Impact of Xenogeneic Silencing on Phage–Host Interactions

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Abstract

Phages, viruses that prey on bacteria, are the most abundant and diverse inhabitants of the Earth. Temperate bacteriophages can integrate into the host genome and, as so-called prophages, maintain a long-term association with their host. The close relationship between host and virus has significantly shaped microbial evolution and phage elements may benefit their host by providing new functions. Nevertheless, the strong activity of phage promoters and potentially toxic gene products may impose a severe fitness burden and must be tightly controlled. In this context, xenogeneic silencing (XS) proteins, which can recognize foreign DNA elements, play an important role in the acquisition of novel genetic information and facilitate the evolution of regulatory networks. Currently known XS proteins fall into four classes (H-NS, MvaT, Rok and Lsr2) and have been shown to follow a similar mode of action by binding to AT-rich DNA and forming an oligomeric nucleoprotein complex that silences gene expression. In this review, we focus on the role of XS proteins in phage–host interactions by highlighting the important function of XS proteins in maintaining the lysogenic state and by providing examples of how phages fight back by encoding inhibitory proteins that disrupt XS functions in the host. Sequence analysis of available phage genomes revealed the presence of genes encoding Lsr2-type proteins in the genomes of phages infecting Actinobacteria. These data provide an interesting perspective for future studies to elucidate the impact of phage-encoded XS homologs on the phage life cycle and phage–host interactions.

> "*You have a grand gift for silence, Watson. It makes you quite invaluable as a companion.*"
> [(Sherlock Holmes)]

Introduction

Phages, viruses that prey on bacteria, represent the most abundant biological entities on this planet and are a major driver of horizontal gene transfer (HGT). Phages are not only present as infectious particles in the environment but are also found as integrated elements (prophages) within the genomes of their bacterial hosts. In some cases, DNA of viral origin accounts for up to 20% of an organism's entire genome [1–3]. Some of this DNA originates from fully functional prophages, which are capable of undergoing a lytic life cycle. However, a considerable part is made up of prophage-like elements, including phage remnants left after incomplete excision events, cryptic (degenerated) prophages or other genetic material acquired by HGT. In fact, this genetic material has significantly shaped microbial evolution due to the development of mutually beneficial interactions between prophage and host [4]. Nevertheless, the safe integration of viral elements into bacterial genomes demands stringent regulation of phage gene expression.

Upon integration into the host genome, a functional phage can exit the prophage state and enter the lytic cycle, which is typically triggered by severe DNA damage that activates the cellular SOS response. Even under non-inducing conditions, cells may encounter spontaneous DNA damage [5], leading

to the SOS-dependent induction of prophages in a small fraction of the lysogenic population [6,7]. Originally, this spontaneous prophage induction (SPI) was considered a potentially detrimental process, but recent research in the fields of microbial biofilms, host–pathogen interaction and population dynamics emphasizes that SPI is an important contributor to the social behavior of microbes [6]. In a recent study, we quantified SPI in populations of *Corynebacterium glutamicum* using reporter promoter fusions. While we observed a positive correlation between SPI and the DNA damage (SOS) response, a significant fraction of the cases also occurred in an SOS-independent manner [8,9]. Thus, the molecular factors influencing SPI in single individuals and how the host modulates its frequency remain largely unknown.

Compared to point mutations or genomic rearrangements, HGT allows bacteria to acquire new traits much more rapidly, but the downside of this medal is that the new information is encoded on foreign genetic material [10,11]. The encounter with xenogeneic (foreign) DNA is a "high risk–high gain" situation: While the acquisition provides the potential for the fast gain of new beneficial traits, the activity of selfish genetic elements or bacterial viruses (bacteriophages) represents a perpetual threat to bacterial cells. Gene expression from xenogeneic material can strongly impair cellular fitness by sequestering RNA polymerase [12,13], by producing toxic proteins and, in the case of phages, by causing cell lysis. With more than 10^{24} productive viral infections on earth [14], the activity of bacteriophages plays a vital role in HGT, which is also reflected by the variety of phage defense mechanisms encoded in bacterial genomes, with restriction modification (RM) systems and CRISPR–Cas being among the most prominent mechanisms [15]. With the expansion of the phage genomic space, many more examples of phage defense systems have been described and have been covered in a number of recent reviews [16,17]. In contrast to the destructive mode of action of RM and CRISPR–Cas systems, where nucleases are employed to wipe out incoming foreign DNA [18], xenogeneic silencing (XS) represents a mechanism promoting tolerance of foreign genetic material [19,20]. The mechanism of XS is based on the activity of small, nucleoid-associated proteins (NAPs) that recognize and bind foreign, AT-rich DNA stretches and silence gene expression due to the formation of a tight nucleoprotein complex [19] (Fig. 1). By this means, XS proteins provide an important basis for the safe acquisition of new genetic material and foster evolutionary network expansion [21]. Hitherto, all known XS proteins fall into one of four different classes: H-NS in Proteobacteria [19,22], MvaT/U in *Pseudomonas* species [23], Rok in *Bacillus subtilis* [24] and Lsr2 in Actinobacteria [25]. Despite the low sequence similarity between different silencers, these proteins appear to fulfill very similar functions in their respective host,

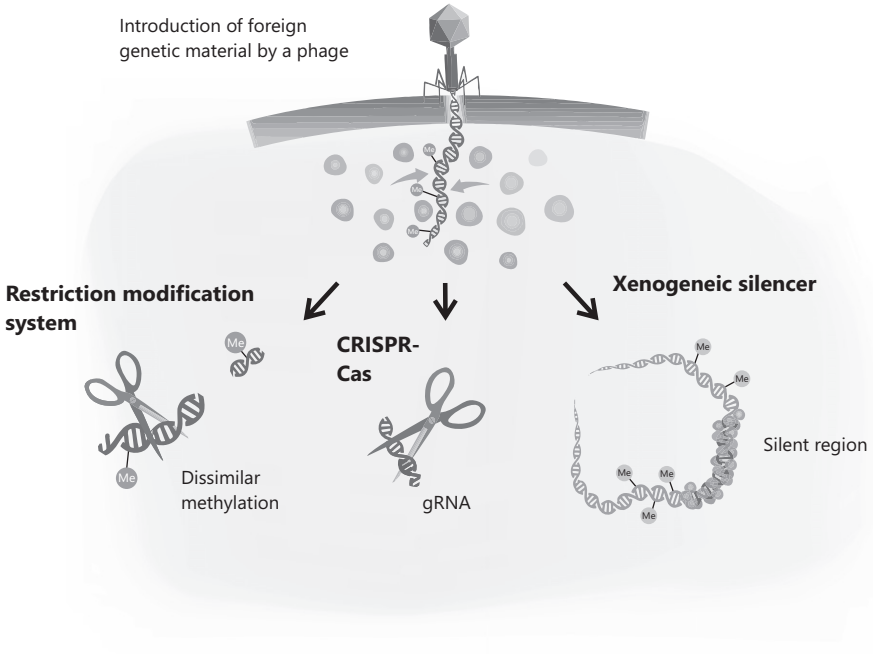

Fig. 1. Xenogeneic silencing of foreign DNA. Microbial cells have evolved a variety of different defense mechanisms to deal with viral DNA and to counteract potential detrimental effects. Schematically included examples are the RM systems and CRISPR–Cas. In contrast, XS proteins are able to recognize foreign, AT-rich DNA and form an oligomeric nucleoprotein complex that silences gene expression at the particular target regions [19].

and examples of cross-complementation have been found for Lsr2, MvaT and H-NS [9,23,25].

Several recent studies focused, in particular, on the impact of H-NS on bacterial genome evolution and network expansion (for recent reviews, see Refs. [19,20,26]). Given the mosaic-like structure of bacterial genomes, (pro-)phages apparently account for a significant fraction of bacterial strain diversification [1]. In a recent study, we provided the first example of a prophage-encoded Lsr2-like protein functioning as an essential silencer of a cryptic prophage in the actinobacterium *Corynebacterium glutamicum*. Considering the generic role of XS proteins in the silencing of foreign DNA, it is reasonable to assume that these proteins play an important role and may adopt different functions in phage–host regulatory interaction. In this review, we summarize the literature focusing on the role of XS proteins on phage–host interactions, including examples of phage-mediated counter-silencing as a defense strategy during infection. Furthermore, we provide a comprehensive bioinformatics analysis of bacterial and phage genomes revealing that genes encoding Lsr2-like proteins are ubiquitously found in the genomes of actinobacteriophages, suggesting an adoption of XS function in the lifestyles of virulent and temperate phages.

XS: Recognition and Binding to Foreign, AT-rich DNA

The basis for XS is provided by the domain organization of the XS proteins, which is remarkably similar among the different classes. Typically, these proteins exhibit a small size of $<$15 kDa and consist of an N-terminal oligomerization domain and a C-terminal DNA-binding domain [20,27].

All silencers have the common feature of preferentially binding DNA regions that are more AT rich than the host genome. This feature appears to be the basis for the targeting of foreign elements since the vast majority of exogenous DNA has been found to be more AT-rich than the host core genome [19,28,29].

High-resolution structural analysis of the C-termini of H-NS and Lsr2 resulted in the identification of the “prokaryotic AT-hook” with the “Q/RGR” motif, which is reminiscent of the “AT-hook” motif found in eukaryotic HMG-I(Y) proteins [30]. AT sequences lack the exocyclic 6-amino group, which consequently allows for a deeper interaction with protein side-chain residues. In addition, the narrower minor groove of AT-rich sequences harbors a surface with a higher electronegative potential (than mixed or GC-rich sequences), enabling a stronger interaction with positively charged residues. From the evolutionary point of view, it is astonishing that although H-NS and Lsr2 do not share any structural similarity, these proteins show the same binding mechanism. In contrast, MvaT/U proteins, found in *Pseudomonas* species, lack the AT-hook motif but instead recognize target DNA via a so-called “AT-pincer” motif consisting of a conserved lysine residue and a downstream KGGN motif interacting with the minor groove [31]. Recently, Duan *et al.* [27] elucidated how the *Bacillus* silencer Rok distinguishes between host and foreign DNA. The authors could show that Rok directly binds to the minor groove of AT-rich sequences in a novel mode that, so far, has not been described for any other winged helix protein. Using *in vitro* protein binding microarrays and comparative genome analysis, the authors concluded that Rok preferentially recognizes a few distinct AT-rich DNA motifs present in horizontally acquired regions, which are significantly underrepresented in *Bacillus* host genomes [27]. However, for the other XS proteins, no sequence-specific recognition has been observed. This characteristic has been, most intensively, studied for H-NS, where AT-rich regions were suggested to function as initial nucleation regions [32]. In a more recent study, a single-molecule counting approach revealed that nucleation sites are crucial for recruiting H-NS molecules [33].

XS: Formation of the Nucleoprotein Complexes

However, binding to DNA is itself not sufficient for XS proteins to fulfill their function. Protein multimerization is an essential step that is required to enable silencing of the target regions. Generally, it is assumed that after the initial nucleation, additional silencing molecules are recruited and concurrently spread along adjacent AT-rich regions (filament formation) [32]. Finally, the high-order oligomerization between distal silencer–DNA complexes leads to strong condensation and DNA compaction that enables silencing of target gene expression [19]. In the current literature, three main mechanisms for silencing are suggested: (i) promoter occlusion, where the filamentous nucleoprotein complex prevents the binding of the RNA polymerase [19,34], (ii) trapping of the RNA polymerase, blocking promoter escape upon binding [34] and (iii) ρ-dependent transcriptional termination that occurs during the pausing of RNA polymerase [35].

Multimerization of XS proteins is facilitated by the N-terminal domains. This effect has been shown, experimentally, for H-NS-, MvaT- and Lsr2-like proteins [9,36–38] and was suggested for Rok [24]. Primarily, the mechanism behind this protein oligomerization was widely studied using H-NS and high-resolution approaches, like atomic force microscopy, electron microscopy and single-molecule magnetic tweezers experiments [37,39,40]. Depending on the concentration of the divalent cations (Mg^{2+} or

Ca^{2+}), atomic force microscopy studies reported either a bridging or stiffened mode of the bound DNA stretches [40,41]. These two different modes were also found for MvaT and Lsr2 [36,38,42,43], underlining a common silencing mechanism among the silencers and explain why these XS proteins are partly able to complement each other in the above-mentioned cross-complementation experiments [9,25]. Nevertheless, the dissimilar AT compositions of host genomes and the differences in DNA binding underline the specialization of the particular XS protein to the requirements in the particular host background.

For H-NS- and MvaT-like silencers, it was shown that XS proteins may form silencing nucleoprotein complexes by interacting with other NAPs. Efficient protein oligomerization is, however, still a prerequisite for the establishment of a silencing nucleoprotein complex. In particular, the small proteins Hha and its paralog YdgT (Cnu) were shown to structurally resemble the N-terminal domain of H-NS and are therefore capable of forming heteromeric complexes [44]. Further studies based on transcriptomics revealed that the binding of Hha to H-NS polymers is required for the efficient repression of a subset of the H-NS regulon [45]. At this point, it is worthwhile to mention that Cnu represents an *oriC* binding protein that binds within a DnaA binding box and has been suggested to contribute to optimal *oriC* activity [46]. In *B. subtilis*, a regulative dependency between the replication initiation protein DnaA and the silencer Rok was recently reported [47], evincing an interesting link between bacterial replication and this XS protein.

In the genomes of *Escherichia coli* and *Salmonella* Typhimurium, paralogs of H-NS, such as StpA, are encoded and are capable of forming heterodimers that in turn can interact again with the aforementioned small proteins Hha/YdgT [44]. Recently, the impact of Hha and StpA on the binding mode of the H-NS protein was investigated by *in vitro* biochemical and biophysical experiments. With their high-resolution approach, Boudreau *et al*. [48], nicely demonstrated how StpA and Hha modify H-NS-polymeric filaments to increase transcriptional pausing and provided evidence showing that the mixed nucleoprotein complexes (consisting of Hha/H-NS or StpA/H-NS, etc.) differentially affect gene regulation. Thus, these experiments further suggest that interactions with paralogs or accessory proteins likely specify the silencing characteristics also depending on the local concentrations of the respective proteins. The formation of heteromeric silencer complexes was also reported for MvaT and its paralog MvaU in *Pseudomonas aeruginosa* [49]. In *Pseudomonas putida*, MvaT-like proteins were shown to control distinct regulons revealing a functional specialization of these proteins [50–52]. Similar scenarios are also conceivable for Lsr2-like proteins, since the genomes of several Actinobacteria, like *Streptomyces coelicolor* A(3), *Streptomyces venezuelae* ATCC 10712 and *Mycobacterium smegmatis* MC^2 155, encode more than one copy of Lsr2-like proteins (based on a BLAST search with the sequence of Lsr2 (Rv3597c) and default parameters, *e*-value $< 1 \times 1010^{-5}$).

The genome compaction of XS proteins resembles the compaction by heteromeric nucleoprotein complexes formed by histones and DNA in eukaryotic cells. Hence, it is reasonable to assume that XS proteins, or in general NAPs, are targets of posttranslational modification (PTM) enzymes. In a very recent review, this topic was elucidated by Dilweg and Dame [53]. Interestingly, the authors reported approximately 29 PTMs for H-NS from *E. coli*; these PTMs were not experimentally investigated, but the physiological implication for DNA condensation and/or silencing was discussed [53].

Silencing of Prophages in Bacteria

The safe integration of viral elements into bacterial genomes demands stringent regulation of expression from highly active phage promoters to avoid the production of potentially toxic proteins. The function of XS proteins thereby provides a basis for integrating foreign genes into host regulatory networks by XS and counter-silencing (the latter is discussed in the next section).

For silencers of all four groups, an influence on the regulation of phage genes has been observed (Table 1). However, this effect is often noted only incidentally in these studies [9,54,56,57]. Interestingly, the essentiality of a particular XS-encoding gene strongly depends on the genetic setup of the particular strain as the induction of mobile genetic elements (MGEs), such as prophages, may lead to cell death. In the case of the Gram-positive actinomycete *C. glutamicum*, the Lsr2-like protein CgpS (*C. glutamicum p*rophage *s*ilencer) was shown to be essential due to its function as a silencer of the cryptic but still inducible prophage CGP3 [9]. Counteracting of CgpS activity led to prophage induction and regional (in situ) replication at the CGP3 locus [58]. In contrast to other XS proteins described so far, CgpS is encoded on the CGP3 prophage itself and appears to act mainly as a silencer of CGP3 gene expression. Genome-wide profiling of CgpS confirmed the binding of this XS protein to AT-rich sequences in the CGP3 element. As a matter of fact, the essentiality of the *cgpS* gene is linked to the presence of CGP3, and *C. glutamicum* strains lacking the prophage do not require CgpS. Interestingly, integration of a second genomic copy of *cgpS* significantly reduced the fraction of spontaneously induced cells, emphasizing the role of XS proteins in the modulation of SPI in

Table 1. Silencing of phage elements in bacterial genomes

Type of silencer	Host strain	GC of host (%)	Prophage-like element	Length (kb) of phage	GC (%) of phage	Reference
H-NS	*E. coli* K-12 BW25113	50.8	Rac (cryptic)	23.1	47.1	[54]
	S. onediensis MR-1	45.9	CP4So (cryptic)	36	43	[55]
MvaT	*P. aeruginosa* PAO1	66.6	Filamentous phage Pf4	15.7	58.7	[56,57]
Rok	*B. subtilis* 168	43.5	Prophage region 4	8	35.8	[24]
			Prophage region 5	20.7	37.5	
			Prophage region 6	34.8	36.1	
			SPβ	134.4	34.6	
Lsr2	*C. glutamicum* ATCC 13032	53.8	Cryptic prophage CGP1	13.5	47.1	[9]
			Cryptic prophage CGP3	186.0	48.4	
	M. tuberculosis H37Rv	65.6	Prophage region 1, Rv1573-1588c, (Rv1582c)	10.5 (1.4[a])	66.2 (62.5[a])	[38]
			Prophage region 2, Rv2645-2664, (Rv2658-2659c)	12.3 (1.5[a])	66.2 (63.5[a])	

[a] In case of *M. tuberculosis*, also the bound genes were considered and are indicated in brackets.

bacterial populations (Frunzke and Pfeifer, unpublished). In addition, for *Mycobacterium tuberculosis*, which is also a member of the Actinobacteria, genome-wide binding studies conducted with a Lsr2-like protein not only confirmed the binding to prophage regions (and other MGEs) [9,38] but also revealed several additional targets in the host genome involved in virulence and immunogenicity [38]. Interestingly, in *B. subtilis*, the Rok protein was also shown to bind prophage genes and to be involved in the control of phage gene expression, as is the case for genes of the prophage SPβ [24,59]. A further interesting example involves *E. coli* H-NS, where a link between H-NS activity and enhanced biofilm formation was recently demonstrated. Here, H-NS was shown to repress the cryptic prophage Rac. Derepression of Rac resulting from *hns* deletion led to prophage induction and cell lysis in a toxin-dependent manner [54]. Moreover, in *Shewanella oneidensis*, H-NS was also reported to be involved in prophage induction during cold adaptation [55]. In addition, the H-NS orthologs MvaT and MvaU were shown to be essential due to the silencing of prophage elements in *P. aeruginosa* strains [56,57]. The depletion of silencers caused increased phage gene expression, the formation of infectious phage particles and cell lysis. Remarkably, only mutants impaired in phage production were capable of compensating the double deletion of *mvaT* and *mvaU* [56,57]. An overview of the studies showing the influence of XS proteins on the control of phage gene expression is provided in Table 1.

How to Overcome Silencing?

The formation of a nucleoprotein complex nucleating at AT-rich regions is a prerequisite for XS. Different studies focusing on counter-silencing mechanisms in various species have revealed that upon activation of gene expression, XS proteins are not released from their target DNA; instead, remodeling of the XS–DNA complex enables RNA polymerase to bind and activate transcription. Different counter-silencing mechanisms are mainly based on other proteins binding in the upstream promoter region, thereby counteracting XS silencing. For instance, this mechanism has been nicely demonstrated by synthetic counter-silencing approaches, where operator sequences of specific transcription factors (TFs) were inserted in the upstream promoter region to counter-silence gene expression upon binding of the particular TF [60]. Several further studies demonstrated that different host-encoded TFs have been coopted—in the course of evolution—to act as counter-silencers. Examples include the response regulator PhoP, an essential activator of *Salmonella* virulence [21], the AraC-family TF ToxT of *Vibrio cholerae* [61], LeuO from *Salmonella enterica* [62], and the two MarR-type regulators RovA and SlyA of *S. enterica* and *Yersinia pseudotuberculosis*, which were shown to antagonize H-NS-dependent silencing of horizontally acquired genes [63,64]. In a recent study, we also could show that the MarR-type regulator MalR of *C. glutamicum*, which controls genes involved in stress-responsive cell envelope remodeling, binds to several regions within the CGP3 prophage and is able to counteract SOS-dependent prophage induction (manuscript submitted, BIORXIV/2019/544056).

An alternative route for counter-silencing lies in the interference between XS proteins belonging to the same protein family. An interesting example has been provided for the unusual H-NS paralog Ler, which functions as a regulator of pathogenicity islands (locus of enterocyte effacement, LEE) in enteropathogenic (EPEC) and enterohemorrhagic *E. coli* strains [65–68]. Structural analysis emphasized that its function as a counter-silencer lies in differences in protein oligomerization as both H-NS and

Ler bind to AT-rich regions [69]. Ler shows two different modes of DNA interaction: At low concentrations, Ler is able to increase DNA folding and wraps DNA; otherwise, with increasing concentration, Ler binds DNA in an unwrapped mode where Ler increases the rigidity of DNA similarly to the nucleoprotein filament formed by H-NS [69]. At these high concentrations, Ler displaces H-NS from the bound DNA and therefore overcomes the silencing of target regions. A further interesting example is provided with H-NST, a truncated derivative of H-NS lacking the DNA-binding domain. This XS protein was found to antagonize H-NS in EPEC and uropathogenic *E. coli* by interfering with its oligomerization domain [70,71]. Remarkably, this principle of silencer interference can be harnessed to study the function of essential XS proteins. Overproduction of the N-terminal oligomerization domain of the Lsr2-type silencer CgpS was used to counteract CgpS silencing *in vivo* in the Actinobacterium *C. glutamicum* [9]. Interference between XS proteins was further demonstrated in this study as the expression of other mycobacterial Lsr2 genes as well as introduction of *E. coli* H-NS led to XS interference at AT-rich regions, resulting in prophage induction. These findings are also supported by a bioinformatics analysis showing that different classes of silencers do not occur in the same species [72].

Altogether, these examples provide important insights how silencing and counter-silencing facilitate the expansion of regulatory networks in bacteria [21]. In the following, we will focus on mechanisms employed by phages to counteract XS proteins in the ongoing arms race between the phage and the host. A few studies, discussed below, already suggest a variety of different mechanisms used by phages to gain control. One example is provided by the 5.5 protein of the *E. coli* phage T7, which is able to antagonize H-NS function upon phage infection [73]. By interfering with the central oligomerization domain of H-NS [37], the 5.5 protein blocks H-NS from forming high-order oligomers, leading to counter-silencing of H-NS-silenced genes [74]. Another example has been reported with the Mip protein (*M*vaT *i*nhibiting *p*rotein), encoded by the LUZ24 phage of *P. aeruginosa* [75]. In 2015, Wagemans *et al.* showed that Mip is able to inhibit the binding of the nucleoid-structuring silencer MvaT to DNA and that Mip and MvaT coprecipitate in pulldown assays. However, the exact mechanism of MvaT inhibition by Mip is not completely understood, yet.

In the case of *E. coli* T4 phage, two different proteins were reported to interfere with H-NS silencing. The protein MotB is a DNA-binding protein that co-purifies with H-NS as well as with the H-NS homolog StpA. Deletion of the *motB* gene led to a decreased burst size [76]. The T4 protein Arn represents an interesting example of a phage-encoded DNA mimic protein and was shown to directly interact with *E. coli* H-NS [77]. While Arn was originally described as an inhibitor of the McrBC restriction enzyme, structural analysis revealed that the shape of the protein mimics the shape and charge of double-stranded DNA, and the authors highlight this DNA mimicry as a mechanistic basis for interfering with the function of DNA-binding proteins, like H-NS [77]. Interestingly, the DNA mimic proteins Ocr of the phage T7 and ArdA of the plasmid CollB-P9 were also reported to antagonize H-NS in a similar way [78]. An overview on described counteracting proteins is provided by Table 2.

Finally, the direct interference with TFs or other proteins likely does not represent the only way to fight off XS. In a very recent study, Kronheim *et al.* [79] highlighted the important role of small molecules secreted by bacterial hosts as weapons against phage infection. A link between these compounds and XS proteins does not necessarily exist, but a few examples suggest that small natural compounds—other than proteins—may also counteract XS. One class of compounds is represented by polyamides containing a biaryl motif. These polyamides especially target the minor groove of AT-rich DNA sequences and manipulate their topology [80]. In their study, Brucoli *et al.* therefore suggest an effect of these compounds on XS. A further example is the antiasthma drug zafirlukast, which was shown to inhibit the DNA-binding ability of Lsr2 in *M. tuberculosis* and *M. smegmatis* [81]. This compound was found to inhibit the growth of both mycobacterial strains and led to clarification of the bacterial cultures after three days. A direct interaction between zafirlukast and Lsr2 was revealed by MALDI-TOF analysis. However, we suggest that such interactions are specific for the particular protein since, in our hands, zafirlukast does not counteract the silencing mediated by the Lsr2-like protein CgpS (unpublished data).

Silencers in Actinobacteriophages

With the Lsr2-like silencer CgpS, we recently provided the first example of a prophage-encoded XS protein [9]. We showed that this protein is crucial for silencing of phage gene expression to maintain the lysogenic state of the large cryptic prophage CGP3 on which it is encoded. Thus far, in the current literature, only one publication, which was based on metagenomics, has reported the presence of an H-NS-like gene in a phage genome [84]. To evaluate how commonly XS-encoding genes are found in phage genomes, we screened phage databases for these genes. Using the actinobacteriophage database PhagesDB [85], we obtained >300 hits (blastp, *e*-value < 0.005) for Lsr2-like proteins. No phages encoding the other types of silencers, H-NS, MvaT or Rok, were found in the genomes of phages infecting actinobacteria, which is not surprising, as members

Table 2. Counteracting XS by phages or other MGEs

Counter-actor	Mechanism	Host	Host XS	Phage or MGE	Source
H-NST	H-NST represents a truncated version of H-NS consisting of the oligomerization domain. H-NST interferes with the correct oligomerization of the native H-NS protein and therefore the correct function.	*E. coli*; uro-pathogenic strain CFT073 and entero-pathogenic strain E2348/69	H-NS	CFT073: UPEC-specific island inserted at *serU*, E2348/69: EPEC-specific island at *asnW*	[70]
T7 protein 5.5	Protein 5.5 is able to interact with the central oligomerization domain and hinders H-NS from forming high-order oligomers.	*E. coli*, e.g., BL21 (DE3)	H-NS	Phage T7 (virulent)	[73,74]
Ler	Ler is a DNA-binding H-NS homologue that is able to increase DNA rigidity similar to the nucleoprotein filament formed by H-NS. This binding leads to a displacement of H-NS and counter-silencing.	*E. coli* (EPEC strain E2348/69)	H-NS	Horizontally acquired pathogenicity island (*LEE1*)	[65–67,69]
Mip	The MvaT-inhibiting protein (Mip) coprecipitates with MvaT and was shown to inhibit the DNA-binding of MvaT.	*P. aeruginosa* PAO1	MvaT	Phage LUZ24 (virulent)	[75]
MotB	MotB copurifies with H-NS and StpA. Deletion of the *motB* gene leads to decreased burst size of T4. Hence, a counter-acting ability against H-NS was suggested.	*E. coli*	H-NS	Phage T4 (virulent)	[76]
Arn	Arn acts as DNA mimicking protein (mimicking the charge and structure of dsDNA) and is able to bind H-NS. Arn binding could be shown to interfere with the binding of H-NS to target regions.	*E. coli*, e.g., BL21 (DE3)	H-NS	Phage T4 (virulent)	[77,78]
Ocr	Ocr is a DNA mimicking protein that mimics B-form DNA. It was shown that Ocr is able to counter-silence H-NS-silenced promoters.	*E. coli* (*i.e.,* C600)	H-NS	Phage T7 (virulent)	[78,82]
ArdA	The crystallization of the ArdA dimer led to the assumption that ArdA is a DNA mimic proteins. Furthermore, it could be shown that increased amounts of ArdA leads to a counter-silencing of H-NS silenced promoters *in vivo*.	Multiple organisms	H-NS	Plasmid Collb-P9	[78,83]

of this bacterial class harbor only Lsr2-like proteins. Thus, we extended the screening to the Virus-Host database (>2500 bacteriophages) [86]. Strikingly, we could not obtain a single hit for H-NS-, MvaT- or Rok-like proteins in >1000 Proteobacterium phages and in >600 phages that infect Firmicutes. These findings illustrate that the function of Lsr2-like proteins has clearly been adopted by actinobacteriophages and that genes are commonly transferred by phages between GC-rich Actinobacteria. In an initial evaluation, we used PHACTS [87] to allocate the >2600 actinophages into temperate (>800) and virulent phages (>1800) and found Lsr2-like proteins in both groups (Fig. 2a, Table S1). Taking into account the respective group sizes (virulent > temperate), Lsr2-like proteins are approximately three times more frequent in temperate than virulent phages (Fig. 2a). An overview of their respective hosts evinced four different genera, *Gordonia*, *Microbacterium*, *Mycobacterium* and *Streptomyces*, in which the mycobacteriophages represent the largest group of Lsr2-encoding phages with 141 members (Fig. 2b). However, mycobacteriophages are strongly overrepresented in PhagesDB (>1600). When considering the overall group sizes of the respective hosts, *lsr2* genes are most likely to be found in genomes of *Streptomyces* phages (18.6%; 32 of 172) (Fig. 2b). Remarkably, comparisons of the GC contents and the genome sizes of Lsr2-encoding phages showed that the genomes were significantly more AT-rich and larger, especially for phages with a putative temperate lifestyle (Fig. 2c). In addition, within the Lsr2 group, genomes of virulent phages exhibit a higher variation with respect to GC content and genome size (Fig. 2c). Furthermore, we performed secondary structure predictions to compare phage- and host-encoded silencer proteins (within the respective group) by their global pairwise identity. While bacterial Lsr2 proteins are highly conserved, we identified a strong variability in terms of the predicted secondary structure within phage-encoded Lsr2-like proteins (Fig. 2d). Taken together, these findings suggest that phage-encoded Lsr2-like proteins have different functions, presumably based on the phage lifestyle.

A hypothesis derived from stealth plasmids (discussed in the section below) and emphasized by the example of CgpS [9] is that XS proteins are involved

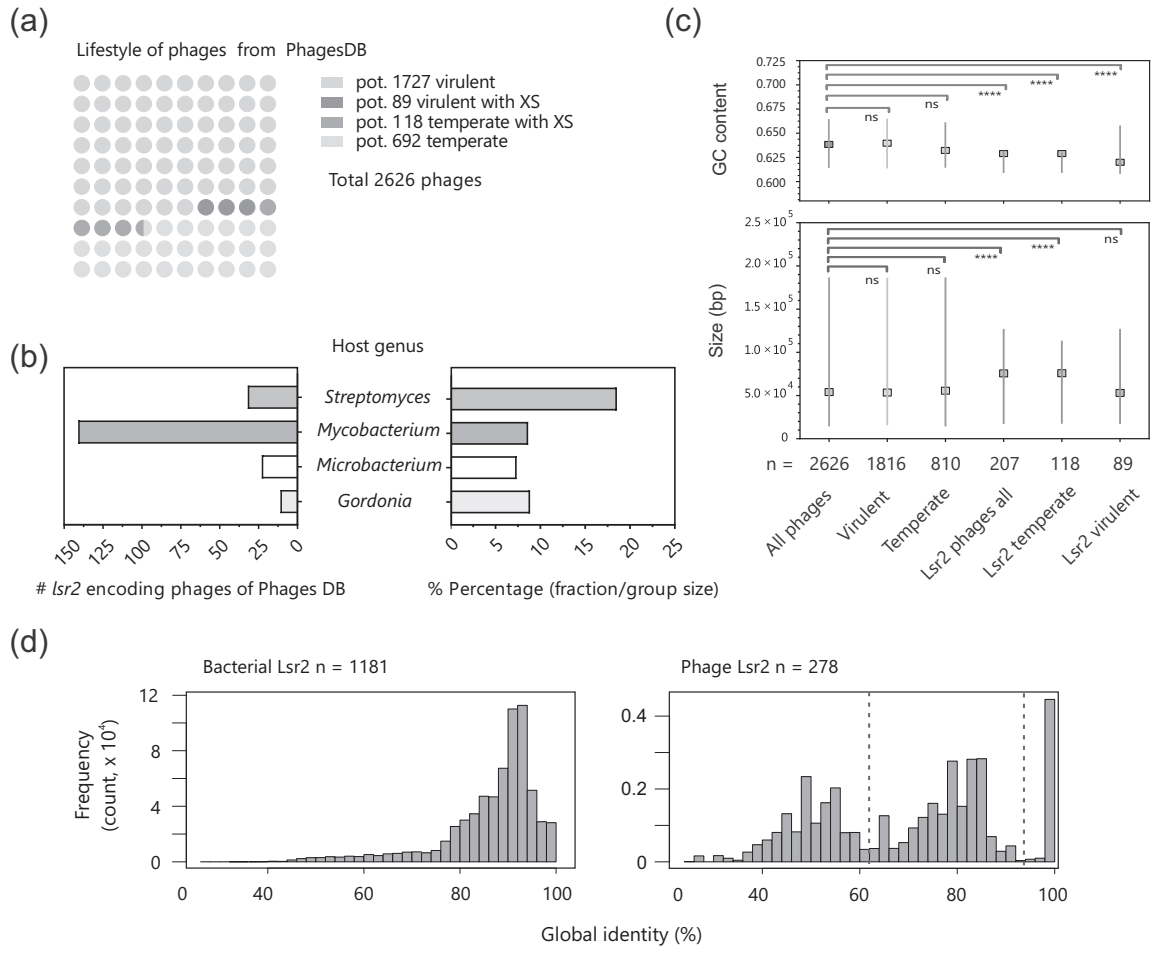

Fig. 2. Lsr2-like proteins are encoded on actinobacteriophage genomes. (a) Distribution of Lsr2-encoding phages is shown among temperate and virulent actinophages. Based on the genomes downloaded from the actinobacteriophage database PhagesDB [85], coding sequences were predicted by prodigal [88] and the lifestyles of phages were predicted using PHACTS [87]. The temperate lifestyle was assigned if the mean minus the standard deviation of the calculated probability was >0.5. Otherwise, a virulent lifestyle was assumed. By this approach, 1816 (1727 corresponding to light blue and 89 corresponding to blue balls) were predicted to be virulent and 810 (692 light orange and 118 corresponding to orange balls) to be temperate (out of 2626 phages, downloaded 19.10.2018). A blastp search (default parameters, *e*-value < 0.005) revealed 207 phages encoding Lsr2-like proteins, of which 89 (blue balls) are virulent phages and 118 are temperate phages (orange balls). Phages containing more than one gene encoding an Lsr2-like protein were counted only once; hits found in draft genomes were excluded. (b) Overview of the host genus of Lsr2-encoding phages. On the left side, the absolute numbers are indicated. The right side of plot shows the proportion of Lsr2-encoding phages among all phages for the respective host genus**.** (c) GC contents and sizes of temperate, virulent and silencer encoding phages were compared with reference group (all phages) by the Kruskal–Wallis test. The medians are indicated with boxes. In the GC-content plot, the lines represent the interquartile ranges, whereas in the size plot, the lines indicate the range of the minimal and maximal values. (d) Global pairwise secondary structure identity between Lsr2 sequences encoded in bacterial (left side) and phage genomes (right side). The identity of Lsr2 structure within bacterial genomes is relative high (mean ~87%) compared to the phage encoded Lsr2 sequences (mean ~70%). Furthermore, the distribution of the phage encoded Lsr2 structure identity evinces a higher diversity by pointing to the existence of particular pairs with high identity to each other and low to the rest of the data set.

in maintaining the lysogenic state, thereby presenting a mechanism of mutual adaptation and integration into host regulatory networks (Fig. 3). In this scenario, XS proteins would work in conjunction with the endogenous phage repression system, stabilizing lysogeny and minimizing the costs for harboring the temperate phage. Furthermore, it is conceivable that XS proteins may influence the lytic-lysogenic decision during phage infection, but this has not yet been addressed experimentally. In virulent phages, silencer proteins may inherit completely different roles. In addition to affecting multiple other targets,

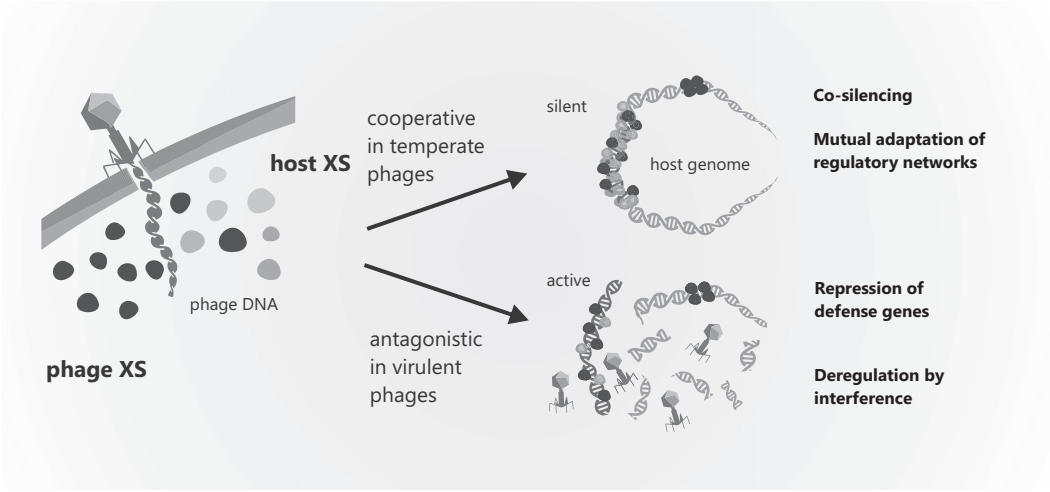

Fig. 3. Model for the functions of phage-encoded silencers depending on the phage lifestyle. Examples like the Lsr2-like silencer CgpS demonstrated that XS proteins may play an important role in maintaining the lysogenic state. Depending on the XS repertoire or the particular host strain, (pro-)phage-encoded XS proteins may also cooperate with the host-encoded protein(s) to form heteromeric complexes. The function of phage-encoded silencers has not been studied experimentally and therefore remains subject to speculation. Nevertheless, it can be postulated that virulent phages might employ XS-like proteins as a weapon to interfere with host XS proteins or to repress other host defense mechanisms.

XS proteins were also found to repress genes encoding different phage defense systems, including CRISPR–Cas genes [89] and an RM system [9]. Furthermore, interference of XS proteins, which are not capable of forming functional heteromeric complexes, may lead to a deregulation of phage genes and counteracting the silencing of phage genes by the host XS. We tested this hypothesis in our previous study where expression of *E. coli hns* as well as other genes encoding mycobacterial Lsr2 proteins led to activation of the CgpS-silenced prophage [9]. Hence, we suggest that virulent phages might employ XS homologs as a weapon to interfere with host defense systems (Fig. 3).

In addition, XS proteins or, in general, NAPs are also used to organize and structure the genome during replication cycles. Hence, it is likely that phages, especially with “larger” genomes, will benefit from efficient DNA packaging proteins that facilitate optimized phage replication and production. Moreover, it is conceivable that phage-encoded silencers may contribute to compaction of the host genome. Here, an analogous example is given by the eukaryotic dinoflagellates. It is suggested that virus-like proteins, termed dinoflagellate/viral nucleoproteins (DVNPs), fulfill the functions of histones by packaging the genomes. This is supported by a recent study in which it was shown that heterologous produced DVNPs outcompete histones in *Saccharomyces cerevisiae* thereby causing toxic effects [90]. Although the dinoflagellate genomes encode histone proteins, the histone expression is strongly reduced, and it is assumed that they provide only regulatory functions [91]. Moreover, based on phylogenetics, it is hypothesized that the histone depletion occurred simultaneously with the acquisition of DVNPs from large-genome viruses (genome size up to 560 kb) and with massive genome expansion [92].

Stealth Silencers Encoded on Plasmids

Usually, the introduction of new MGEs, such as plasmids, imposes high fitness costs on the host organism [93]. The magnitude of the costs depends on many factors, such as plasmid-specific characteristics (replication, plasmid reception, integration and conjugation, encoded traits), expression level of plasmid-borne genes and the genetic background of the host organism [93]. Interestingly, it is assumed that the main burden arises from the expression of plasmid-encoded genes that comes from transcription, translation, or the interactions between plasmid- and host-encoded proteins [94]. One way to reduce the cost is to use “stealth genes,” particularly genes encoding silencer proteins. The H-NS-like protein Sfh was one of the first characterized plasmid encoded stealth proteins. Doyle *et al.* [95] investigated the costs of the AT-rich pSf-R27 plasmid and evinced a significant biosynthetic burden in the absence of *sfh*. Therefore, the authors concluded that these proteins are quite useful in infiltrating a new host by reducing the metabolic burden to a minimum. Strikingly, transcriptome and genome-wide binding analysis revealed that the regulons

from the chromosomally and plasmid-encoded H-NS-like silencers are completely different [96–98], although the proteins are closely related. While plasmid-encoded variants typically exhibit a specific and narrow target spectrum (mostly with a focus on HGT-acquired regions), the host H-NS is known to act as a global regulator modulating the expression of both HGT-acquired and core genes [97,98]. The basis for this selectivity was addressed by chimeric protein fusions, and the experimental results suggest a correlation between higher flexibility of the linker-domain (connecting the N-terminal oligomerization part to the C-terminal DNA-binding domain) and a decrease in selectivity due to stable binding to broader ranges of DNA geometries [99]. Astonishingly, in a more recent study, a known H-NS target, the *gadAB* operon, was examined in *Shigella flexneri*. Here, reduced expression was observed in the presence of the pSf-R27 plasmid, leading to reduced acid resistance and showing that a plasmid- and host-encoded silencer also co-regulate core genes [100].

Bioinformatics analyses revealed that genes encoding H-NS- and MvaT-like silencers are over-represented on large plasmids. In addition, H-NS-encoding plasmids are found to be more AT-rich than other NAP-harboring plasmids [101]. To also evaluate the distribution of Lsr2- and Rok-like silencers, we performed a BLASTp search (*e*-value < 0.005) using a plasmid database that we retrieved from the NCBI nucleotide database (>24000 plasmid sequences, source database RefSeq, Fig. 4a). Here, we identified 408 hits for H-NS-like proteins, 35 for MvaT-like proteins, 63 for Lsr2-like proteins and 18 for Rok-like proteins. A comparison of the average sizes revealed that silencer-encoding plasmids are at least five times larger than the average plasmid sequence deposited in the database (Fig. 4a). Furthermore, H-NS-encoding *E. coli* plasmids displayed a significantly higher AT content (96 of the 408 H-NS plasmids) (Fig. 4b). This trend was, however, not observed for GC-rich *Streptomycetes* (Fig. 4b) or *P. aeruginosa* and *B. subtilis* plasmids (data not shown), but the sequence data in these databases feature a strong bias, and only a few sequences are available for some species (e.g., n = 13 for *Streptomycetes* plasmids). In line with previous studies, our findings emphasize that especially large plasmids appear to benefit from DNA-organizing proteins and that self-silencing may represent a strategy for host infiltration harnessed by phages and plasmids alike.

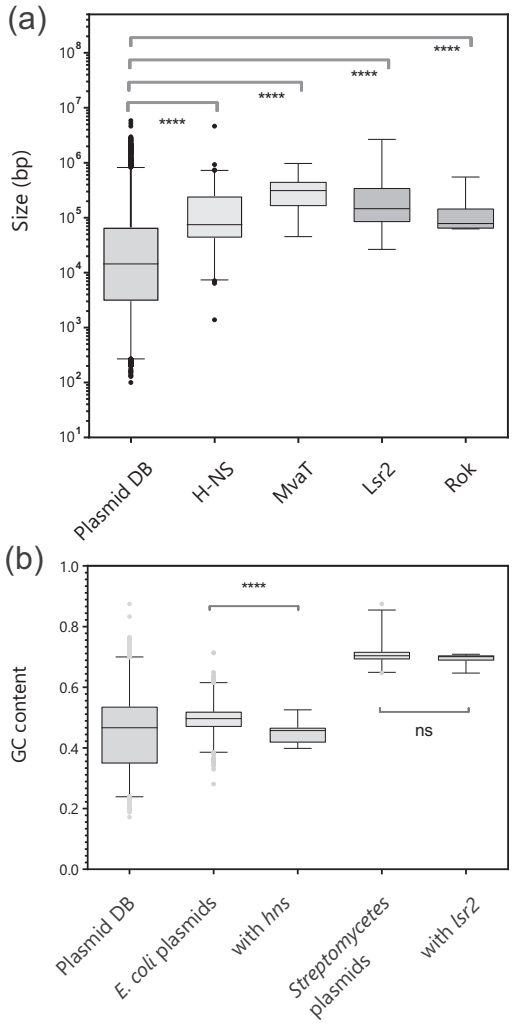

Fig. 4. Distribution of silencer-encoding genes on plasmids. (a) Overall, 24197 sequences for plasmids were retrieved from the NCBI nucleotide database using RefSeq as the source data base (filter criteria: bacteria, genomic DNA, plasmid, RefSeq on 29.10.2018). Via a local blastp search (*e*-value < 0.005) with the amino acid sequences of H-NS (WP_001287378.1), MvaT (WP_003093888.1), Lsr2 (WP_003419513.1) and Rok (WP_003232378.1), approximately 408, 35, 63 and 18 hits, respectively, were found. The sizes were compared in a boxplot with ranges from 1% to 99% and evaluated by Kruskal–Wallis tests. (b) GC content of the plasmids, including all sequences, *E. coli* plasmids (n = 2600), with *hns* (n = 95) and *Streptomycetes* plasmids (n = 147) with *lsr2* (13) were compared in a boxplot (range, 1%–99%; evaluated by Kruskal–Wallis tests).

Future Perspectives

In conclusion, several recent studies highlight the important role of XS proteins in phage–host interactions, for example, by silencing expression of genomically integrated prophages or phage remnants. The first examples, like the T7 5.5 protein or Mip [74,75] encoded by a *Pseudomonas* phage, demonstrate that counteracting XS represents an important aspect of lytic infection. As illustrated by the examples of Lsr2-like proteins encoded by various actinophages, the function of XS proteins

apparently has been adopted by phages as well. Nevertheless, many gaps remain in the prokaryotic and phage sequence space as for the majority of prokaryotic phyla, no XS protein has been identified so far. Furthermore, it is striking that while homologs of H-NS, MvaT, Rok and Lsr2 are encoded by plasmids, only *lsr2* homologs were identified in phage genomes. Considering the high sequence variability of phage-encoded silencers, these proteins most likely perform many different functions depending on the particular lifestyle of the phage, which needs to be addressed in future studies.

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Abbreviations used:
HGT, horizontal gene transfer; SPI, spontaneous prophage induction; RM, restriction modification; XS, xenogeneic silencing; NAP, nucleoid-associated protein; PTM, posttranslational modification; MGE, mobile genetic element; TF, transcription factor; EPEC, enteropathogenic; DVNP, dinoflagellate/viral nucleoprotein.

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