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► **To cite this version:**

Adeline Pastuszka, Geneviève M Rousseau, Vincent Somerville, Sebastien Levesque, Jean-Philippe Fiset, et al.. Dairy phages escape CRISPR defence of *Streptococcus thermophilus* via the anti-CRISPR AcrIIA3. *International Journal of Food Microbiology*, 2023. hal-04448765

HAL Id: hal-04448765

<https://amu.hal.science/hal-04448765>

Submitted on 9 Feb 2024

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Dairy phages escape CRISPR defence of *Streptococcus thermophilus* via the anti-CRISPR AcrIIA3

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 The corrections made in this section will be reviewed and approved by a journal production editor.

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Abstract

Bacterial community collapse due to phage infection is a major risk in cheese making processes. As virulent phages are ubiquitous and diverse in milk fermentation factories, the use of phage-resistant lactic acid bacteria (LAB) is essential to obtain high-quality fermented dairy products. The LAB species *Streptococcus thermophilus* contains two type II-A CRISPR-Cas systems (CRISPR1 and CRISPR3) that can effectively protect against phage infection. However, virulent streptococcal phages carrying anti-CRISPR proteins (ACR) that block the activity of CRISPR-Cas systems have emerged in yogurt and cheese environments. For example, phages carrying AcrIIA5 can impede both CRISPR1 and CRISPR3 systems, while AcrIIA6 stops only CRISPR1. Here, we explore the activity and diversity of a third streptococcal phage anti-CRISPR protein, namely AcrIIA3. We were able to demonstrate that AcrIIA3 is efficiently active against the CRISPR3-Cas system of *S. thermophilus*. We used AlphaFold2 to infer the structure of AcrIIA3 and we predicted that this new family of functional ACR in virulent streptococcal phages has a new α -helical fold, with no previously identified structural homologs. Because ACR proteins are being explored as modulators in genome editing applications, we also tested AcrIIA3 against SpCas9. We found that AcrIIA3 could block SpCas9 in bacteria but not in human cells. Understanding the diversity and functioning of anti-defence mechanisms will be of importance in the design of long-term stable starter cultures.

Keywords:

Anti-CRISPR, Bacteriophages, CRISPR-Cas system, SpCas9, Lactic acid bacteria

Data availability

1 Introduction

Streptococcus thermophilus is a lactic acid bacterium (LAB) widely used as a starter culture by the dairy industry, especially for the manufacture of fermented milk products such as yogurt and speciality cheeses. However, the presence of virulent streptococcal phages in the dairy environment poses a significant industrial risk as they can potentially infect and lysed the selected bacterial strains, leading to disrupted milk fermentation processes and to low-quality fermented products (Gameau and Moineau, 2011).

Bacteria have evolved several defence systems to protect themselves against phage infections (Labrie et al., 2010). Among these, CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR associated proteins) system is now well-documented, particularly in *S. thermophilus*, as it plays a central role in its antiphage arsenal (Barrangou et al., 2007; Deveau et al., 2008; Gameau et al., 2010). A CRISPR-Cas system is composed of a CRISPR array, consisting of several repeated nucleotide sequences separated by variable sequences called spacers, and of their associated *cas* genes. Together, they act as a protective and adaptive immune memory, which relies on three general steps for its mode of action. The first step is the acquisition of a new spacer, originating from invading DNA such as phage genomes, into the CRISPR array (Barrangou et al., 2007; Yosef et al., 2012). The CRISPR array is then transcribed and processed into mature CRISPR RNAs (crRNAs), involving different elements of the CRISPR-Cas machinery and host factors (Deltcheva et al., 2011). Finally, during the last but crucial interference step, crRNAs will guide Cas nucleases to recognize and cleave matching invading DNA sequences (Barrangou et al., 2007; Brouns et al., 2008). The sequence complementarity between the crRNA and the foreign element leads to a sequence-specific immunity (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005; Barrangou et al., 2007; Gameau et al., 2010).

The significant diversity in the *cas* gene composition associated with the CRISPR arrays has led to a classification scheme that has identified so far two major classes, six types (I-VI) and several sub-types of CRISPR-Cas systems (Makarova et al., 2020). *S. thermophilus* strains can carry in their genome up to four different CRISPR-Cas systems, belonging to the type II-A (two), type I-E, and type III-A (Horvath et al., 2008; Horvath and Barrangou, 2010). Typically, *S. thermophilus* strains heavily depend on the CRISPR1-Cas and CRISPR3-Cas systems, which are both type II-A systems relying on Cas9 for interference, to defend against phages and to acquire new immunities (Horvath et al., 2008; Somerville et al., 2022).

- Q4 As phages and their host strains are in a perpetual arms race (Philippe et al., 2023), phages use a plethora of mechanisms to counteract bacterial defences, among which anti-CRISPR proteins (ACRs) have been found to block CRISPR-Cas activity in numerous bacterial genera (Bondy-Denomy et al., 2013). Over 90 ACR families have been described and this diversity is linked to their distinct modes of action as well as to the existence of many subtypes of CRISPR-Cas systems in bacteria and archaea (Bondy-Denomy et al., 2015; Pawluk et al., 2018). In addition, these viral ACRs proteins rapidly became of biotechnological interest as they could be exploited to modulate Cas9 cleavage activity in genome editing applications (Pawluk et al., 2016a). Since the discovery of AcrIIA1-A4 in various prophages, several ACR acting on type II-A CRISPR-Cas systems have been found in virulent phages and metagenomes (Hynes et al., 2017b; Rauch et al., 2017; Hynes et al., 2018; Eitzinger et al., 2020; Song et al., 2022).

- To date, two families of ACRs proteins have been found and characterised in virulent *S. thermophilus* phages (Hynes et al., 2017a; Hynes et al., 2017b; Hynes et al., 2018; Philippe et al., 2022). AcrIIA5 was found to be very effective in impeding Cas9 activity of CRISPR1 system, with also an activity against CRISPR3 system (Hynes et al., 2017a; Hynes et al., 2017b). It has also been demonstrated that AcrIIA5 can also hinder other Cas9 nucleases such as the one from *Streptococcus pyogenes* (Hynes et al., 2017a; Hynes et al., 2017b). AcrIIA6 is more specialized as it can only block the Cas9 of the CRISPR1 system of *S. thermophilus* (Hynes et al., 2018). More recently, nine new ACRs families (AcrIIA24-32) were identified in streptococcal mobile genetic elements (MGEs) and showed activity against both SpCas9 and *S. thermophilus* CRISPR3-Cas9 or against *S. thermophilus* CRISPR1-Cas9 only (Song et al., 2022).

Interestingly, an uncharacterized AcrIIA3 homolog was recently identified by metagenomic analyses in two streptococcal phages, RMK202_1 and RMK202_2, found in an undefined starter culture used to make Swiss hard cheeses (Somerville et al., 2021). AcrIIA3 was first described in *Listeria monocytogenes* prophages (Rauch et al., 2017) and a previous phylogenetic analysis showed that they were only found in phages infecting *Listeria* and *Streptococcus*, including *S. pyogenes*. AcrIIA3 activity against the CRISPR-Cas system of *L. monocytogenes* has been demonstrated but not against SpCas9 due to toxicity issues in *Escherichia coli* (Rauch et al., 2017).

- It seems to be an advantage for phages, particularly streptococcal virulent phages ~~found~~ found in a cheese environment, to possess an ACR as it allows them to coexist with *S. thermophilus* strains containing CRISPR spacers targeting them (Philippe et al., 2023). Here, we report the first characterisation of an AcrIIA3 from a *S. thermophilus* virulent phage and found that this protein, which defines an ACR family with a new α -helical fold, was effective in blocking the CRISPR3-Cas system of *S. thermophilus* as well as SpCas9.

2 Experimental procedures

2.1.2.1 Bioinformatics

Basic local alignment search tool was used to search for AcrIIA3 homologs in the non-redundant protein database. Multi sequence protein alignment was implemented with the R packages *msa* (Bodenhofer et al., 2015) and *ggmsa* (Zhou et al., 2022). The corresponding nucleotide phylogeny was implemented with a neighbour joining algorithm from the *ape* package (Paradis et al., 2004).

2.2.2.2 Bacterial strains and growth conditions

All bacterial strains, phages, and plasmids used in this study are listed in Supplementary Table S1. *S. thermophilus* strains were cultured in M17 broth (Oxoid) supplemented with 0.5% (wt/vol) lactose (LM17). Cultures were incubated at 37°C or 42°C without shaking, depending on whether they were overnight or same day cultures, respectively. For the experiments using pTRKL2, 5 µg/ml erythromycin was added to the medium. *Lactococcus lactis* MG1363 and derivatives were grown in M17 broth supplemented with 0.5% (wt/vol) glucose (GM17) and incubated at 30°C, without shaking. For the experiments using pL2Cas9 and pNT1, 5 µg/ml erythromycin and 5 µg/ml chloramphenicol were added to the medium. For all phage assays, 10 mM CaCl₂ were added to the medium.

2.3.2.3 Phage amplification

Scrapings of phage lysates stored at -80 °C with 15% glycerol were co-inoculated with the appropriate host strain and grown until complete lysis. The phage lysates were then filtered through a 0.45 µm polyethersulfone (PES) filter. One hundred microliters of these lysates were used to infect the same host strain grown to an OD_{600nm} between 0.1 and 0.2 at 42°C for *S. thermophilus* or 30°C for *L. lactis*. After complete cell lysis had occurred, these second phage lysates were also filtered (0.45 µm PES filter) and stored at 4°C until use.

2.4.2.4 Generation of a CRISPR3-immunised strain

The detailed protocol to generate a targeted phage-resistant strain of *S. thermophilus* is already available elsewhere (Hynes et al., 2016). Briefly, oligos were designed to match a protospacer found in the genome of the virulent streptococcal phage 2972, without ACR and in the genome of the virulent streptococcal phage D5691 carrying an AcrIIA3 (Supplementary Table S2). We also considered the presence of an appropriate PAM to guide the acquisition of the new spacer in the CRISPR3 locus of *S. thermophilus*. Annealed oligos were diluted with the digested pNZ123 to ligate them together. The resulting vector was then transformed into electrocompetent Gram-positive *L. lactis* cells (Holo and Nes, 1989). After confirmation of the presence of the desired insert by colony PCR and sequencing, the plasmid was then electroporated into *S. thermophilus* DGCC7710 cells, that were made competent through a glycine shock-based protocol (Hynes, Lemay et al., 2017 (Hynes et al., 2017a)). The presence of the plasmid was again confirmed by colony PCR and sequencing. A transformant was grown overnight in LM17 medium with 5 µg/ml chloramphenicol at 37°C, and 100 µl of this culture was used to inoculate 10 ml of fresh LM17 broth without any selective pressure. Phage lysate was then freshly diluted in phage buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM MgSO₄) to obtain a multiplicity of infection (MOI) of 5. One hundred microliters of the diluted phage lysate were mixed with 300 µl of the *S. thermophilus* culture grown to an OD_{600nm} of 0.6, supplemented with 10 mM CaCl₂, and incubated for 5 min at 42°C. The phage-infected culture was then added to 3 ml of molten 0.75% agar medium at 55°C supplemented with 10 mM CaCl₂ and poured onto a plate of LM17 containing 1% agar and 10 mM CaCl₂. Plates were incubated overnight at 42°C. Surviving colonies were then screened for presence of the desired spacer in the CRISPR3 array by colony PCR using a spacer-specific primer (Supplementary Table S2).

2.5.2.5 Cloning Acr

A gBlock™ (Integrated DNA Technologies), corresponding to the gene coding for the AcrIIA3 found in *S. thermophilus* phage CHPC640 (AcrIIA3_{CHPC640}), with nearly 60 additional base pairs upstream and downstream, was ordered. The gene coding for the AcrIIA5 of virulent streptococcal phage D1126 was also PCR-amplified to serve as a positive control for anti-CRISPR activity. We added 30-nt extensions overlapping the pTRKL2 cloning site to facilitate Gibson assembly (Supplementary Table S2). Plasmid pTRKL2 was digested with KpnI-HF (New England Biolabs) and the Gibson assemblies performed. The resulting Gibson reactions were then transformed into electrocompetent *L. lactis* cells, as described above. The plasmids were purified and after confirmation of the desired sequence, transformed into freshly prepared electrocompetent *S. thermophilus* cells. Finally, the sequence of the insert was reconfirmed by sequencing. Approximately the same process was followed for cloning the AcrIIA3_{Sp} allele, but the coding sequence was inserted into the SmaI cut site of the low-copy vector pNT1 (Supplementary Table S2).

2.6.2.6 Phage plaque assays

Five hundred microliters (standard plate) or 1 ml (square plate) of an overnight culture of the desired *S. thermophilus* strain were inoculated into 3 ml (standard plate) or 7 ml (square plate) of molten 0.75% agar medium kept at 55°C. The molten mix was then rapidly poured onto a plate of the same medium with 1% agar, containing the appropriate antibiotic. Plates were allowed to set and dry. Phage lysates were serially diluted in phage buffer and 5 µl of each

dilution were spotted onto the dry overlay. When the phage spots were dry, plates were incubated at 42°C overnight. Plaques were counted at the lowest dilution at which they were visible. Lactococcal phage p2 lysate was also serially diluted in phage buffer. Three hundred microliters of an overnight culture of the desired *L. lactis* strain and 100 µl of diluted phages were co-inoculated into 3 ml of molten 0.75% agar medium at 55°C. The molten mix was rapidly poured onto a plate of the same medium with 1% agar, containing the appropriate antibiotics. The plates were then incubated overnight at 30°C, and plaques counted from plates with between 30 and 300 plaques.

2.7.2.7 Protein structure prediction and analysis

We used a Github notebook (<https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb#scrollTo=XUo6foMQxwS2>) to perform structure predictions. Predicted protein structures were submitted to the Dali server to identify the closest structural homologs in the PDB (Holm, 2020). Structure analyses and visual representations were performed using ChimeraX (Pettersen et al., 2021).

2.8.2.8 Cell culture, transfection, and genotyping

K562 cell line was obtained from the ATCC (CCL-243) and maintained at 37°C under 5% CO₂ in RPMI medium supplemented with 10% FBS, 1% penicillin-streptomycin (Gibco™), and 1% GlutaMAX (Gibco™). K562 cells (2 × 10⁵ cells/transfection) were transfected with an Amaxa 4D-nucleofector™ (Lonza) using the SF nucleofection kit (program FF-120) per manufacturer's recommendations. Genomic DNA was extracted with QuickExtract DNA extraction solution (EpiCentre) following manufacturer's recommendations. Sanger sequencing was performed on PCR amplicons to quantify the percentage of edited alleles using TIDE (Brinkman et al., 2014). Primers used are provided in Supplementary Table S2.

2.9.2.9 Genome editing vectors

SpCas9 and associated sgRNAs were expressed from pSpCas9(BB)-2A-GFP (PX458) (Addgene plasmid #48138) (Ran et al., 2013), a gift from Feng Zhang (Massachusetts Institute of Technology, Cambridge, MA 02139, USA). Spacer (guide) sequences are provided in Supplementary Table 2. Two genes were targeted for this experiment, *EMXI* and *RUNXI*. Untagged AcrIIA5 and AcrIIA6 were expressed from pVAX_CBI_AcrIIA5_D1126 (Addgene plasmid #136663) and pVAX_CBI_AcrIIA6_D1024 (Addgene plasmid #136666) (Hynes et al., 2018). The AcrIIA3 ORFs from *L. monocytogenes* (Lmo) (Rauch et al., 2017) and *S. pyogenes* (Sp) (Rauch et al., 2017) prophages, and *S. thermophilus* (St) phage CHPC640 were codon-optimized for expression in human cells using the GenSmart™ codon optimization tool, synthesized as gBlocks™, and cloned into the pVAX_CBI_AcrIIA5_D1126 (Addgene plasmid #136663) backbone using NotI and XhoI. Codon-optimized AcrIIA3 sequences are provided in Supplementary Table S3.

3.3 Results

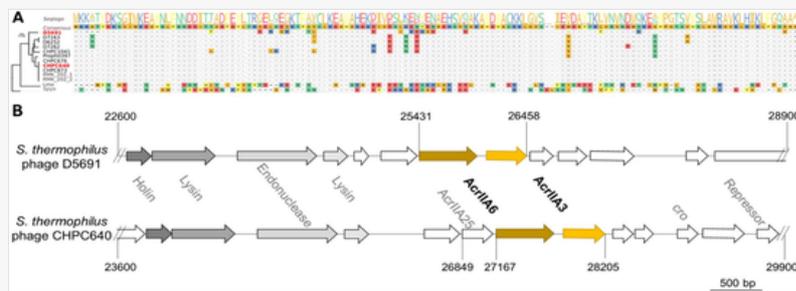
3.3.1 AcrIIA3, an ACR rarely found in phages infecting *S. thermophilus*

From the AcrIIA3 identified in phages RMK202_1 and RMK202_2, we first searched for additional AcrIIA3s homologs in the genomes of *S. thermophilus* phages available in GenBank and in an industrial database. We found AcrIIA3 homologs in 11 out of 278 phage genomes (Fig. 1A). An AcrIIA3 homolog was also found in a prophage (proph0397, Fig. 1A). Regarding the viral classification, six *S. thermophilus* phages carrying an AcrIIA3 belonged to the *Moineauvirus* genera (*cos*-group), three belonged to the *Brüssowvirus* genera (*pac* group) and the last one belonged to the 987 group. Thus, as of now, the prevalence of AcrIIA3 among the *S. thermophilus* phage population is 4%. In comparison, the prevalence of AcrIIA5 is 5% and the prevalence of AcrIIA6 is 32%, specifically 14 phages carrying an AcrIIA5 homolog and 90 phages carrying an AcrIIA6 homolog out of 278 phage genomes (Hynes et al., 2018).

 Images are optimised for fast web viewing. Click on the image to view the original version.

alt-text: Fig. 1

 Fig. 1 Fig. 1



AcrIIA3 in *S. thermophilus* phages. (A) Protein sequence of the AcrIIA3 alleles found in *S. thermophilus* phages and prophages. They have been compared to those initially described in *L. monocytogenes* (Lmo) and *S. pyogenes* (Spyo) prophages. (B) Genomic context of the genes encoding Acrs in *S. thermophilus* phages D5691 and CHPC640. Gene function is annotated when known. Genes with the same predicted function in both phage genomes are displayed with the same color. The relative position of the Acrs cluster in both phage genomes is indicated above and below the figure.

Among the 11 phages in which an AcrIIA3 homolog was found, we identified six alleles (Fig. 1A). The size of these AcrIIA3 ranged from 128 amino acids in streptococcal phage D5691 to 131 or 132 amino acids in the others. This size range is similar to what was previously observed in AcrIIA3 from prophages of *L. monocytogenes* (Lmo) and *S. pyogenes* (Spyo), whose sizes were 125 and 124 amino acids, respectively (Fig. 1A). Overall, the AcrIIA3 alleles found in *S. thermophilus* phages are similar and differ by only a few amino acids, with the AcrIIA3 from the virulent phage D5691 being the most distant (Fig. 1A).

Of note, *S. thermophilus* phages possessing an AcrIIA3 were also found to carry an AcrIIA6, the latter blocking only the CRISPR1-Cas system. Of interest, an AcrIIA25 homolog was also predicted in eight out of the eleven phages. The genes coding for the AcrIIA6 and AcrIIA25 were usually located immediately upstream or downstream of the gene coding for the newly identified AcrIIA3, perhaps clustering in an “anti-defence island” (Fig. 1B).

3.2.3.2 AcrIIA3 inhibits the CRISPR3-Cas system of *S. thermophilus*

As mentioned above, AcrIIA3 have been previously described in *L. monocytogenes* and *S. pyogenes* prophages, and the type II-A CRISPR-Cas systems found in these bacteria are closer to the CRISPR3-Cas system of *S. thermophilus* (Supplementary Table S4; Hynes et al., 2017a; Hynes et al., 2017b; Rauch et al., 2017). In fact, it has already been demonstrated that an ACR inhibiting SpCas9 can also block the CRISPR3-Cas system of *S. thermophilus* but not the CRISPR1-Cas system (Song et al., 2022). Thus, it was tempting to speculate that the AcrIIA3s from streptococcal virulent phages were active against the *S. thermophilus* CRISPR3-Cas system.

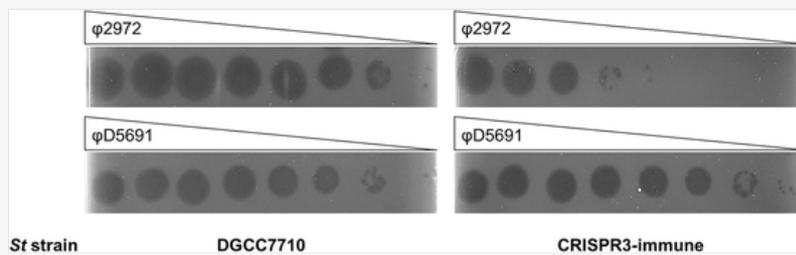
To test this hypothesis, we first generated a CRISPR3-immunised (phage-resistant) strain possessing a new spacer in its CRISPR3 array targeting a region of the virulent phage D5691. We selected the virulent phage D5691 among the *S. thermophilus* phages carrying an AcrIIA3 because it infects the *S. thermophilus* strain DGCC7710, which is a well-characterised strain used worldwide for yogurt production (Hynes et al., 2017a; Hynes et al., 2017b; Hynes et al., 2018; Philippe et al., 2022). This phage also carries a functional AcrIIA6 immediately upstream of AcrIIA3 (Fig. 1B). We selected a spacer targeting the gene coding for the receptor binding protein (RBP) because this region shares homology with that of the virulent phage 2972, which also infects the strain DGCC7710, but has no known AcrIIA.

Then, we determined the phage sensitivity profile of the wild-type strain DGCC7710 and its CRISPR3-immune derivative against phages 2972 and D5691 (Fig. 2). As expected, the wild-type strain was highly sensitive to both phages, with titres estimated at 10^9 pfu/ml for both phages 2972 and D5691. Also as expected, the RBP-targeting spacer added in the CRISPR3-immune strain conferred resistance to phage 2972, with a 4- \log_{10} reduction in the phage titre, compared to the wild-type strain. On the other hand, the CRISPR3-immune strain was still sensitive to phage D5691, strongly suggesting that this phage carries an ACR against the CRISPR3-Cas system of *S. thermophilus* DGCC7710.

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Phage D5691 possesses an Acr blocking the *S. thermophilus* CRISPR3-Cas system. Phage plaque assays were performed with 10-fold dilutions of phage 2972 or phage D5691 (10^0 to 10^{-7} from left to right) to determine the sensitivity profile of the *S. thermophilus* wild-type strain DGCC7710 and the CRISPR3-immune strain derivative. We spotted 5 μ l of each phage dilution on the dry overlay. Representative images of at least three biological replicates are shown.

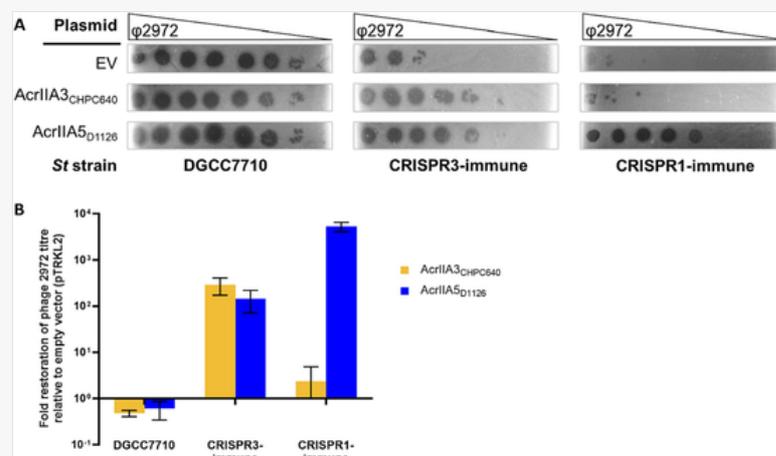
To confirm these results, the *acrIIA3* gene of phage D5691 was cloned into the high-copy plasmid pNZ123 to test its activity against the two *S. thermophilus* type II-A CRISPR-Cas systems. However, we could not obtain any clones, either in *E. coli* or in *L. lactis*, presumably due to the previously reported AcrIIA3 toxicity (Rauch et al., 2017). The *acrIIA3* gene of phage D5691 was then cloned into low-copy number vectors pNT1 and pTRKL2, but again, we faced toxicity issues. A gBlock™ corresponding to the AcrIIA3 allele found in *S. thermophilus* phage CHPC640 (Szymczak et al., 2019) was ordered as this allele is identical to the one discovered by metagenomic analysis in phages present in an undefined starter culture used to make hard cheeses (Somerville et al., 2021). AcrIIA3_{CHPC640} was successfully cloned into pTRKL2. The *acrIIA5* gene of phage D1126 was also cloned into pTRKL2, as this allele has been previously shown to be effective against both CRISPR1-Cas and CRISPR3-Cas systems of *S. thermophilus* (Hynes et al., 2017a; Hynes et al., 2017b).

Phage 2972 was tested on the wild-type strain and the CRISPR3-immunised strain carrying either the empty vector pTRKL2, the AcrIIA3_{CHPC640}-containing vector or the AcrIIA5_{D1126}-containing vector (Fig. 3A). As expected, the wild-type strains exhibited the same phage sensitivity profile, regardless of the plasmid carried. In the CRISPR3-immune strains, the presence of AcrIIA3 partially restores phage sensitivity, increasing the phage titre by 2 log₁₀, as does AcrIIA5. These results indicated that AcrIIA3_{CHPC640} is capable of blocking CRISPR3-Cas interference. AcrIIA3_{CHPC640} was also tested in a CRISPR1-immune strain (Hynes et al., 2016) and it had no effect, unlike AcrIIA5 which was able to restore phage sensitivity by approximately 4 log₁₀ (Fig. 3B). These results confirm that the streptococcal AcrIIA3 specifically blocks the CRISPR3-Cas system of *S. thermophilus*.

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alt-text: Fig. 3

Fig. 3 Fig. 3

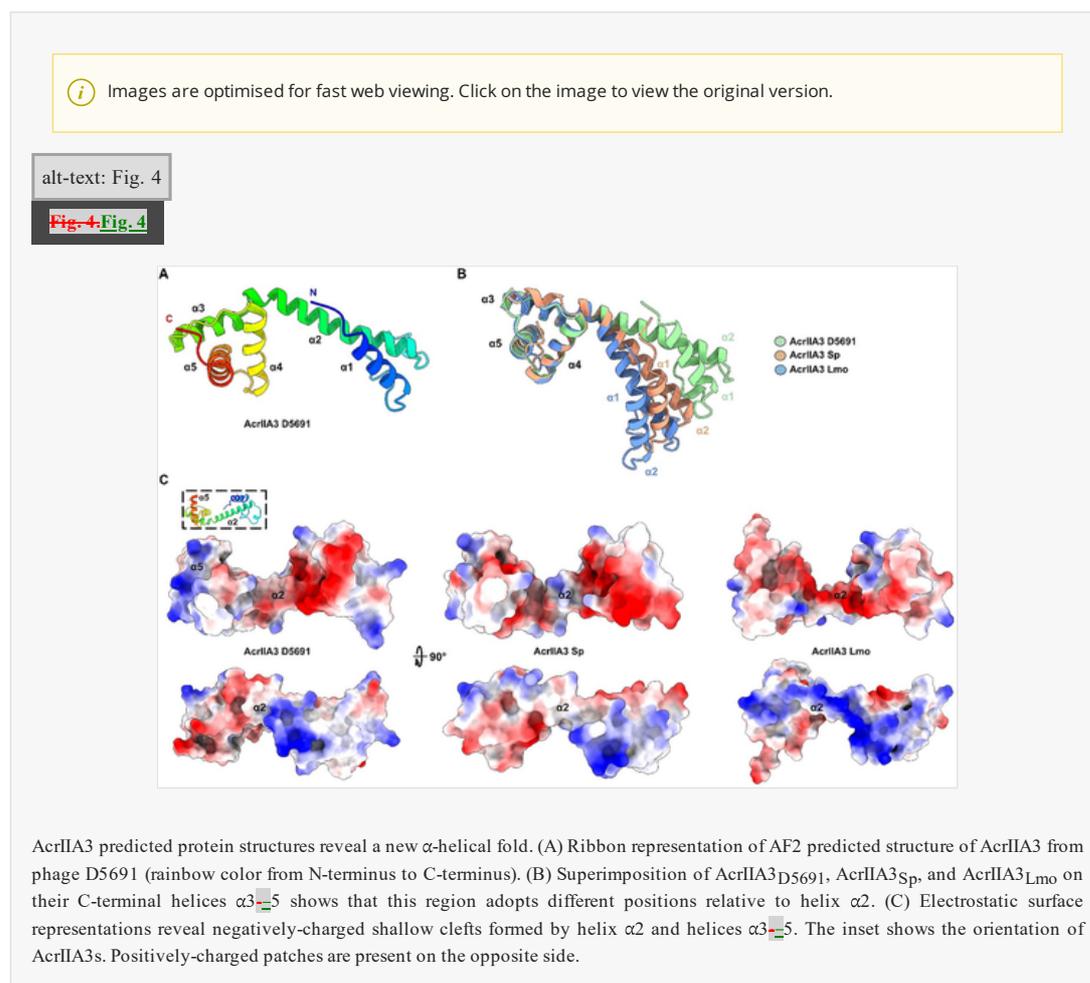


AcrIIA3 restores sensitivity to phage 2972 to a CRISPR3-immune strain. (A) Ten-fold dilutions of phage 2972 (10^0 to 10^{-7} from left to right) were spot on the phage sensitive strain *S. thermophilus* DGCC7710 and its CRISPR-immune derivatives, carrying either the empty vector pTRKL2 (EV) or the vector expressing AcrIIA3 (AcrIIA3_{CHPC640}) or AcrIIA5 (AcrIIA5_{D1126}). We spotted 5 μ l of each phage dilution on the dry overlay. Representative images of at least three biological replicates are shown. (B) Fold restoration of phage 2972 titre on strains carrying an Acr, not immunized/immunised or CRISPR-immunized/immunised, over ones carrying only the empty vector. Error bars show the mean \pm SD (n = 3 biological replicates).

3.3.3.3 AcrIIA3 reveals a new α -helical fold

As AcrIIA3 does not share significant sequence similarities with proteins of known function and [with the aim aimed](#) to gain insight into its mode of action, we predicted the 3D structure of AcrIIA3_{D5691}, as representative of the AcrIIA3 homologs found in *S. thermophilus* phages using the machine-learning AlphaFold2 (AF2) protein structure prediction algorithm ([Jumper et al., 2021](#)). It has been acknowledged that AF2-predicted structures can be used equally well as experimental structures when carefully considering confidence metrics ([Akdel et al., 2022](#); [Goulet and Cambillau, 2022](#)). We also used AlphaFold2 to predict the structure of AcrIIA3_{Sp}, and AcrIIA3_{Lmo} for comparison purposes.

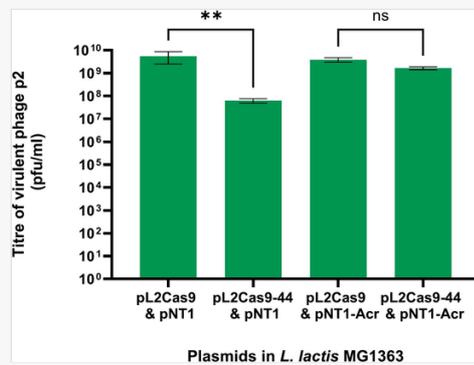
The three AcrIIA3 structures, predicted with high confidence scores (Supplementary Fig. S2A), contain five α -helices assembled into an elongated conformation in which helix α 1 and helices α 3–5 flank both ends of the longest helix α 2 (Fig. 4A and B). The search for structural homologs using the Dali server ([Holm, 2020](#)) returned non-significant hits indicating that AcrIIA3 has a new α -helical fold (Supplementary Fig. S2C). Although these structures are overall similar, variations are observed in the position of helices α 3–5 relative to helix α 2 (Fig. 4B). The confidence of predicted structural packing is high (Supplementary Fig. S2B) suggesting that this conformational variability could reflect a molecular adaptation of AcrIIA3 proteins to Cas9 homologs. Interestingly, in the three AcrIIA3 structures, a negatively charged shallow cleft is formed by the α 3–5 helix-bundle and helix α 2, while the opposite side is mostly positively charged (Fig. 4C). These structural features are likely key determinants of AcrIIA3 molecular mechanism that remains to be determined.



3.4.3.4 AcrIIA3 blocks SpCas9 in *Lactococcus lactis*

Previously, we showed that Cas9 from *Streptococcus pyogenes* and a gRNA cloned into a single plasmid can provide phage resistance to another lactic acid bacteria namely *L. lactis* ([Lemay et al., 2017](#)). A *L. lactis* strain carrying SpCas9 and a gRNA targeting the *orf44* of the virulent lactococcal phage p2 (pL2Cas9-44) ([Hynes et al., 2017a](#); [Hynes et al., 2017b](#)) provided a 2-log₁₀ reduction of the phage titre compared to a strain carrying the non-targeting plasmid (pL2Cas9) (Fig. 5). Thus, we tested the activity of AcrIIA3 against SpCas9. Also, AcrIIA3 homologs have been found previously in *S. pyogenes* prophages ([Rauch et al., 2017](#)).





The AcrIIA3 allele of *S. pyogenes* prophage restores sensitivity to phage p2 in *L. lactis*. Determination of the titre of the virulent phage p2 on its host *L. lactis* MG1363 carrying either pL2Cas9 or pL2Cas9 targeting the phage (pL2Cas9-44) and either the empty vector pNT1 or the vector expressing AcrIIA3_{Sp} (pNT1-Acr). Error bars show the mean \pm SD (n = 3 biological replicates) and statistical difference was calculated by one-way ANOVA with Bonferroni's Multiple Comparisons Test (** $p < 0.01$, ns: $p > 0.05$).

The gene coding for AcrIIA3_{Sp} was successfully cloned into the low-copy plasmid pNT1 and introduced into a *L. lactis* strain already containing another plasmid carrying SpCas9 and a phage targeting spacer (pL2Cas9-44). The titre of phage p2 on the strain carrying SpCas9-44 and AcrIIA3 was almost the same as the one on the wild-type strain. These results demonstrate that AcrIIA3_{Sp} can block SpCas9 activity in this heterologous system.

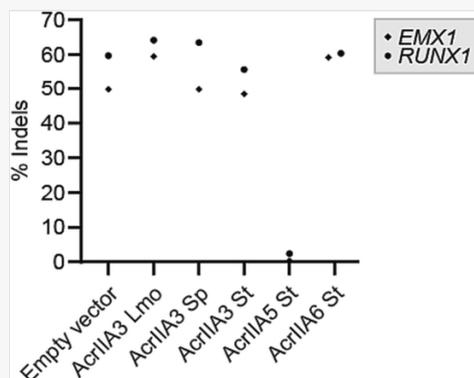
3.5.3.5 AcrIIA3 does not block SpCas9 in human cells

As SpCas9 is also widely used in various genome editing applications, we evaluated the activity of three viral AcrIIA3 (from *S. thermophilus*, *S. pyogenes* and *L. monocytogenes*) in human cells. Following transient overexpression of SpCas9 and ACRs, we observed near complete inhibition of SpCas9 nuclease activity with AcrIIA5, as determined by TIDE (Brinkman et al., 2014) analysis from Sanger sequencing (Fig. 6 and Supplementary Fig. S12). In agreement with our previous findings, AcrIIA6 did not inhibit SpCas9 activity (Hynes et al., 2018; Fuchsbaauer et al., 2019). No inhibition of SpCas9 nuclease activity was observed with all three AcrIIA3 proteins (Fig. 6 and Supplementary Fig. S12), corroborating previous findings based on transient overexpression of SpCas9 and an AcrIIA3 homolog from a *S. pyogenes* prophage in human HEK293T cells (Rauch et al., 2017).

Images are optimised for fast web viewing. Click on the image to view the original version.

alt-text: Fig. 6

Fig. 6, Fig. 6



Anti-CRISPR activity of AcrIIAs against SpCas9 in human cells. Small indels quantification as determined by TIDE from Sanger sequencing. K562 cells were transfected with 500 ng of Cas9 nuclease expression vector and 500 ng of human codon-optimized anti-CRISPR expression vector, and genomic DNA was harvested three days post-transfection. An empty pVAX vector was used as a control to normalize DNA concentrations. n = 2 independent biological replicates performed at different times give equivalent results (see Supplementary Fig. S12). Lmo, *Listeria monocytogenes*. Sp, *Streptococcus pyogenes*. St, *Streptococcus thermophilus*.

4.4 Discussion

The Gram-positive lactic acid bacterium *S. thermophilus* possesses highly efficient type II-A CRISPR-Cas systems that protect the cells against phage infections during the milk fermentation process. On the other hand, some streptococcal phages have evolved to replicate in these CRISPR+ strains by acquiring genes that code for ACR proteins to bypass CRISPR-Cas systems, particularly by blocking Cas9 activities. A knowledge of the Acr arsenal found in these phages is essential for the dairy industry to choose the most suitable strains. While streptococcal phages have been shown

previously to code for AcrIIA5 or AcrIIA6, it was only recently that AcrIIA3 was identified in a few *S. thermophilus* phages. Due to the limited AcrIIA3 distribution in known streptococcal phages, and the fact that phages rarely maintain expendable genes, we set out to explore the activity of AcrIIA3 proteins.

A set of 11 *S. thermophilus* phages (out of 278 genomes) was found to carry an AcrIIA3. The presence of nucleotide polymorphisms led to the identification of six *acrIIA3* alleles in these dairy phages, but these polymorphisms did not result in major changes in the predicted putative domains. The number of different AcrIIA3 alleles was not surprising as it was also previously observed for AcrIIA5 and AcrIIA6 (Hynes et al., 2018). For AcrIIA5, 11 different alleles have been identified out of 14 streptococcal phages while 48 alleles were found out of 90 phages for AcrIIA6. It has been argued that this ACR diversity results from an adaptation to the host Cas9, which also exhibits some diversity (Fuchsbaauer et al., 2019).

Interestingly, AcrIIA3 is usually associated with other Acrs like AcrIIA6 and AcrIIA25 in *S. thermophilus* phages. Similarly, AcrIIA3 has been associated with AcrIIA1 and AcrIIA2 in *L. monocytogenes* prophages (Rauch et al., 2017). Such ACR clustering may be explained by their activity spectrum. AcrIIA3 and AcrIIA25 were found effective against the type II-A CRISPR3 system, AcrIIA6 was effective against the type II-A CRISPR1 system of *S. thermophilus*. As *S. thermophilus* strains can encode different CRISPR-Cas systems, carrying specialized Acrs allow virulent phages to counteract these bacterial defence systems, allowing viral replication and persistence in the milk fermentation environments.

Another reason might be the presence of a strong regulation of the AcrIIA3 expression. It has been shown previously that *acr* genes are often associated with Acr-associated (*aca*) genes, which act as regulators (Pawluk et al., 2016a; Pawluk et al., 2016b). Phage *acr* genes are usually transcribed early during the phage infection process, which allows rapid accumulation of ACR, but also often leads to cell toxicity, which could ultimately also hinder phage replication. To minimize this deleterious effect, *aca* genes bind to the *acr* promoter and regulate its expression (Birkholz et al., 2019; Stanley et al., 2019; Osuna et al., 2020). A similar regulatory mechanism might exist for AcrIIA3 as we have encountered several toxicity issues when cloning the *acrIIA3* gene alone without any regulator. This genomic environment could also explain the strong anti-CRISPR activity observed with phage D5691 naturally carrying an AcrIIA3 (Fig. 2) compared to weaker anti-CRISPR activity observed when the *acrIIA3* gene was cloned alone into the, albeit low-copy, vector pTRKL2 (Fig. 3). Moreover, these results have been obtained with two distinct alleles of the *acrIIA3* gene, which could also potentially lead to variations in the observed anti-CRISPR activity.

We have also demonstrated that AcrIIA3 can block SpCas9-mediated interference in *L. lactis*, which is consistent with the relative closeness between SpCas9 and *S. thermophilus* CRISPR3-Cas9 (St3Cas9). But no visible ACR effect was noticed when tested in human cells. Similar results have already been observed, with ACRs having less efficiency when tested in human cells than *in vitro* (Hynes et al., 2018; Song et al., 2022). It would have been interesting to test AcrIIA3 activity against the St3Cas9 nuclease in human cells, but we failed to observe high levels of editing activity with our expression systems (data not shown).

Lastly, the predicted structure of AcrIIA3s revealed a new fold, but did not bring any insight into AcrIIA3 molecular mechanism. Further biochemical and structural work is therefore required to determine if this phage ACR family uses a novel structure to achieve previously observed CRISPR-Cas9 inhibition mechanisms such as occluding the DNA-binding site or the nuclease active site, perturbing Cas9 functional dynamics, and degrading the guide RNA (Pawluk et al., 2018; Fuchsbaauer et al., 2019; Jia and Patel, 2021; Wang et al., 2022), or if it uses an unprecedented mode of action to inactivate CRISPR-Cas system.

In conclusion, we have uncovered a third family of functional ACR with a new fold in virulent streptococcal phages. AcrIIA3 was found to block the interference step of the CRISPR3-Cas system of *S. thermophilus*. With the emergence of ACR-carrying phages in dairy factories, additional phage control strategies are needed for *S. thermophilus*.

CRedit Author Contribution statement

Adeline Pastuszka: Conceptualization, Formal analysis, Investigation, Writing – original draft. **Geneviève M. Rousseau:** Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Vincent Somerville, Sébastien Levesque, Jean-Philippe Fiset, Adeline Goulet, Yannick Doyon:** Formal analysis, Investigation, Writing. **Sylvain Moineau:** Conceptualization, Formal analysis, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank IFF for access to their streptococcal phage genome database and for phage D5691. We acknowledge ChimeraX for molecular graphics developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from [National Institutes of Health](#) and the Office of Cyber Infrastructure and Computational Biology, [National Institute of Allergy and Infectious Diseases](#). S.M. acknowledges funding by the [Natural Sciences and Engineering Research Council of Canada](#) (Discovery program). S.M. holds a T1 Canada Research Chair in Bacteriophages. [This study was supported by a grant from the Natural Sciences and Engineering Research Council of Canada \(RGPIN-2014-059680\) to Y.D. V.S. acknowledges Postdoc.Mobility funding by the Swiss National Science Foundation \(P500PB_214419\).](#)

~~Appendix A~~ Appendix A Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2023.110414>.

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 The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

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Highlights

- Phages carrying AcrIIA3 can bypass the CRISPR3-Cas system of *Streptococcus thermophilus*.
 - AcrIIA3 blocks the interference step of *S. thermophilus* CRISPR3 system.
 - AcrIIA3 has a new α -helical fold.
 - AcrIIA3 was difficult to study due to its toxicity.
-

~~Appendix A~~ [Appendix A](#) Supplementary data

 [Multimedia Component 1](#)

Supplementary material

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Queries and Answers

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