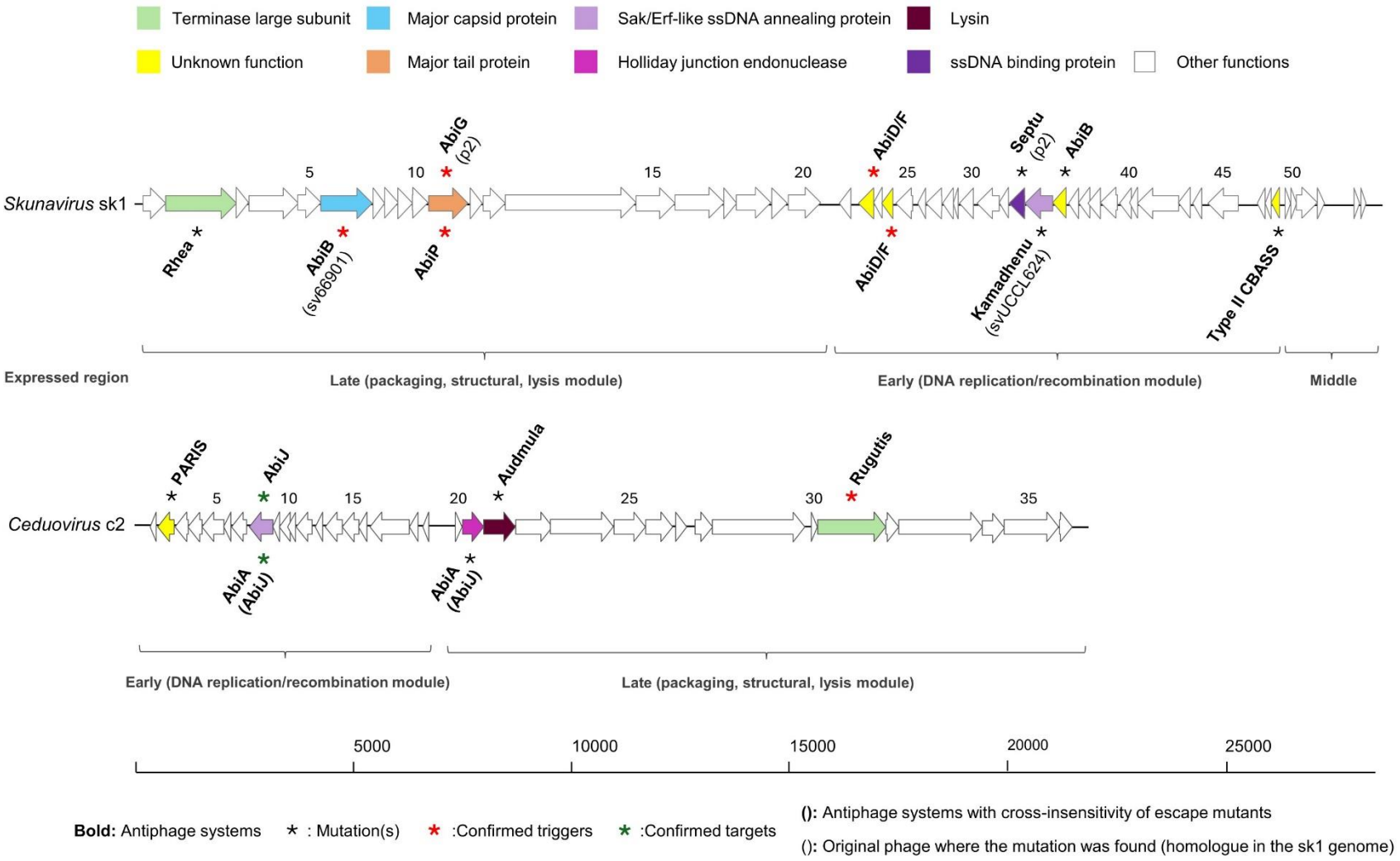

Terminase large subunit
Major capsid protein
Sak/Erf-like ssDNA annealing protein
Lysin
Unknown function
Major tail protein
Holliday junction endonuclease
ssDNA binding protein
Other functions
Skunavirus sk1
Rhea
AbiB (sv66901)
AbiG (p2)
AbiP
AbiD/F
AbiD/F
Septu (p2)
AbiB
Kamadhenu (svUCCL624)
Type II CBASS
Expressed region
Late (packaging, structural, lysis module)
Early (DNA replication/recombination module)
Middle
Ceduovirus c2
PARIS
AbiJ
Audmula
Rugutis
AbiA (AbiJ)
AbiA (AbiJ)
Early (DNA replication/recombination module)
Late (packaging, structural, lysis module)
5000
10000
15000
20000
25000
Bold: Antiphage systems
: Mutation(s)
:Confirmed triggers
:Confirmed targets
(): Antiphage systems with cross-insensitivity of escape mutants
(): Original phage where the mutation was found (homologue in the sk1 genome)

Figure 2. Schematic representation summarizing the phage escape mutant analysis, trigger assay and cross-insensitivity results of escape mutants. *Skunavirus* (sk1) or *Ceduovirus* (c2) phage genes mutated (indicated by an asterisk) for phage escape mutants to overcome the activity of the antiphage system (bold). Red asterisks denote confirmed phage-derived triggers, while green asterisks denote confirmed phage-derived targets (related to Figure 3). Where escape mutants were isolated from a different *Skunavirus* member, the homologue in the sk1 genome is depicted, with the original phage shown in parentheses. AbiJ was insensitive towards the AbiA escape mutants and is also depicted in parentheses.

To determine whether the presence of the single point mutation in the phage genes identified as triggers would alleviate the observed toxicity of the induced gene to the antiphage system compared to the one of the control strain, the mutated version of the gene (as found in the escape mutant genomes) was introduced into the strain expressing the antiphage system and the control strain. Expression of the mutated variants of the genes revealed that in four out of six cases toxicity was not observed in the strain carrying the antiphage system (sk1_ORF24 and sv62601_ORF26 for AbiD/F, p2_mtp for AbiG and sk713_mtp for AbiP) (Figure 3B). The mutated variants of c2_ter (Rugutis) and sv66901_mcp (AbiB) still displayed the same toxicity profiles as observed for the wild-type encoded protein (Figure 3A).

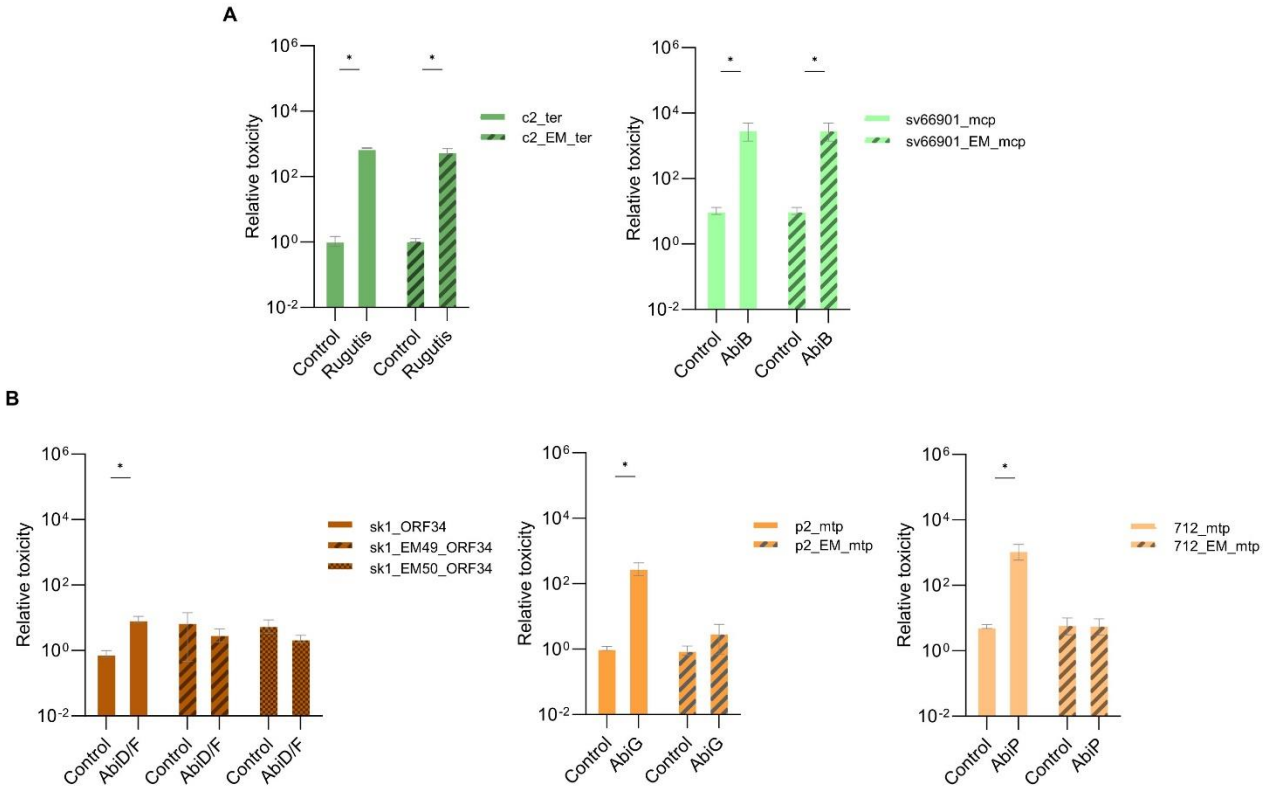


Figure 3. Relative toxicity of mutated phage triggers to control strains and strains expressing the antiphage systems. (CFU/ml before induction divided by CFU/ml after induction). A) Same toxicity profiles upon induction of the mutated variants; B) Neutralised toxicity upon induction of the mutated variants of the genes in the strain carrying the antiphage system. Asterisk marks statistically significant differences in relative toxicity of the phage proteins to the relevant antiphage system compared to the control (unpaired t-test, P-value < 0.05). EM, escape mutant.

4.4.6 Audmula exhibits a novel non-abortive infection mode of action

It has previously been established that Audmula renders *L. cremoris* resistant to phages of the genus *Ceduovirus* and that it is active post phage DNA injection (22). Here it is reported that one single point mutation in the muramidase domain of its lysin-encoding gene allows ceduoviruses to overcome the activity of Audmula. Additionally, Audmula was previously found to represent a predicted transmembrane glycosyltransferase and therefore likely involved in cell wall biosynthesis. In this study, transmission electron microscopy (TEM) also revealed that the cell wall of *L. cremoris* NZ9000 expressing Audmula was statistically significant thicker than the cell wall of the wild-type strain [27.22 (± 2.85) nm; N = 119 and 22.56 (± 3.25) nm; N = 93 respectively; two-tailed, unpaired P-value < 0.0001; (Figure 4A)].

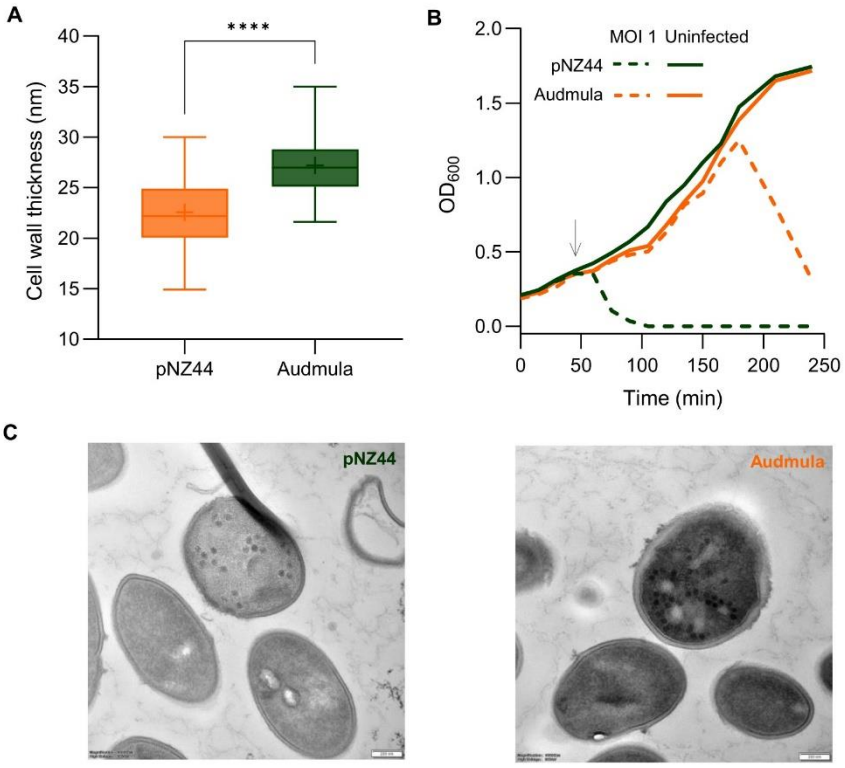


Figure 4. Cell wall thickness and phage visualization in the presence of Audmula. A) Box plots of wall thickness of *L. cremoris* NZ9000::pNZ44 and *L. cremoris* NZ9000::pNZ44 cells expressing Audmula antiphage system measured using TEM. Asterisks mark statistically significant differences in cell wall thickness (unpaired t-test, P-value < 0.0001). B) Monitored growth (OD_{600nm}) of *L. cremoris* NZ9000::pNZ44 and and *L. cremoris* NZ9000::pNZ44 expressing Audmula antiphage system with or without infection with phage c2 for TEM sampling at 45 min (imaging at panel C). C) TEM images of *L. cremoris* NZ9000::pNZ44 and *L. cremoris* NZ9000::pNZ44 expressing Audmula antiphage system infected with phage c2 at 45 min.

Previously, adsorption and transduction assays revealed that c2 retains the ability to adsorb and inject its DNA into *L. cremoris* NZ9000 expressing Audmula (22). In addition, it was also observed that Audmula confers a delay in culture lysis during infection with c2 at a low MOI (0.1), indicating that Audmula functions through a non-Abi mechanism (22). To investigate if Audmula affects the maturation of viral particles, two *L. cremoris* NZ9000 cultures (one expressing Audmula, the second carrying the empty vector as a control) were infected with phage c2 at an MOI of 0.1. OD_{600} of the cultures was recorded every 15 minutes for the cultures post-infection to validate the growth pattern (Figure 4B). Whereas in the control strain the culture collapsed 60 minutes post-infection, lysis occurred after 180 minutes for the strain expressing Audmula (Figure 4B). At 45 minutes post-infection, samples were taken for analysis by TEM. In both samples of the control strain and of the Audmula culture 45 minutes post infection, intracellular phages were visualised (Figure 4C), proving that Audmula allows the maturation of viral particles.

These findings indicate that the mode of action of Audmula relies on modifying the thickness and perhaps composition of the peptidoglycan layer, which allows c2 to infect the cell expressing the antiphage system, yet apparently prevents appropriately timed cell lysis by the c2 lysin. A single mutation in the muramidase domain of the c2 lysin as found in the escape mutants seems to allow such a phage to overcome the Audmula-modified peptidoglycan barrier (Figure 5).

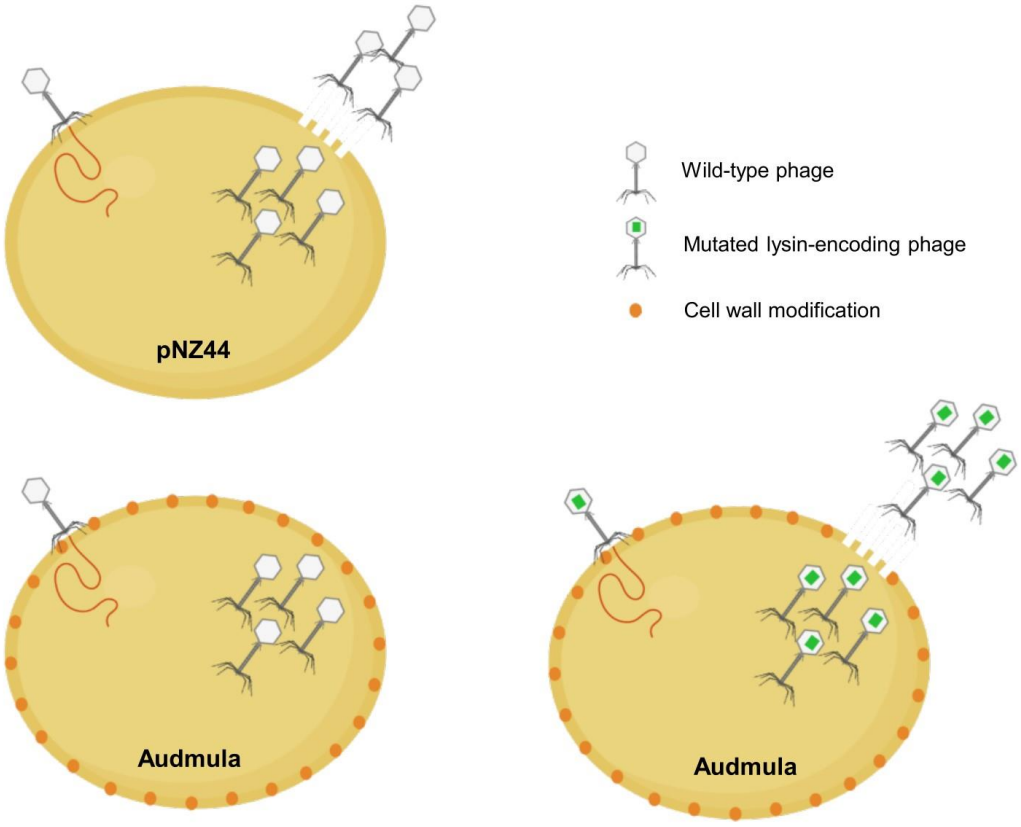


Figure 5. Audmula proposed mode of action. In the case of phage sensitive *L. cremoris* NZ9000::pNZ44 (top), phage c2 is able to adsorb to the cell wall, inject its DNA, replicate and lyse the cell. In the case of *L. cremoris* NZ9000::pNZ44 expressing Audmula antiphage system (bottom), modifications in the sugar composition of the cell wall due to the activity of Audmula do not allow c2 to lyse the cell wall after replication. The presence of small numbers of escape mutants carrying a mutation in the active site of their lysin allow them to cleave the modified cell wall and spread the infection to neighbouring cells.

4.5 Discussion

In the current study, we endeavoured to establish additional mechanistic insights for the recently confirmed plasmid-encoded antiphage systems present in *Lactococcus* (22). Escape mutants were successfully isolated for 13 of the 16 tested antiphage systems in this study. A single point mutation was sufficient in most instances for a phage to circumvent the antiphage system, consistent with previous studies (26, 29, 31, 67, 68). Inability to isolate escape mutants for some antiphage systems may be due to technical factors, such as the employed panel of phages or the presumably higher (artificial) expression of the systems compared to the natural state. Interestingly, the success rate for escape mutant isolation in a previous systematic escape mutant isolation study was significantly lower (isolation for 15 out of 54 systems). This was attributed to biological factors relevant to this study, such as the possibility of multiple mutations being required, which would occur at a much lower frequency, or the inability of the phage to mutate an essential component, necessary for evading the antiphage system (33).

The same gene was typically mutated among escape mutants isolated for a given antiphage system, resulting in a total of 15 mutated genes for the 13 antiphage systems. These genes were grouped into four categories based on (predicted) functionality. Six of the 15 proteins (i.e. the large terminase, the major capsid protein, two tail proteins, and two proteins of unknown function), were found to apparently activate the toxicity of various antiphage systems. In most instances, no toxicity was observed when the

mutated version of the phage gene was expressed along with the antiphage system, explaining how the escape mutants bypass the antiphage systems. Additionally, two phage proteins (SSAPs) were apparently found to act as targets for AbiA and AbiJ, which also share an additional mechanistic similarity (escape mutant cross-insensitivity) as described below. Interestingly, SSAP diversity has previously been linked to the evolution of antiphage systems targeting SSAPs (60)

The terminase large subunit is known to be involved in phage DNA packaging with its ATPase domain being responsible for providing the energy required for DNA translocation into the empty capsids (69). The large terminase, the major capsid and tail proteins have previously been reported to activate particular antiphage systems such as Avs (70), CapRel^{SJ46} (32) and DSR2 (71), respectively, indicating similarities in the activation of different antiphage systems despite their host origin and their sequence, structural and functional diversity. Although no system in this study was found to be activated by an SSB or SSAP, several defence systems such as Hna, AbpAB and Retron-Eco8 have been shown to be activated by SSB (33, 72, 73).

Sak is an SSAP recombinase with ssDNA binding properties that are known to be involved in dsDNA break repair, in genome circularization and in DNA replication (74, 75). A Sak-like SSAP-encoding gene was mutated in different phages to circumvent three different antiphage systems of this study. Interestingly, the AbiA escape mutants were also insensitive to AbiJ, confirming a greater mechanistic similarity in this instance and proposing an approach for revealing such mechanisms. This information is vital for

designing starter cultures for the dairy industry, as it is necessary to select strains with antiphage systems that cannot be circumvented by the same phage escape mutants, thus creating a robust and phage-resistant starter blend. Notably, this was not observed for other combinations, where the same gene was mutated in different phages to bypass different antiphage systems in this study, suggesting that while different systems are governed by general principles, critical differences exist. These differences likely underpin the presence of multiple antiphage systems in many lactococcal dairy strains (and in some cases on the same plasmid, see Kamadhenu-carrying plasmid, (22)) from an evolutionary perspective to provide several biological hurdles to phage infection.

To our knowledge, this is the first report of phages bypassing an antiphage system (Audmula) by mutating the lysin-encoding gene, which is responsible for digesting the cell wall and allowing phage particle release as the final step of an infection cycle (76). We propose that Audmula due to its transglycosylase properties is modifying the composition of the cell wall to prohibit the release of the mature phage particles. However, a mutation in the active site of the phage lysin is sufficient to circumvent the protection provided by Audmula and release the progeny. We propose that the c2 lysin mutants capable of degrading the Audmula-modified cell wall are already present in the lysate at a frequency of 10^{-4} (obtained escape mutants frequency) and they are responsible for eventual collapse of the culture after many cycles of re-infection. As cell death in the case of Audmula appears to be caused by the phage rather than self-induced, antiphage system Audmula is likely functioning through a non-abortive infection pathway

(based on the current Abi definition), which agrees with the previously delayed (non-Abi-like) lysis in broth profile obtained (22). However, notably, Audmula still demonstrates an altruistic nature by preventing the spread of phage progeny, similar to Abi systems. The suggested mode of action may explain the relatively narrow spectrum of phages (ceduoviruses) targeted by Audmula, as the *Ceduovirus* lysins are known to have different active domains compared to *Skunavirus*, each recognising and cleaving different sugars (77). Such mechanisms are reminiscent of the *Bacillus subtilis* DynA system, which forms large clusters on the cell membrane to support its integrity, resulting in delayed lysis for the benefit of the bacterial population (78).

All in all, we envision that the complex landscape of bacterial immunity, now understood to be governed by some general activation/targeting principles, will gradually be decoded through similar studies. We expect that the findings of this study will provide practical advice for the dairy fermentation industry, while the ongoing exploration of the antiphage systems mechanisms will keep providing invaluable biotechnological and biomedical tools, such as the restriction enzymes, CRISPR-Cas-based technologies or retron-derived DNA for genome editing (34–37).

4.6 Data availability

Genomes of the wild-type phages used in this study for escape mutant isolation and as references for the SNP analysis were deposited in Genbank under the accession numbers PP906165 (P008), PP906166 (p2),

PP906167 (sk1), PP906168 (sv66901), PP906169 (c2), PP906170 (sk713), PP906171 (sv62601), PP906172 (svUCCL624), 56006 (PP906173) and PP906174 (50501).

Coordinates of predicted structures are accessible on Zenodo (https://doi.org/10.5281/zenodo.12740547).

Any additional information required to reanalyse the data reported in this study is available from the corresponding author upon request.

4.7 References

1. Mahony,J., Bottacini,F. and van Sinderen,D. (2023) Towards the diversification of lactococcal starter and non-starter species in mesophilic dairy culture systems. *Microb Biotechnol*, 16, 1745–1754.

2. Hayes,S., Murphy,J., Mahony,J., Lugli,G.A., Ventura,M., Noben,J.P., Franz,C.M.A.P., Neve,H., Nauta,A. and van Sinderen,D. (2017) Biocidal inactivation of *Lactococcus lactis* bacteriophages: Efficacy and targets of commonly used sanitizers. *Front Microbiol*, 8, 107.

3. Garneau,J.E. and Moineau,S. (2011) Bacteriophages of lactic acid bacteria and their impact on milk fermentations. *Microb Cell Fact*, 10, 1–10.

4. Ortiz Charneco,G., de Waal,P.P., van Rijswijck,I.M.H., van Peij,N.N.M.E., van Sinderen,D. and Mahony,J. (2023) Bacteriophages in the dairy industry: A problem solved? *Annu Rev Food Sci Technol*, 14, 367–385.

5. Mahony,J., Cambillau,C. and Van Sinderen,D. (2017) Host recognition by lactic acid bacterial phages. *FEMS Microbiol Rev*, 41, S16–S26.

6. Oliveira,J., Mahony,J., Hanemaaijer,L., Kouwen,T.R.H.M. and van Sinderen,D. (2018) Biodiversity of bacteriophages infecting *Lactococcus lactis* starter cultures. *J Dairy Sci*, 101, 96–105.

7. Chmielewska-Jeznach,M., Bardowski,J.K. and Szczepankowska,A.K. (2018) Molecular, physiological and phylogenetic traits of *Lactococcus* 936-type phages from distinct dairy environments. *Sci Rep*, 8, 12540.

8. Marcelli,B., de Jong,A., Janzen,T., Serrano,M., Kok,J. and Kuipers,O.P. (2020) Complete genome sequences of 28 lactococcal bacteriophages isolated from failed dairy fermentation processes. *Microbiol Resour Announc*, 9.

9. Chopin,M.C., Chopin,A. and Bidnenko,E. (2005) Phage abortive infection in lactococci: Variations on a theme. *Curr Opin Microbiol*, 8, 473–479.

10. Ainsworth,S., Stockdale,S., Bottacini,F., Mahony,J. and van Sinderen,D. (2014) The *Lactococcus lactis* plasmidome: much learnt, yet still lots to discover. *FEMS Microbiol Rev*, 38, 1066–1088.

11. Georjon,H. and Bernheim,A. (2023) The highly diverse antiphage defence systems of bacteria. *Nat Rev Microbiol*, 21, 686–700.

12. Labrie,S.J., Samson,J.E. and Moineau,S. (2010) Bacteriophage resistance mechanisms. *Nat Rev Microbiol*, 8, 317–327.

13. Lopatina,A., Tal,N. and Sorek,R. (2020) Abortive infection: Bacterial suicide as an antiviral immune strategy. *Annu Rev Virol*, 7, 371–384.

14. Bernheim,A. and Sorek,R. (2020) The pan-immune system of bacteria: antiviral defence as a community resource. *Nat Rev Microbiol*, 18, 113–119.

15. Doron,S., Melamed,S., Ofir,G., Leavitt,A., Lopatina,A., Keren,M., Amitai,G. and Sorek,R. (2018) Systematic discovery of antiphage defense systems in the microbial pangenome. *Science (1979)*, 359.

16. Gao,L., Altae-Tran,H., Böhning,F., Makarova,K.S., Segel,M., Schmid-Burgk,J.L., Koob,J., Wolf,Y.I., Koonin,E. V. and Zhang,F. (2020) Diverse enzymatic activities mediate antiviral immunity in prokaryotes. *Science (1979)*, 369, 1077–1084.

17. Millman,A., Melamed,S., Leavitt,A., Doron,S., Bernheim,A., Hör,J., Garb,J., Bechon,N., Brandis,A., Lopatina,A., *et al.* (2022) An expanded arsenal of immune systems that protect bacteria from phages. *Cell Host Microbe*, 30, 1556-1569.e5.

18. Vassallo,C.N., Doering,C.R., Littlehale,M.L., Teodoro,G.I.C. and Laub,M.T. (2022) A functional selection reveals previously undetected anti-phage defence systems in the *E. coli* pangenome. *Nat Microbiol*, 7, 1568–1579.

19. Patel,P.H., Taylor,V.L., Zhang,C., Getz,L.J., Fitzpatrick,A.D., Davidson,A.R. and Maxwell,K.L. (2024) Anti-phage defence through inhibition of virion assembly. *Nat Commun*, 15, 1644.

20. Bravo,J.P.K., Aparicio-Maldonado,C., Nobrega,F.L., Brouns,S.J.J. and Taylor,D.W. (2022) Structural basis for broad anti-phage immunity by DISARM. *Nat Commun*, 13, 2987.

21. Tamulaitiene,G., Sabonis,D., Sasnauskas,G., Ruksenaite,A., Silanskas,A., Avraham,C., Ofir,G., Sorek,R., Zaremba,M. and Siksnys,V. (2024) Activation of Thoeris antiviral system via SIR2 effector filament assembly. *Nature*, 627, 431–436.

22. Grafakou,A., Mosterd,C., Beck,M.H., Kelleher,P., McDonnell,B., de Waal,P.P., van Rijswijck,I.M.H., van Peij,N.N.M.E., Cambillau,C.,

Mahony,J., *et al.* (2024) Discovery of antiphage systems in the lactococcal plasmidome. *Nucleic Acids Res*, 52, 9760–9776..

23. Bidnenko,E., Ehrlich,D. and Chopin,M.C. (1995) Phage operon involved in sensitivity to the *Lactococcus lactis* abortive infection mechanism AbiD1. *J Bacteriol*, 177, 3824–3829.

24. Dinsmore,P.K. and Klaenhammer,T.R. (1997) Molecular characterization of a genomic region in a *Lactococcus* bacteriophage that is involved in its sensitivity to the phage defense mechanism AbiA. *J Bacteriol*, 179, 2949–2957.

25. Bidnenko,E., Chopin,M.C., Ehrlich,S.D. and Anba,J. (2002) *Lactococcus lactis* AbiD1 abortive infection efficiency is drastically increased by a phage protein. *FEMS Microbiol Lett*, 214, 283–287.

26. Bouchard,J.D. and Moineau,S. (2004) Lactococcal phage genes involved in sensitivity to AbiK and their relation to single-strand annealing proteins. *J Bacteriol*, 186, 3649–3652.

27. Haaber,J., Rousseau,G.M., Hammer,K. and Moineau,S. (2009) Identification and characterization of the phage gene *sav*, involved in sensitivity to the lactococcal abortive infection mechanism AbiV. *Appl Environ Microbiol*, 75, 2484–2494.

28. Labrie,S.J., Tremblay,D.M., Moisan,M., Villion,M., Magadán,A.H., Campanacci,V., Cambillau,C. and Moineau,S. (2012) Involvement of the major capsid protein and two early-expressed phage genes in the activity of the lactococcal abortive infection mechanism AbiT. *Appl Environ Microbiol*, 78, 6890–6899.

29. Depardieu,F., Didier,J.-P., Bernheim,A., Sherlock,A., Molina,H., Duclos,B. and Bikard,D. (2016) A eukaryotic-like serine/threonine kinase protects staphylococci against phages. *Cell Host Microbe*, 20, 471–481.

30. Millman,A., Bernheim,A., Stokar-Avihail,A., Fedorenko,T., Voichek,M., Leavitt,A., Oppenheimer-Shaanan,Y. and Sorek,R. (2020) Bacterial retrons function in anti-phage defense. *Cell*, 183, 1551-1561.e12.

31. Rousset,F., Depardieu,F., Miele,S., Dowding,J., Laval,A.-L., Lieberman,E., Garry,D., Rocha,E.P.C., Bernheim,A. and Bikard,D. (2022) Phages and their satellites encode hotspots of antiviral systems. *Cell Host Microbe*, 30, 740-753.e5.

32. Zhang,T., Tamman,H., Coppieters ’t Wallant,K., Kurata,T., LeRoux,M., Srikant,S., Brodiazhenko,T., Cepauskas,A., Talavera,A., Martens,C., *et al.* (2022) Direct activation of a bacterial innate immune system by a viral capsid protein. *Nature*, 612, 132–140.

33. Stokar-Avihail,A., Fedorenko,T., Hör,J., Garb,J., Leavitt,A., Millman,A., Shulman,G., Wojtania,N., Melamed,S., Amitai,G., *et al.* (2023) Discovery of phage determinants that confer sensitivity to bacterial immune systems. *Cell*, 186, 1863-1876.e16.

34. Loenen,W.A.M., Dryden,D.T.F., Raleigh,E.A., Wilson,G.G. and Murray,N.E. (2014) Highlights of the DNA cutters: a short history of the restriction enzymes. *Nucleic Acids Res*, 42, 3–19.

35. Adli,M. (2018) The CRISPR tool kit for genome editing and beyond. *Nat Commun*, 9, 1911.

36. Singh,M., Bindal,G., Misra,C.S. and Rath,D. (2022) The era of Cas12 and Cas13 CRISPR-based disease diagnosis. *Crit Rev Microbiol*, 48, 714–729.

37. Lopez,S.C., Crawford,K.D., Lear,S.K., Bhattarai-Kline,S. and Shipman,S.L. (2022) Precise genome editing across kingdoms of life using retron-derived DNA. *Nat Chem Biol*, 18, 199–206.

38. Linares,D.M., Kok,J. and Poolman,B. (2010) Genome sequences of *Lactococcus lactis* MG1363 (revised) and NZ9000 and comparative physiological studies. *J Bacteriol*, 192, 5806–5812.

39. Erazo Garzon,A., Mahony,J., Bottacini,F., Kelleher,P. and van Sinderen,D. (2019) Complete genome sequence of *Lactococcus lactis* subsp. *cremoris* 3107, host for the model lactococcal P335 bacteriophage TP901-1. *Microbiol Resour Announc*, 8, e01635-18.

40. Bolotin,A., Wincker,P., Mauger,S., Jaillon,O., Malarme,K., Weissenbach,J., Ehrlich,S.D. and Sorokin,A. (2001) The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res*, 11, 731–753.

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

42. O'Driscoll,J., Glynn,F., Cahalane,O., O'Connell-Motherway,M., Fitzgerald,G.F. and van Sinderen,D. (2004) Lactococcal plasmid pNP40 encodes a novel, temperature-sensitive restriction-modification system. *Appl Environ Microbiol*, 70, 5546–5556.

43. Chandry,P.S., Moore,S.C., Boyce,J.D., Davidson,B.E. and Hillier,A.J. (1997) Analysis of the DNA sequence, gene expression, origin of replication and modular structure of the *Lactococcus lactis* lytic bacteriophage sk1. *Mol Microbiol*, 26, 49–64.

44. Bebeacua,C., Tremblay,D., Farenc,C., Chapot-Chartier,M.P., Sadovskaya,I., van Heel,M., Veesler,D., Moineau,S. and Cambillau,C. (2013) Structure, adsorption to host, and infection mechanism of virulent lactococcal phage p2. *J Virol*, 87, 12302–12312.

45. Higgins,D.L., Sanozky-Dawes,R.B. and Klaenhammer,T.R. (1988) Restriction and modification activities from *Streptococcus lactis* ME2 are encoded by a self-transmissible plasmid, pTN20, that forms cointegrates during mobilization of lactose-fermenting ability. *J Bacteriol*, 170, 3435–3442.

46. Loof,M., Lembke,J. and Teuber,M. (1983) Characterization of the genome of the *Streptococcus lactis* “subsp. *diacetylactis*” bacteriophage P008 wide-spread in German cheese factories. *Syst Appl Microbiol*, 4, 413–423.

47. Lillehaug,D. (1997) An improved plaque assay for poor plaque-producing temperate lactococcal bacteriophages. *J Appl Microbiol*, 83, 85–90.

48. Gish,W. and States,D.J. (1993) Identification of protein coding regions by database similarity search. *Nat Genet*, 3, 266–272.

49. Hyatt,D., Chen,G.-L., LoCascio,P.F., Land,M.L., Larimer,F.W. and Hauser,L.J. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics*, 11, 1–8.

50. Altschul,S. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*, 25, 3389–3402.

51. Lugli,G.A., Milani,C., Mancabelli,L., van Sinderen,D. and Ventura,M. (2016) MEGAnnotator: A user-friendly pipeline for microbial genomes assembly and annotation. *FEMS Microbiol Lett*, 363, 1–8.

52. Bateman,A., Coin,L., Durbin,R., Finn,R.D., Hollich,V., Griffiths-Jones,S., Khanna,A., Marshall,M., Moxon,S., Sonnhammer,E.L.L., *et al.* (2004) The Pfam protein families database. *Nucleic Acids Res*, 32, 138–141.

53. Marchler-Bauer,A., Lu,S., Anderson,J.B., Chitsaz,F., Derbyshire,M.K., DeWeese-Scott,C., Fong,J.H., Geer,L.Y., Geer,R.C., Gonzales,N.R., *et al.* (2011) CDD: A Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res*, 39, 225–229.

54. Söding,J., Biegert,A. and Lupas,A.N. (2005) The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res*, 33.

55. Mirdita,M., Schütze,K., Moriwaki,Y., Heo,L., Ovchinnikov,S. and Steinegger,M. (2022) ColabFold: making protein folding accessible to all. *Nat Methods*, 19, 679–682.

56. Holm,L. (2022) Dali server: structural unification of protein families. *Nucleic Acids Res*, 50, W210–W215.

57. Pettersen,E.F., Goddard,T.D., Huang,C.C., Meng,E.C., Couch,G.S., Croll,T.I., Morris,J.H. and Ferrin,T.E. (2021) UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Science*, 30, 70–82.

58. Scaltriti,E., Tegoni,M., Rivetti,C., Launay,H., Masson,J., Magadan,A.H., Tremblay,D., Moineau,S., Ramoni,R., Lichière,J., *et al.* (2009) Structure and function of phage p2 ORF34(p2), a new type of single-stranded DNA binding protein. *Mol Microbiol*, 73, 1156–1170.

59. Szczepańska,A.K. (2009) Bacteriophage-encoded functions engaged in initiation of homologous recombination events. *Crit Rev Microbiol*, 35, 197–220.

60. Steczkiewicz,K., Prestel,E., Bidnenko,E. and Szczepankowska,A.K. (2021) Expanding diversity of Firmicutes single-strand annealing proteins: A putative role of bacteriophage-host arms race. *Front Microbiol*, 12.

61. Weaver,S. and Levine,M. (1977) Recombinational circularization of *Salmonella* phage P22 DNA. *Virology*, 76, 29–38.

62. Poteete,A.R. and Fenton,A.C. (1983) DNA-binding properties of the Erf protein of bacteriophage P22. *J Mol Biol*, 163, 257–275.

63. Aravind,L., Makarova,K.S. and Koonin,E.V. (2000) Holliday junction resolvases and related nucleases: identification of new families, phyletic

distribution and evolutionary trajectories. *Nucleic Acids Res*, 28, 3417–3432.

64. Iyer,L.M., Koonin,E. V and Aravind,L. (2002) Classification and evolutionary history of the single-strand annealing proteins, RecT, Redβ, ERF and RAD52. *BMC Genomics*, 3, 8.

65. Altschul,S.F., Gish,W., Miller,W., Myers,E.W. and Lipman,D.J. (1990) Basic local alignment search tool. *J Mol Biol*, 215, 403–410.

66. Sayers,E.W., Beck,J., Bolton,E.E., Bourexis,D., Brister,J.R., Canese,K., Comeau,D.C., Funk,K., Kim,S., Klimke,W., *et al.* (2021) Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res*, 49, D10–D17.

67. Samson,J.E., Bélanger,M. and Moineau,S. (2013) Effect of the abortive infection mechanism and type III toxin/antitoxin system AbiQ on the lytic cycle of *Lactococcus lactis* phages. *J Bacteriol*, 195, 3947–56.

68. Blower,T.R., Chai,R., Przybilski,R., Chindhy,S., Fang,X., Kidman,S.E., Tan,H., Luisi,B.F., Fineran,P.C. and Salmond,G.P.C. (2017) Evolution of *Pectobacterium* bacteriophage ΦM1 to escape two bifunctional type III toxin-antitoxin and abortive infection systems through mutations in a single viral gene. *Appl Environ Microbiol*, 83.

69. Feiss,M. and Rao,V.B. (2012) The bacteriophage DNA packaging machine. *Adv Exp Med Biol*, 726, 489–509.

70. Gao,L.A., Wilkinson,M.E., Strecker,J., Makarova,K.S., Macrae,R.K., Koonin,E. V. and Zhang,F. (2022) Prokaryotic innate immunity through pattern recognition of conserved viral proteins. *Science (1979)*, 377.

71. Garb,J., Lopatina,A., Bernheim,A., Zaremba,M., Siksnys,V., Melamed,S., Leavitt,A., Millman,A., Amitai,G. and Sorek,R. (2022) Multiple phage resistance systems inhibit infection via SIR2-dependent NAD+ depletion. *Nat Microbiol*, 7, 1849–1856.

72. Sasaki,T., Takita,S., Fujishiro,T., Shintani,Y., Nojiri,S., Yasui,R., Yonesaki,T. and Otsuka,Y. (2023) Phage single-stranded DNA-binding protein or host DNA damage triggers the activation of the AbpAB phage defense system. *mSphere*, 8.

73. Sather,L.M., Zamani,M., Muhammed,Z., Kearsley,J.V.S., Fisher,G.T., Jones,K.M. and Finan,T.M. (2023) A broadly distributed predicted helicase/nuclease confers phage resistance via abortive infection. *Cell Host Microbe*, 31, 343-355.e5.

74. Bouchard,J.D. and Moineau,S. (2004) Lactococcal phage genes involved in sensitivity to AbiK and their relation to single-strand annealing proteins. *J Bacteriol*, 186, 3649–3652.

75. Dy,R.L., Richter,C., Salmond,G.P.C. and Fineran,P.C. (2014) Remarkable mechanisms in microbes to resist phage infections. *Annu Rev Virol*, 1, 307–331.

76. Young,R., Wang,I.-N. and Roof,W.D. (2000) Phages will out: strategies of host cell lysis. *Trends Microbiol*, 8, 120–128.

77. Labrie,S., Vukov,N., Loessner,M.J. and Moineau,S. (2004) Distribution and composition of the lysis cassette of *Lactococcus lactis* phages and functional analysis of bacteriophage ul36 holin. *FEMS Microbiol Lett*, 233, 37–43.

78. Guo,L., Sattler,L., Shafqat,S., Graumann,P.L. and Bramkamp,M. (2022) A bacterial dynamin-like protein confers a novel phage resistance strategy on the population level in *Bacillus subtilis*. *mBio*, 13.

4.8 Supplementary data

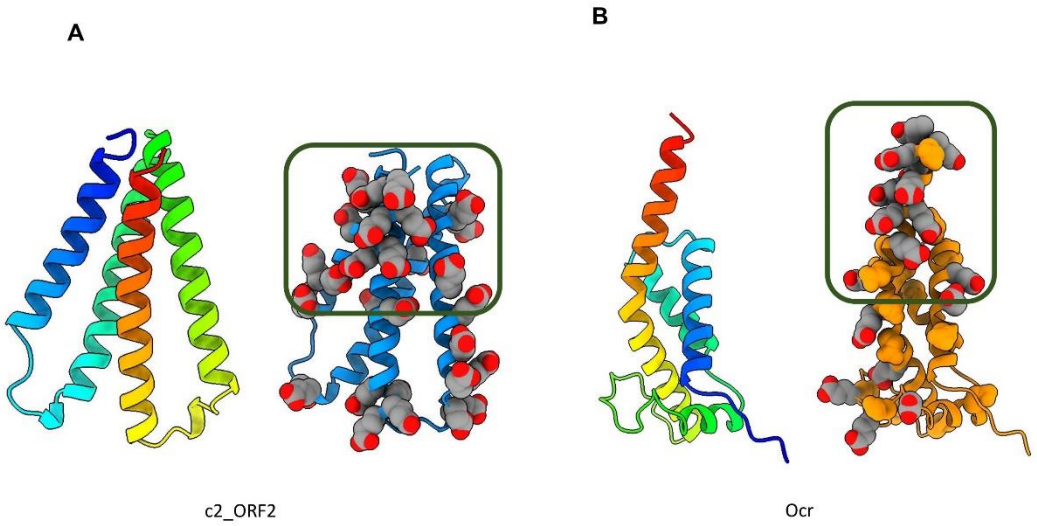


Figure S1: Charge distribution of c2 ORF2 and Ocr versus their putative partner, the PARIS-ii dimers from *L. lactis* and *E. coli*, respectively. (A) Left: c2_ORF2 ribbon view rainbow colored (Nt, blue to Ct, red). Right: with the cluster of positively charged residues (Asp and Glu) with their atoms represented as spheres (C:grey, O:red). (B) Left: Ocr ribbon view rainbow colored (Nt, blue to Ct, red). Right: with the cluster of positively charged residues (Asp and Glu) with their atoms represented as spheres (C:grey, O:red). The green rectangles identify the charged clusters

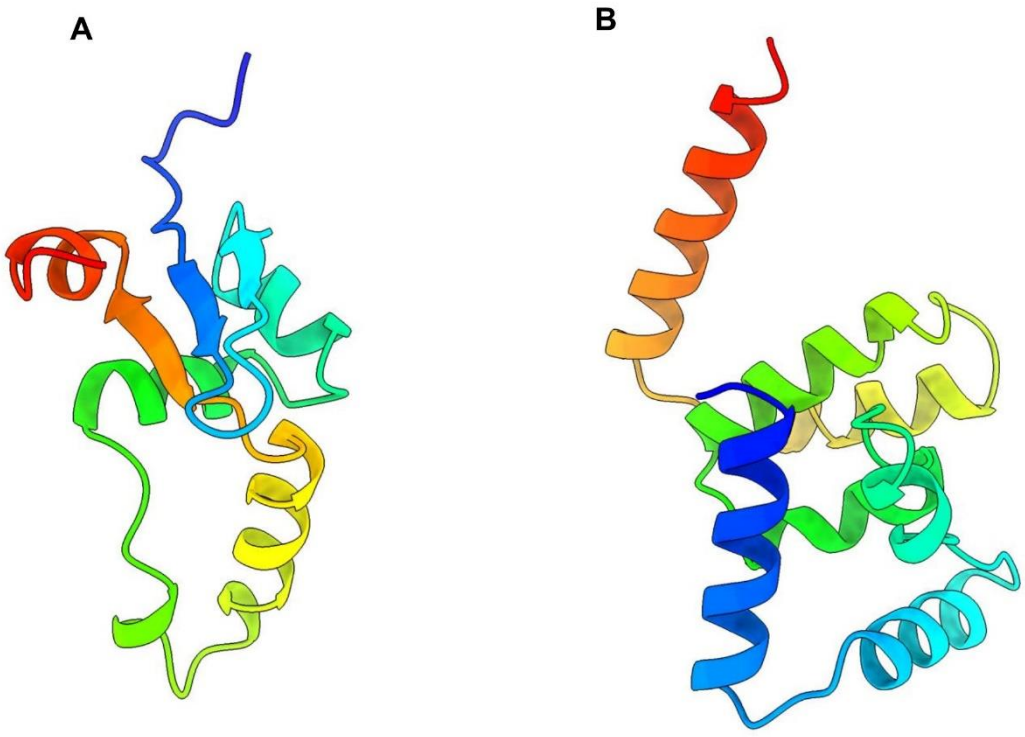


Figure S2: Ribbon views rainbow colored (Nt, blue to Ct, red) of two hypothetical proteins [(A) sk1_ORF24 and (B) sv62601_ORF26] in which AbiD/F-insensitive phage escape mutants of two different phages had acquired a mutation.

Table S1. Cloning details of the candidate phage-derived trigger genes

Phage gene	Phage	NCBI Taxonomy ID	Locus_tag	Associated antiphage system	Enzymes	Vector
Terminase large subunit	sk1	PP906167	sk1p02	Rhea (pNZ44)	BamHI/SalI	pPTPi
Sak-like ssDNA annealing protein	svUCCL624	PP906172	svUCCL624_39	Kamadhenu (pNZ44)	BamHI/SalI	pPTPi
Lysin	c2	PP906169	c2p22	Audmula (pNZ44)	BamHI/SalI	pPTPi
Terminase large subunit	c2	PP906169	c2p31	Rugutis (pNZ44)	BamHI/SalI	pPTPi
Hypothetical protein	c2	PP906169	c2p02	PARIS (pPEPi)	BamHI/SalI	pPTPi
Hypothetical protein	sk1	PP906167	sk1p48	Type II CBASS (pNZ44)	BamHI/SalI	pPTPi
ssDNA binding protein	p2	PP906166	FDI51_gp32	Septu (pPTPi)	BamHI/SalI	pPEPi
Holliday junction resolvase	c2	PP906169	c2p21	AbiA (pPTPi)	BamHI/SalI	pPEPi
Erf-like ssDNA annealing protein	c2	PP906169	c2p08	AbiA (pPTPi)	BamHI/SalI	pPEPi
Hypothetical protein	sk1	PP906167	sk1p35	AbiB (pNZ44)	BamHI/SalI	pPTPi

Major capsid protein	sv66901	PP906168	sv66901_07	AbiB (pNZ44)	BamHI/SalI	pPTPi
Hypothetical protein	sk1	PP906167	sk1p24	AbiD-like (pNZ44)	BamHI/SalI	pPTPi
Hypothetical protein	sv62601	PP906171	sv62601_26	AbiD-like (pNZ44)	BamHI/SalI	pPTPi
Major tail protein	p2	PP906166	FDI51_gp11	AbiG (pNZ44)	BamHi/EcoRI	pPTPi
Sak-like ssDNA annealing protein	sk713	PP906170	sk713_37	AbiJ (pNZ44)	BamHI/SalI	pPTPi
Major tail protein	sk713	PP906170	sk713_12	AbiP (pNZ44)	BamHI/SalI	pPTPi

Table S1. Cloning details of the candidate phage-derived trigger genes (continued).

Phage gene	F sequence	R sequence
Terminase large subunit	AGCAGCGGATCCAGGAGGCACTCACCATGTATTATTTAAATAAAATGTTG	AGCAGCGTCGACTTATACTTCATCTGACACC
Sak-like ssDNA annealing protein	AGCAGCGGATCCAGGAGGCACTCACCATGAGCGTATTTGAACAACT	AGCAGCGTCGACTTATTTTCCCTCTGTTGCTT
Lysin	AGCAGCGGATCCAGGAGGCACTCACCATGTTCCCTTATAAAAAAACAATAAT	AGCAGCGTCGACCTATTTCACTTCTCCTGAAG

Terminase large subunit	AGCAGCGGATCCAGGAGGCACTCACCATGAGTTTAATTCAAGACTGGA	AGCAGCGTCGACTTAAATGAAATAGTCCTCACTTT
Hypothetical protein	AGCAGCGGATCCAGGAGGCACTCACCATGAAATTAAAAAATCAAATCGA	AGCAGCGTCGACTTACACCTCCTCCATTTC
Hypothetical protein	AGCAGCGGATCCAGGAGGCACTCACCATGGCTAGAGACAAATATTTGAT	AGCAGCGTCGACTTATCTATTATCTCCTAAATCAAAAT
ssDNA binding protein	AGCAGCGGATCCAGGAGGCACTCACCATGGCAATCATCACAGTAAC	AGCAGCGTCGACCTAGAAAGGTAAATCTTCCG
Holliday junction resolvase	AGCAGCGGATCCAGGAGGCACTCACCATGAAAAAAATTTTAGCTATTGACTT	AGCAGCGTCGACCTAACCCAAGTGTTCGCAA
Erf-like ssDNA annealing protein	AGCAGCGGATCCAGGAGGCACTCACCATGGAAAGCAAAGTTCTAAAATTA	AGCAGCGTCGACTTAAGCATTCATATTTACTTTTCC
Hypothetical protein	AGCAGCGGATCCAGGAGGCACTCACCATGGAAACAATTAACATTAAATTTG	AGCAGCGTCGACTCATTCGATAACCTCTTCTTT
Major capsid protein	AGCAGCGGATCCAGGAGGCACTCACCATGAATAAACCTGATTTAATCGAA	AGCAGCGTCGACTTACTTCCTCCATTTTATTCTTAT
Hypothetical protein	AGCAGCGGATCCAGGAGGCACTCACCATGGCTTTGAATTATTTACAATTAAG	AGCAGCGTCGACGTACGCTAGTGATTCTTTCATA
Hypothetical protein	AGCAGCGGATCCAGGAGGCACTCACCATGACAAATGAAGAATTATATGAAAG	AGCAGCGTCGACTTATCTCATTGCCTCCCTTTG
Major tail protein	AGCAGCGGATCCAGGAGGCACTCACCATGAAATTAGATTATAATTCACGTG	AGCAGCGAATTCTTATGAATGGTCAGTTACTGAA
Sak-like ssDNA annealing protein	AGCAGCGGATCCAGGAGGCACTCACCATGAGCGTATTTGAACAGCTT	AGCAGCGTCGACTTATTCCCCTTTTTCTGTTTCTT
Major tail protein	AGCAGCGGATCCAGGAGGCACTCACCATGAAATTAGATTATAATTCACGTG	AGCAGCGTCGACCTAGGGTCTATCTGTTACAG

Table S2. Detailed information of the isolated phage escape mutants for each antiphage system.

Escape mutant	Antiphage system	Phage	Gene function	NCBI accession number	Locus tag	Number of mutations	Predicted effect of mutation on protein/Mutation(s)
1	Rhea	sv66901	Terminase large subunit	PP906168	sv66901_03	1	H204Q
2	Rhea	sv66901	Terminase large subunit	PP906168	sv66901_03	2	T58A & S108I
3	Rhea	sv66901	Terminase large subunit	PP906168	sv66901_03	3	S108I
			Terminase small subunit	PP906168	sv66901_01		E101K
			Receptor-binding protein	PP906168	sv66901_20		G78W
4	Rhea	sv62601	Terminase large subunit	PP906171	sv62601_02	1	A132G
5	Rhea	sv62601	Terminase large subunit	PP906171	sv62601_02	2	A132G
			Terminase small subunit	PP906171	sv62601_01		E101K
6	Rhea	sv62601	Terminase large subunit	PP906171	sv62601_02	2	A132G
			Terminase small subunit	PP906171	sv62601_01		E101K
7	Rhea	P008	Terminase large subunit	PP906165	LPPV008_gp02	1	G233V
8	Rhea	P008	Terminase large subunit	PP906165	LPPV008_gp02	1	G233V
9	Rhea	P008	Terminase large subunit	PP906165	LPPV008_gp02	1	G233V
10	Rhea	P008	Terminase large subunit	PP906165	LPPV008_gp02	1	G233V
11	Rhea	P008	Terminase large subunit	PP906165	LPPV008_gp02	1	G233V
12	Rhea	P008	Terminase large subunit	PP906165	LPPV008_gp02	1	H204N
13	Rhea	sk1	Terminase large subunit	PP906167	sk1p02	1	G233V
14	Rhea	sk1	Terminase large subunit	PP906167	sk1p02	1	G233V

15	Kamadhenu	svUCCL624	Sak-like ssDNA annealing protein	PP906172	svUCCL624_39	1	G22D
16	Kamadhenu	svUCCL624	Sak-like ssDNA annealing protein	PP906172	svUCCL624_39	1	G22A
17	Kamadhenu	svUCCL624	Sak-like ssDNA annealing protein	PP906172	svUCCL624_39	1	G22D
18	Audmula	c2	Lysin	PP906169	c2p22	1	G49S
19	Audmula	c2	Lysin	PP906169	c2p22	1	L140F
20	Audmula	c2	Lysin	PP906169	c2p22	1	G49S
21	Audmula	50501	Lysin	PP906174	50501_22	1	Y96C
22	Audmula	50501	Lysin	PP906174	50501_22	1	A33E
23	Audmula	56006	Lysin	PP906173	56006_22	1	A33T
24	Audmula	56006	Lysin	PP906173	56006_22	1	D34Y
25	Audmula	56006	Lysin	PP906173	56006_22	1	A64E
26	Rugutis	c2	Intergenic region	PP906169	-	2	C12570A
			Terminase large subunit	PP906169	c2p31		F79L
27	Rugutis	c2	Terminase large subunit	PP906169	c2p31	1	F79L
28	Rugutis	c2	Terminase large subunit	PP906169	c2p31	1	A88S
29	PARIS	c2	Hypothetical protein	PP906169	c2p02	1	M1T
30	PARIS	c2	AAA family ATPase	PP906169	c2p17	2	Q290E
			Hypothetical protein	PP906169	c2p02		M121T
31	PARIS	c2	Intergenic region	PP906169	-	1	G221T
32	PARIS	c2	Intergenic region	PP906169	-	1	C208A
33	PARIS	c2	Hypothetical protein	PP906169	c2p02	1	M121T

34	PARIS	c2	Hypothetical protein	PP906169	c2p02	3	M121I, E122N, E123G, V124G
35	Type II CBASS	sk1	Hypothetical protein	PP906167	sk1p48	2	E46stop
			Intergenic region	PP906167	-		C26134T
36	Type II CBASS	sk1	Intergenic region	PP906167	-	1	T26140C
37	Type II CBASS	sk1	Intergenic region	PP906167	-	1	T26140C
38	Septu	p2	ssDNA binding protein	PP906166	FDI51_gp32	1	D58N
39	Septu	p2	Tape measure protein	PP906166	FDI51_gp14	4	E117D
			ssDNA binding protein	PP906166	FDI51_gp32		D58N
			Sak-like ssDNA annealing protein	PP906166	FDI51_gp33		F108L
			Terminase large subunit	PP906166	FDI51_gp02		G261V
40	Septu	p2	ssDNA binding protein	PP906166	FDI51_gp32	1	D58N
41	AbiA	c2	Holliday junction resolvase	PP906169	c2p21	2	W111C
			Erf-like ssDNA annealing protein	PP906169	c2p08		W84L
42	AbiA	c2	Holliday junction resolvase	PP906169	c2p21	2	D141Y
			Erf-like ssDNA annealing protein	PP906169	c2p08		W84L
43	AbiB	sk1	Hypothetical protein	PP906167	sk1p35	1	L86F
44	AbiB	sk1	Hypothetical protein	PP906167	sk1p35	3	L86F
			Intergenic region	PP906167	-		C18719A
			Intergenic region	PP906167	-		T28221G
45	AbiB	sk1	Hypothetical protein	PP906167	sk1p35	3	L86F

			Intergenic region	PP906167	-		C18719A
			Intergenic region	PP906167	-		T28221G
46	AbiB	sv66901	Major capsid protein	PP906168	sv66901_07	1	S97F
47	AbiB	sv66901	Middle expressed protein 4	PP906168	sv66901_48	1	T41I
48	AbiB	sv66901	Major capsid protein	PP906168	sv66901_07	1	L124I
49	AbiD/F	sk1	Hypothetical protein	PP906167	sk1p24	1	E62stop
50	AbiD/F	sk1	Hypothetical protein	PP906167	sk1p24	1	V10G
51	AbiD/F	sv62601	Hypothetical protein	PP906171	sv62601_26	1	Q17stop
52	AbiD/F	sv62601	Hypothetical protein	PP906171	sv62601_26	1	Q17stop
53	AbiG	p2	Intergenic region	PP906166	-	3	G6551T
			Major tail protein	PP906166	FDI51_gp11		V38A
			Major tail protein	PP906166	FDI51_gp11		A167P
54	AbiG	p2	Major tail protein	PP906166	FDI51_gp11	2	V38A
			Major tail protein	PP906166	FDI51_gp11		A167P
55	AbiJ	sk713	Sak-like ssDNA annealing protein	PP906170	sk713_37	1	G22E
56	AbiJ	sk713	Sak-like ssDNA annealing protein	PP906170	sk713_37	1	G22E
57	AbiJ	sk713	Sak-like ssDNA annealing protein	PP906170	sk713_37	1	G22E
58	AbiJ	sk713	Sak-like ssDNA annealing protein	PP906170	sk713_37	1	G22E
59	AbiJ	c2	Erf-like ssDNA annealing protein	PP906169	c2p08	1	K24N
60	AbiJ	c2	Erf-like ssDNA annealing protein	PP906169	c2p08	2	K24N

			Hypothetical protein	PP906169	c2p05		A102S
61	AbiJ	c2	Erf-like ssDNA annealing protein	PP906169	c2p08	2	K24N
			Terminase large subunit	PP906169	c2p31		E385D
62	AbiJ	p2	Sak-like ssDNA annealing protein	PP906166	FDI51_gp33	1	G22E
63	AbiJ	p2	Sak-like ssDNA annealing protein	PP906166	FDI51_gp33	3	G22E
			AAA family ATPase	PP906166	FDI51_gp41		A109S
			Major capsid protein	PP906166	FDI51_gp06		Q167K
64	AbiP	sk1	Major tail protein	PP906167	sk1p11	1	Y133S
65	AbiP	sk713	Major tail protein	PP906170	sk713_12	1	T213K
66	AbiP	sk713	Major tail protein	PP906170	sk713_12	1	T213A

Table S3. Combinations of antiphage systems with shared genes mutated to circumvent their activity and escape mutants used to test their insensitivity towards distinct systems (cross-insensitivity of escape mutants).

Shared mutated gene to circumvent distinct antiphage systems	Escape mutant used [phage(s)_antiphage system origin]	Antiphage system tested	Escape mutant insensitivity
Terminase large subunit	sk1_Rhea	Rhea	Yes
		Rugutis	No
Major tail protein	p2_AbiG	AbiG	Yes
		AbiP	No
	sk1/sk713_AbiP	AbiP	Yes
		AbiG	No
ssDNA annealing or binding protein	sk713/c2_AbiJ	AbiJ	Yes
		Septu/Kamadhenu/AbiA	No
	c2_Septu	Septu	Yes
		AbiJ/Kamadhenu/AbiA	No
	c2_AbiA	AbiA	**Yes**
		AbiJ	**Yes**
		Kamadhenu/Septu	No

Table S4. EOP values obtained from cross-checking AbiA escape mutants against both AbiA and AbiJ.

Antiphage system	Phage		
	c2	EM41	EM42
AbiA	≤ 1.09 (± 0.28) $\times 10^{-6}$	3.89 (± 0.84) $\times 10^{-1}$	9.17 (± 2.89) $\times 10^{-1}$
AbiJ	1.61 (± 0.92) $\times 10^{-6}$	0.99 (± 0.16) $\times 10^{-1}$	1.57 (± 0.05) $\times 10^{-1}$

Chapter V

General discussion and future perspectives

Lactococcus lactis and *Lactococcus cremoris* strains are widely used in starter cultures for dairy fermentations. However, the omnipresence of bacteriophages in non-sterile dairy environments poses a constant threat to the industry. Phage infections of these starter cultures can result in slowed or even failed fermentations, leading to detrimental environmental and economic consequences. The persistent threat of phages, coupled with the growing global demand for dairy and fermented food products, underscores the need for phage-resistant starter cultures that can reliably and robustly withstand these challenges.

While genetically modified organisms (GMOs) may in principle address this issue, their use is heavily regulated and often faces consumer opposition. However, many traits, including phage resistance, can be encoded by conjugative or mobilizable plasmids, which can be transferred or co-transferred between strains through natural processes. Antiphage systems are present in most if not all bacteria to provide a diversity of naturally evolved strategies for bacteria to defend against phages. Indeed, as demonstrated in this thesis and other studies, genetically transferable antiphage activities can be harnessed to develop phage-robust strains. Therefore, the discovery and application of novel antiphage systems within the lactococcal plasmidome could offer a natural and enduring solution to the ongoing phage problem in dairy fermentations.

Despite significant research efforts on antiphage systems in lactococci up until the 2000s, and the discovery of some of the earliest antiphage systems in these bacteria (such as abortive infection systems, AbiA to AbiZ), the recent identification of numerous, previously unknown antiphage systems

in other bacteria suggested that many remain undiscovered. Although most of the currently known lactococcal Abi systems are plasmid-encoded, consistent with reports indicating that mobile genetic elements often carry antiphage systems, plasmids had not been systematically investigated for the presence of novel antiphage systems.

The discovery of seven novel, plasmid-encoded lactococcal antiphage systems, which appear to be widely distributed across various bacterial species, along with five systems whose homologues have been identified in other bacteria, highlights plasmids as a rich source of phage-resistance mechanisms and positions *Lactococcus* as an excellent model to uncover these systems. These findings significantly contribute to the field of bacterial phage immunity, particularly by expanding our understanding of defence mechanisms beyond the well-studied species, *E. coli* and *B. subtilis*. This knowledge can be leveraged to improve dairy fermentations by either directly selecting strains that carry these antiphage systems as starters or by enhancing intrinsic phage resistance of strains through the natural transfer of plasmids encoding these mechanisms to such strains.

The majority of newly identified antiphage systems described in this thesis have been classified as Abi systems, primarily based on phenotypic observations. Similarly, most of the previously established lactococcal Abi systems (AbiA to AbiZ) have been categorized in this way, in many cases relying on limited phenotypic data. However, since only a few of these mechanisms have been extensively characterized, and direct evidence linking the system's activity to phage-induced cell dormancy/death is often

lacking, further studies are needed to confirm that these systems truly operate through abortive infection.

Additionally, the majority of identified lactococcal Abi-like systems are plasmid-encoded, with only a few exceptions. This might lead to the assumption that lactococcal defences against phages are primarily plasmid-based. However, there is a discovery bias towards plasmid-encoded antiphage systems, as plasmids are more frequently studied for phage resistance due to their potential transfer by conjugation to other strains to enhance phage robustness and other desirable traits. While it would not be surprising to find that defence systems are primarily plasmid-borne in (particular representatives of) this bacterial genus -since lactococci are known to carry up to twelve plasmids with various beneficial traits, and a higher copy number associated with plasmid-encoded genes may facilitate increased levels of phage resistance- future studies are needed to determine whether lactococcal Abi-like systems are indeed primarily plasmid-encoded.

Functional and structural analyses of antiphage systems using AlphaFold2 and Dali have revealed potential modes of action for several newly identified as well as previously discovered systems. These tools also enabled the grouping of several antiphage systems based on structural superimposition, in addition to previously established sequence similarity. Such approaches will remain instrumental in elucidating the functions, structures, complexes and classification of emerging antiphage systems. We anticipate that conducting structural searches of proteins with unknown

functions against an antiphage system database will allow discovery of previously unrecognized antiphage systems.

Despite the expansion of the lactococcal antiphage system repertoire, phages may in many cases bypass these defences through specific mutations. This underscores the need to not only continue expanding the arsenal of known antiphage systems but also to deepen our understanding of the diversity of their mode of action. Thoroughly characterized antiphage systems with varying mechanisms could be combined to develop starter cultures with enhanced phage resistance, making it more difficult for phages to bypass them through single-point mutations. In fact, synergistic protection of combined systems has been demonstrated in *E. coli*, and future studies will likely confirm this for other bacteria.

Further characterisation of a given lactococcal antiphage system typically involves assessment of the effect of that antiphage system on specific steps of the phage lytic cycle, which was employed as part of this thesis. However, this approach does not differentiate if inhibition of this phage proliferation step is a direct or indirect target for the system. Additionally, this approach provides general mechanistic information and can be combined with phage escape mutant isolation and analysis, which may for example identify phage-encoded proteins sensed by a given antiphage system.

Indeed, the isolation and analysis of escape mutants was crucial in identifying phage-encoded proteins involved in the mechanisms of antiphage systems. It was revealed that some antiphage systems are activated by phage proteins with similar functions, suggesting mechanistic

commonalities across different systems. Moreover, certain phage escape mutants were found to resist two distinct antiphage systems, further highlighting mechanistic similarities and offering a potential approach for uncovering such mechanisms. This knowledge is essential in order to develop robust starter cultures, as it underscores the importance of selecting strains with antiphage systems that cannot be bypassed by the same phage escape mutants.

However, no cross-resistance was observed in other cases where different phages mutated the same gene to overcome distinct antiphage systems. This indicates that, although various systems may operate under similar principles, they possess crucial differences. These differences likely explain why many lactococcal dairy strains may carry multiple antiphage systems, as this diversity creates several biological barriers to phage infection. Finally, the phage escape mutant isolation and analysis, combined with the predicted functional domain information, led to a proposed novel model of action for one of the antiphage systems. This further validates the escape mutant isolation and analysis as an experimental approach for uncovering mechanistic details of antiphage systems.

While much remains to be discovered, the research presented in this thesis has significantly advanced our understanding of the lactococcal phage resistome, providing practical solutions and insights for the dairy fermentation industry. Given that bacterial antiphage systems, such as restriction enzymes, CRISPR-Cas, and retron-based DNA technologies, are increasingly used as biotechnology and biomedical tools, the knowledge generated here holds potential beyond the food industry. Future research

should continue to explore and decode the diverse bacterial phage immunity landscape, not only in the biotechnologically relevant species belonging to the genus *Lactococcus* but also across various bacterial species, which is now recognized to be governed by general activation and targeting principles.

Acknowledgements

First of all, I would like to thank Douwe and Jenn from the bottom of my heart for once more. Thank you for giving me this opportunity and for warmly welcoming me into your team. For explaining anything with humbleness and respect, and for always being available to answer emails or knocks on your door. Thank you for creating a positive and supportive atmosphere and for being by my side as I grew into a mature scientist.

I extend my heartfelt thanks to Cas. Thank you for being my second brain during this project, for always retaining your humorous and positive attitude, for being an excellent and supportive teammate as well as a friend. I am deeply grateful and proud to have shared the Abi family moments and achievements with you.

Special thanks to our industrial partners, especially Paul, Noël and Irma, for consistently showing a genuine interest in all our findings and for their supportive, positive, professional, and friendly attitude.

I wish to express my appreciation to Christian for his interest in the project and for his constant enthusiasm for performing AlphaFold analyses at any time. Thank you for the harmonious and constructive collaboration and for the added value you brought to the project with your findings.

I am indebted to Matthias, for patiently teaching me everything from scratch, when I did not even know what a primer is! Thank you for imparting me all your knowledge, for being a “scientific dad”, for setting the experimental bases for this fruitful project, and for sharing the initial excitement of the results.

I would like to acknowledge Science Foundation Ireland and dsm-firmenich for the financial support that enabled the success of this PhD project, along with the School of Microbiology and APC Microbiome Ireland for all the resources and assistance provided.

I am thankful for the constant support of Brian. Thank you for answering all of my last-minute questions during the first year (every day at 4 pm) and for always being helpful in every matter throughout the four years.

I deeply appreciate Sofia and Katherine for their love and passion for science. I'm especially grateful for their help with result interpretation during those late, challenging (desperate) evenings. I will miss our scientific and non-scientific talks during sunny coffee breaks.

Deeply grateful for Kelsey and Jun, who co-stressed (or co-decomposed) with me during the last months in our office corner.

Many thanks to the rest of the 335-340 lab members, both old and new, for their constant assistance, friendliness, and kindness; and special thanks to Guillermo for sharing moments and experience in (the best) 339 with me.

I am deeply grateful for finding in the laboratory, amidst novel antiphage systems, the best friends I could ever ask for: Sofia, Rocio, Jun, Luiza, and Elisa. Thank you for all the stimulating scientific conversations and the fun lunch breaks we shared. Thank you for all the unforgettable and fun weekends and moments. You made my time in Ireland fleeting, yet deeply memorable and full of joy. This journey would not have been the same without you.

Of course, I could have not done without the support of my family; my mum Anti, dad Kostas and sister Nena. I will always appreciate the values you have instilled in me, which have been a shield throughout this journey. Thank you for providing anything for our education nurturing curiosity and fostering a lifelong love of learning. I'm deeply grateful of your constant prioritization and love. Thank you for always being proud of me, giving me strength and encouraging me. I am equally proud of you all.

Heartfelt thanks to my best cousin and soulmate Eleanna, for being just a text away, despite the miles.

Many thanks to Marina, my first lab supervisor, who patiently answered all my questions and taught me to work independently and stay well-

organized. Thank you for showing me the joy of investigation, believing in me, and encouraging me to pursue a PhD abroad.

I would like to extend my heartfelt thanks to my favourite housemates and enduring friends: Kimia, Laura, Nieves, Ailsa and Judith. Thank you for the long kitchen conversations about failed experiments and PhD dramas (as well as the more enjoyable chats!). Thank you for being a shoulder to cry on and for making everyday life during these four years a true pleasure.

Many thanks to Noeleen and Philip for warmly welcoming me into their home and generously including me as me part of their family during my first Christmas away from home in the pandemic.

Finally, many thanks to my Greek “friends-islands” in Ireland, Alexandra and Homer. Thank you for being a “Greek escape” on the tough days and for all the fun moments!

And as they say in Ireland “Thanks a million”!