1 **Title:**

2 **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.

37   *"You have a grand gift for silence, Watson. It makes you quite invaluable as a companion."*

38   (Sherlock Holmes)

39

40   **Introduction**

41   Phages, viruses that prey on bacteria, represent the most abundant biological entities on this planet and

42   are a major driver of horizontal gene transfer (HGT). Phages are not only present as infectious particles in

43   the environment but are also found as integrated elements (prophages) within the genomes of their

44   bacterial hosts. In some cases, DNA of viral origin accounts for up to 20% of an organism's entire genome

45   [1-3]. Some of this DNA originates from fully functional prophages, which are capable of undergoing a lytic

46   life cycle. However, a considerable part is made up of prophage-like elements, including phage remnants

47   left after incomplete excision events, cryptic (degenerated) prophages or other genetic material acquired

48   by HGT. In fact, this genetic material has significantly shaped microbial evolution due to the development

49   of mutually beneficial interactions between prophage and host [4]. Nevertheless, the safe integration of

50   viral elements into bacterial genomes demands stringent regulation of phage gene expression.

51   Upon integration into the host genome, a functional phage can exit the prophage state and enter the lytic

52   cycle, which is typically triggered by severe DNA damage that activates the cellular SOS response. Even

53   under non-inducing conditions, cells may encounter spontaneous DNA damage [5], leading to the SOS-

54   dependent induction of prophages in a small fraction of the lysogenic population [6, 7]. Originally, this

55   spontaneous prophage induction (SPI) was considered a potentially detrimental process, but recent

56   research in the fields of microbial biofilms, host-pathogen interaction and population dynamics

57   emphasizes that SPI is an important contributor to the social behavior of microbes [6]. In a recent study,

58   we quantified SPI in populations of *C. glutamicum* using reporter promoter fusions. While we observed a

59   positive correlation between SPI and the DNA damage (SOS) response, a significant fraction of the cases

60   also occurred in an SOS-independent manner [8, 9]. Thus, the molecular factors influencing SPI in single

61   individuals and how the host modulates its frequency remain largely unknown.

62   Compared to point mutations or genomic rearrangements, HGT allows bacteria to acquire new traits much

63   more rapidly, but the downside of this medal is that the new information is encoded on foreign genetic

64   material [10, 11]. The encounter with xenogeneic (foreign) DNA is a 'high risk - high gain' situation: While

65   the acquisition provides the potential for the fast gain of new beneficial traits, the activity of selfish genetic

66   elements or bacterial viruses (bacteriophages) represents a perpetual threat to bacterial cells. Gene

67   expression from xenogeneic material can strongly impair cellular fitness by sequestering RNA polymerase

68   [12, 13], by producing toxic proteins and, in the case of phages, by causing cell lysis. With more than 10^{24}

69   productive viral infections on earth [14], the activity of bacteriophages plays a vital role in HGT, which is

70   also reflected by the variety of phage defense mechanisms encoded in bacterial genomes, with restriction

71   modification (RM) systems and CRISPR-Cas being among the most prominent mechanisms [15]. With the

72   expansion of the phage genomic space, many more examples of phage defense systems have been

73   described and have been covered in a number of recent reviews [16, 17]. In contrast to the destructive

74   mode of action of RM and CRISPR-Cas systems, where nucleases are employed to wipe out incoming

75   foreign DNA [18], xenogeneic silencing (XS) represents a mechanism promoting tolerance of foreign

76   genetic material [19, 20]. The mechanism of XS is based on the activity of small, nucleoid-associated

77   proteins (NAPs) that recognize and bind foreign, AT-rich DNA stretches and silence gene expression due

78   to the formation of a tight nucleoprotein complex [19] (Figure 1). By this means, XS proteins provide an

79   important basis for the safe acquisition of new genetic material and foster evolutionary network expansion

80   [21]. Hitherto, all known XS proteins fall into one of four different classes: H-NS in Proteobacteria [19, 22],

81   MvaT/U in *Pseudomonas* species [23], Rok in *Bacillus subtilis* [24] and Lsr2 in Actinobacteria [25]. Despite

82   the low sequence similarity between different silencers, these proteins appear to fulfill very similar

83 functions in their respective host, and examples of cross-complementation have been found for Lsr2, MvaT

84 and H-NS [9, 23, 25].

85 Several recent studies focused, in particular, on the impact of H-NS on bacterial genome evolution and

86 network expansion (for recent reviews, see [19, 20, 26]). Given the mosaic-like structure of bacterial

87 genomes, (pro-)phages apparently account for a significant fraction of bacterial strain diversification [1].

88 In a recent study, we provided the first example of a prophage-encoded Lsr2-like protein functioning as an

89 essential silencer of a cryptic prophage in the actinomycete *Corynebacterium glutamicum*. Considering the

90 generic role of XS proteins in the silencing of foreign DNA, it is reasonable to assume that these proteins

91 play an important role and may adopt different functions in phage-host regulatory interaction. In this

92 review, we summarize the literature focusing on the role of XS proteins on phage-host interactions,

93 including examples of phage-mediated counter-silencing as a defense strategy during infection.

94 Furthermore, we provide a comprehensive bioinformatics analysis of bacterial and phage genomes

95 revealing that genes encoding Lsr2-like proteins are ubiquitously found in the genomes of

96 actinobacteriophages, suggesting an adoption of XS function in the lifestyles of virulent and temperate

97 phages.

98

99 **Xenogeneic silencing: Recognition and binding to foreign, AT-rich DNA**

100 The basis for XS is provided by the domain organization of the XS proteins, which is remarkably similar

101 among the different classes. Typically, these proteins exhibit a small size of <15 kDa and consist of an N-

102 terminal oligomerization domain and a C-terminal DNA-binding domain [20, 27]. All silencers have the

103 common feature of preferentially binding DNA regions that are more AT rich than the host genome. This

104 feature appears to be the basis for the targeting of foreign elements since the vast majority of exogenous

105 DNA has been found to be more AT rich than the host core genome [19, 28, 29].

106   High-resolution structural analysis of the C-termini of H-NS and Lsr2 resulted in the identification of the

107   'prokaryotic AT-hook' with the 'Q/RGR' motif, which is reminiscent of the 'AT-hook' motif found in

108   eukaryotic HMG-I(Y) proteins [30]. AT-sequences lack the exocyclic 6-amino group, which consequently

109   allows a deeper interaction with protein side-chain residues. In addition, the narrower minor groove of

110   AT-rich sequences harbors a surface with a higher electronegative potential (than mixed or GC-rich

111   sequences), enabling a stronger interaction with positively charged residues. From the evolutionary point

112   of view, it is astonishing that although H-NS and Lsr2 do not share any structural similarity, these proteins

113   show the same binding mechanism. In contrast, MvaT/U proteins, found in *Pseudomonas* species, lack the

114   AT-hook motif but instead recognize target DNA via a so-called 'AT-pincer' motif consisting of a conserved

115   lysine residue and a downstream KGGN motif interacting with the minor groove [31]. Recently, Duan *et al.*

116   elucidated how the *Bacillus* silencer Rok distinguishes between host and foreign DNA [27]. The authors

117   could show that Rok directly binds to the minor groove of AT-rich sequences in a novel mode that, so far,

118   has not been described for any other winged helix protein. Using *in vitro* protein binding microarrays and

119   comparative genome analysis, the authors concluded that Rok preferentially recognizes a few distinct AT-

120   rich DNA motifs present in horizontally acquired regions, which are significantly underrepresented in

121   *Bacillus* host genomes [27]. However, for the other XS proteins, no sequence-specific recognition has been

122   observed. This characteristic has been, most intensively, studied for H-NS, where AT-rich regions were

123   suggested to function as initial nucleation regions [32]. In a more recent study, a single-molecule counting

124   approach revealed that nucleation sites are crucial for recruiting H-NS molecules [33].

125

Xenogeneic silencing: Formation of the nucleoprotein complexes

126

127   However, binding to DNA is itself not sufficient for XS proteins to fulfill their function. Protein

128   multimerization is an essential step that is required to enable silencing of the target regions. Generally, it

129   is assumed that after the initial nucleation, additional silencing molecules are recruited and concurrently

130   spread along adjacent AT-rich regions (filament formation) [32]. Finally, the high-order oligomerization

131   between distal silencer-DNA complexes leads to strong condensation and DNA compaction that enables

132   silencing of target gene expression [19]. In the current literature, three main mechanisms for silencing are

133   suggested: (i) Promoter occlusion, where the filamentous nucleoprotein complex prevents the binding of

134   the RNA polymerase [19, 34], (ii) trapping of the RNA polymerase, blocking promoter escape upon binding

135   [34] and, (iii) ρ-dependent transcriptional termination that occurs during the pausing of RNA polymerase

136   [35].

137   Multimerization of XS proteins is facilitated by the N-terminal domains. This effect has been shown,

138   experimentally, for H-NS-, MvaT- and Lsr2-like proteins [9, 36-38] and was suggested for Rok [24].

139   Primarily, the mechanism behind this protein oligomerization was widely studied using H-NS and high-

140   resolution approaches, like atomic force microscopy (AFM), electron microscopy and single-molecule

141   magnetic tweezers experiments [37, 39, 40]. Depending on the concentration of the divalent cations (Mg^{2+}

142   or Ca^{2+}), AFM studies reported either a bridging or stiffened mode of the bound DNA stretches [40, 41].

143   These two different modes were also found for MvaT and Lsr2 [36, 38, 42, 43], underlining a common

144   silencing mechanism among the silencers and explain why these XS proteins are partly able to complement

145   each other in the abovementioned cross-complementation experiments [9, 25]. Nevertheless, the

146   dissimilar AT compositions of host genomes and the differences in DNA binding underline the

147   specialization of the particular XS protein to the requirements in the particular host background.

148   For H-NS- and MvaT-like silencers, it was shown that XS proteins may form silencing nucleoprotein

149   complexes by interacting with other NAPs. Efficient protein oligomerization is, however, still a prerequisite

150   for the establishment of a silencing nucleoprotein complex. In particular, the small proteins Hha and its

151   paralog YdgT (Cnu) were shown to structurally resemble the N-terminal domain of H-NS and are therefore

152   capable of forming heteromeric complexes [44]. Further studies based on transcriptomics revealed that

153   the binding of Hha to H-NS polymers is required for the efficient repression of a subset of the H-NS regulon

154   [45]. At this point, it is worthwhile to mention that Cnu represents an *oriC* binding protein that binds within

155   a DnaA binding box and has been suggested to contribute to optimal *oriC* activity [46]. In *B. subtilis*, a

156   regulative dependency between the replication initiation protein DnaA and the silencer Rok was recently

157   reported [47], evincing an interesting link between bacterial replication and this XS protein.

158   In the genomes of *E. coli* and *Salmonella* Typhimurium, paralogs of H-NS, such as StpA, are encoded and

159   are capable of forming heterodimers that in turn can interact again with the aforementioned small

160   proteins Hha/YdgT [44]. Recently, the impact of Hha and StpA on the binding mode of the H-NS protein

161   was investigated by *in vitro* biochemical and biophysical experiments. With their high-resolution approach,

162   Boudreau et al., nicely demonstrated how StpA and Hha modify H-NS-polymeric filaments to increase

163   transcriptional pausing and provided evidence showing that the mixed nucleoprotein complexes

164   (consisting of Hha/H-NS or StpA/H-NS, etc.) differentially affect gene regulation [48]. Thus, these

165   experiments further suggest that interactions with paralogs or accessory proteins likely specify the

166   silencing characteristics also depending on the local concentrations of the respective proteins. The

167   formation of heteromeric silencer complexes was also reported for MvaT and its paralog MvaU in

168   *Pseudomonas aeruginosa* [49]. In *P. putida*, MvaT-like proteins were shown to control distinct regulons

169   revealing a functional specialization of these proteins [50-52]. Similar scenarios are also conceivable for

170   Lsr2-like proteins, since the genomes of several Actinobacteria, like *Streptomyces coelicolor* A(3), *S.*

171   *venezuelae* ATCC 10712 and *M. smegmatis* MC² 155, encode more than one copy of Lsr2-like proteins

172   (based on a BLAST search with the sequence of Lsr2 (Rv3597c) and default parameters, e-value $< 1 \times 10^{-5}$).

173   The genome compaction of XS proteins resembles the compaction by heteromeric nucleoprotein

174   complexes formed by histones and DNA in eukaryotic cells. Hence, it is reasonable to assume that XS

175   proteins, or in general NAPs, are targets of posttranslational modification (PTM) enzymes. In a very recent

176   review, this topic was elucidated by Dilweg and Dame [53]. Interestingly, the authors reported

177   approximately 29 PTMs for H-NS from *E. coli*; these PTMs were not experimentally investigated, but the

178   physiological implication for DNA condensation and/or silencing was discussed [53].

179

180   **Silencing of prophages in bacteria**

181   The safe integration of viral elements into bacterial genomes demands stringent regulation of expression

182   from highly active phage promoters to avoid the production of potentially toxic proteins. The function of

183   XS proteins thereby provides a basis for integrating foreign genes into host regulatory networks by XS and

184   counter-silencing (the latter is discussed in the next section).

185   For silencers of all four groups, an influence on the regulation of phage genes has been observed (Table

186   1). However, this effect is often noted only incidentally in these studies [9, 54-56]. Interestingly, the

187   essentiality of a particular XS-encoding gene strongly depends on the genetic setup of the particular strain

188   as the induction of mobile genetic elements, such as prophages, may lead to cell death. In the case of the

189   Gram-positive actinomycete *Corynebacterium glutamicum*, the Lsr2-like protein CgpS (*C̲. g̲lutamicum*

190   p̲rophage s̲ilencer) was shown to be essential due to its function as a silencer of the cryptic but still

191   inducible prophage CGP3 [9]. Counteracting of CgpS activity led to prophage induction and regional (in

192   situ) replication at the CGP3 locus [57]. In contrast to other XS proteins described so far, CgpS is encoded

193   on the CGP3 prophage itself and appears to act mainly as a silencer of CGP3 gene expression. Genome-

194   wide profiling of CgpS confirmed the binding of this XS protein to AT-rich sequences in the CGP3 element.

195   As a matter of fact, the essentiality of the *cgpS* gene is linked to the presence of CGP3, and *C. glutamicum*

196   strains lacking the prophage do not require CgpS. Interestingly, integration of a second genomic copy of

197   *cgpS* significantly reduced the fraction of spontaneously induced cells, emphasizing the role of XS proteins

198   in the modulation of SPI in bacterial populations (Frunzke and Pfeifer, unpublished). Additionally, for

199   *Mycobacterium tuberculosis*, which is also a member of the Actinobacteria, genome-wide binding studies

200   conducted with a Lsr2-like protein not only confirmed the binding to prophage regions (and other mobile

201   genetic elements (MGEs)) [9, 38] but also revealed several additional targets in the host genome involved

202   in virulence and immunogenicity [38]. Interestingly, in *Bacillus subtilis*, the Rok protein was also shown to

203   bind prophage genes and to be involved in the control of phage gene expression, as is the case for genes

204   of the prophage SPβ [24, 58]. A further interesting example involves *E. coli* H-NS, where a link between H-

205   NS activity and enhanced biofilm formation was recently demonstrated. Here, H-NS was shown to repress

206   the cryptic prophage Rac. Derepression of Rac resulting from *hns* deletion led to prophage induction and

207   cell lysis in a toxin-dependent manner [56]. Moreover, in *Shewanella oneidensis,* H-NS was also reported

208   to be involved in prophage induction during cold adaptation [59]. Additionally, the H-NS orthologs MvaT

209   and MvaU were shown to be essential due to the silencing of prophage elements in *P. aeruginosa* strains

210   [54, 55]. The depletion of silencers caused increased phage gene expression, the formation of infectious

211   phage particles and cell lysis. Remarkably, only mutants impaired in phage production were capable of

212   compensating the double deletion of *mvaT* and *mvaU* [54, 55]. An overview of the studies showing the

213   influence of XS proteins on the control of phage gene expression is provided in Table 1.

214

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 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 *S. 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 (EHEC) *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

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239   to increase DNA folding and wraps DNA; otherwise, with increasing concentration, Ler binds DNA in an

240   unwrapped mode where Ler increases the rigidity of DNA similarly to the nucleoprotein filament formed

241   by H-NS [69]. At these high concentrations, Ler displaces H-NS from the bound DNA and therefore

242   overcomes the silencing of target regions. A further interesting example is provided with H-NST, a

243   truncated derivative of H-NS lacking the DNA-binding domain. This XS protein was found to antagonize H-

244   NS in enteropathogenic (EPEC) and uropathogenic *E. coli* by interfering with its oligomerization domain

245   [70, 71]. Remarkably, this principle of silencer interference can be harnessed to study the function of

246   essential XS proteins. Overproduction of the N-terminal oligomerization domain of the Lsr2-type silencer

247   CgpS was used to counteract CgpS silencing *in vivo* in the Actinobacterium *C. glutamicum* [9]. Interference

248   between XS proteins was further demonstrated in this study as the expression of other mycobacterial Lsr2

249   genes as well as introduction of *E. coli* H-NS led to XS interference at AT-rich regions, resulting in prophage

250   induction. These findings are also supported by a bioinformatics analysis showing that different classes of

251   silencers do not occur in the same species [72].

252   Altogether, these examples provide important insights how silencing and counter-silencing facilitate the

253   expansion of regulatory networks in bacteria [21]. In the following, we will focus on mechanisms employed

254   by phages to counteract XS proteins in an ongoing arms race between the phage and the host. A few

255   studies, discussed below, already suggest a variety of different mechanisms used by phages to gain control.

256   One example is provided by the 5.5 protein of the *E. coli* phage T7, which is able to antagonize H-NS

257   function upon phage infection [73]. By interfering with the central oligomerization domain of H-NS [37],

258   the 5.5 protein blocks H-NS from forming high-order oligomers, leading to counter-silencing of H-NS-

259   silenced genes [74]. Another example has been reported with the Mip protein (<u>M</u>vaT <u>i</u>nhibiting <u>p</u>rotein),

260   encoded by the LUZ24 phage of *P. aeruginosa* [75]. In 2015, Wagemans et al. showed that Mip is able to

261   inhibit the binding of the nucleoid-structuring silencer MvaT to DNA and that Mip and MvaT coprecipitate

262   in pulldown assays. However, the exact mechanism of MvaT inhibition by Mip is not completely

263   understood, yet.

264   In the case of *E. coli* T4 phage, two different proteins were reported to interfere with H-NS silencing. The

265   protein MotB is a DNA-binding protein that co-purifies with H-NS as well as with the H-NS homolog StpA.

266   Deletion of the *motB* gene led to a decreased burst size [76]. The T4 protein Arn represents an interesting

267   example of a phage-encoded DNA mimic protein and was shown to directly interact with *E. coli* H-NS [77].

268   While Arn was originally described as an inhibitor of the McrBC restriction enzyme, structural analysis

269   revealed that the shape of the protein mimics the shape and charge of double-stranded DNA, and the

270   authors highlight this DNA mimicry as a mechanistic basis for interfering with the function of DNA-binding

271   proteins, like H-NS [77]. Interestingly, the DNA mimic proteins Ocr of the phage T7 and ArdA of the plasmid

272   Collb-P9 were also reported to antagonize H-NS in a similar way [78].

273   Finally, the direct interference with TFs or other proteins likely does not represent the only way to fight

274   off XS. In a very recent study, Kronheim et al. highlighted the important role of small molecules secreted

275   by bacterial hosts as weapons against phage infection [79]. A link between these compounds and XS

276   proteins does not necessarily exist, but a few examples suggest that small natural compounds – other than

277   proteins – may also counteract XS. One class of compounds is represented by polyamides containing a

278   biaryl motif. These polyamides especially target the minor groove of AT-rich DNA sequences and

279   manipulate their topology [80]. In their study, Brucoli et al. therefore suggest an effect of these chemical

280   compounds on XS. A further example is the antiasthma drug zafirlukast, which was shown to inhibit the

281   DNA-binding ability of Lsr2 in *M. tuberculosis* and *M. smegmatis* [81]. This compound was found to inhibit

282   the growth of both mycobacterial strains and led to clarification of the bacterial cultures after three days.

283   A direct interaction between zafirlukast and Lsr2 was revealed by MALDI-TOF analysis. However, we

284   suggest that such interactions are specific for the particular protein since, in our hands, zafirlukast does

285   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 [82]. To evaluate how commonly XS-encoding genes are found in phage genomes, we screened phage databases for these genes. Using the actinobacteriophage database PhagesDB [83], 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 of this bacterial class harbor only Lsr2-like proteins. Thus, we extended the screening to the Virus-Host database (>2500 bacteriophages) [84]. 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 [85] to allocate the >2600 actinophages into temperate (>800) and virulent phages (>1800) and found Lsr2-like proteins in both groups (Figure 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 (Figure 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 (Figure 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 *Streptomyce*s phages (18.6 %, 32 of 172) (Figure 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 (Figure 2C). In addition,

14

311   within the Lsr2 group, genomes of virulent phages exhibit a higher variation with respect to GC content

312   and genome size (Figure 2C). Furthermore, we performed secondary structure predictions to compare

313   phage- and host-encoded silencer proteins (within the respective group) by their global pairwise identity.

314   While bacterial Lsr2 proteins are highly conserved, we identified a strong variability in terms of the

315   predicted secondary structure within phage-encoded Lsr2-like proteins (Figure 2D). Taken together, these

316   findings suggest that phage-encoded Lsr2-like proteins have different functions, presumably based on the

317   phage lifestyle.

318   A hypothesis derived from stealth plasmids (discussed in the section below) and emphasized by the

319   example of CgpS [9] is that XS proteins are involved in maintaining the lysogenic state, thereby presenting

320   a mechanism of mutual adaptation and integration into host regulatory networks (Figure 3). In this

321   scenario XS proteins would work in conjunction with the endogenous phage repression system, stabilizing

322   lysogeny and minimizing the costs for harboring the temperate phage. Furthermore, it is conceivable that

323   XS proteins may influence the lytic-lysogenic decision during phage infection, but this has not yet been

324   addressed experimentally. In virulent phages, silencer proteins may inherit completely different roles. In

325   addition to affecting multiple other targets, XS proteins were also found to repress genes encoding

326   different phage defense systems, including CRISPR-*cas* genes [86] and an RM system [9]. Furthermore,

327   interference of XS proteins, which are not capable of forming functional heteromeric complexes, may lead

328   to a deregulation of phage genes and counteracting the silencing of phage genes by the host XS. We tested

329   this hypothesis in our previous study where expression of *E. coli hns* as well as other genes encoding

330   mycobacterial Lsr2 proteins led to activation of the CgpS-silenced prophage [9]. Hence, we suggest that

331   virulent phages might employ XS homologs as a weapon to interfere with host defense systems (Figure 3).

332   In addition, XS proteins or, in general, NAPs are also used to organize and structure the genome during

333   replication cycles. Hence, it is likely that phages, especially with 'larger' genomes, will benefit from efficient

334   DNA packaging proteins that facilitate optimized phage replication and production. Moreover, it is

335   conceivable that phage-encoded silencers may contribute to compaction of the host genome. Here, an

336 analogous example is given by the eukaryotic dinoflagellates. It is suggested that virus-like proteins,

337 termed Dinoflagellate/Viral NucleoProteins (DVNPs), fulfill the functions of histones by packaging the

338 genomes. This is supported by a recent study in which it was shown that heterologous produced DVNPs

339 outcompete histones in *Saccharomyces cerevisiae* by causing toxic effects [87]. Although the dinoflagellate

340 genomes encode histone proteins, the histone expression is strongly reduced, and it is assumed that they

341 provide only regulatory functions [88]. Moreover, based on phylogenetics, it is hypothesized that the

342 histone depletion occurred simultaneously with the acquisition of DVNPs from large-genome viruses

343 (genome size up to 560 kb) and with massive genome expansion [89].

344

345 **Stealth silencers encoded on plasmids**

346 Usually, the introduction of new MGEs, such as plasmids, imposes high fitness costs on the host organism

347 [90]. The magnitude of the costs depends on many factors, such as plasmid-specific characteristics

348 (replication, plasmid reception, integration & conjugation, encoded traits), expression level of plasmid-

349 borne genes and the genetic background of the host organism [90]. Interestingly, it is assumed that the

350 main burden arises from the expression of plasmid-encoded genes that comes from transcription,

351 translation, or the interactions between plasmid- and host-encoded proteins [91]. One way to reduce the

352 cost is to use 'stealth genes', particularly genes encoding silencer proteins. The H-NS-like protein Sfh was

353 one of the first characterized plasmid encoded stealth proteins. Doyle et al. investigated the costs of the

354 AT-rich pSf-R27 plasmid and evinced a significant biosynthetic burden in the absence of *sfh* [92]. Therefore,

355 the authors concluded that these proteins are quite useful in infiltrating a new host by reducing the

356 metabolic burden to a minimum. Strikingly, transcriptome and genome-wide binding analysis revealed

357 that the regulons from the chromosomally- and plasmid-encoded H-NS-like silencers are completely

358 different [93-95], although the proteins are closely related. While plasmid-encoded variants typically

359 exhibit a specific and narrow target spectrum (mostly with a focus on HGT-acquired regions), the host H-

360 NS is known to act as a global regulator modulating the expression of both HGT-acquired and core genes

361   [94, 95]. The basis for this selectivity was addressed by chimeric protein fusions, and the experimental

362   results suggest a correlation between higher flexibility of the linker-domain (connecting the N-terminal

363   oligomerization part to the C-terminal DNA-binding domain) and a decrease in selectivity due to stable

364   binding to broader ranges of DNA geometries [96]. Astonishingly, in a more recent study, a known H-NS

365   target, the *gadAB* operon, was examined in *Shigella flexneri*. Here, reduced expression was observed in

366   the presence of the pSf-R27 plasmid, leading to reduced acid resistance and showing that a plasmid- and

367   host- encoded silencer also co-regulate core genes [97].

368   Bioinformatics analyses revealed that genes encoding H-NS- and MvaT-like silencers are overrepresented

369   on large plasmids. In addition, H-NS-encoding plasmids are found to be more AT-rich than other NAP-

370   harboring plasmids [98]. To also evaluate the distribution of Lsr2- and Rok-like silencers, we performed a

371   BLASTp search (e-value < 0.005) using a plasmid database that we retrieved from the NCBI nucleotide

372   database (>24000 plasmid sequences, source database RefSeq, Figure 4A). Here, we identified 408 hits for

373   H-NS-like proteins, 35 for MvaT-like proteins, 63 for Lsr2-like proteins and 18 for Rok-like proteins. A

374   comparison of the average sizes revealed that silencer-encoding plasmids are at least five times larger than

375   the average plasmid sequence deposited in the database (Figure 4A). Furthermore, H-NS-encoding *E. coli*

376   plasmids displayed a significantly higher AT content (96 of the 408 H-NS plasmids) (Figure 4B). This trend

377   was, however, not observed for GC-rich *Streptomycetes* (Figure 4B) or *P. aeruginosa* and *B. subtilis*

378   plasmids (data not shown), but the sequence data in these databases feature a strong bias, and only a few

379   sequences are available for some species (e.g. n=13 for *Streptomycetes* plasmids). In line with previous

380   studies, our findings emphasize that especially large plasmids appear to benefit from DNA-organizing

381   proteins and that self-silencing may represent a strategy for host infiltration harnessed by phages and

382   plasmids alike.

383

384

385 **Future perspectives**

386 In conclusion, several recent studies highlight the important role of XS proteins in phage-host interactions,

387 e.g., by silencing expression of genomically integrated prophages or phage remnants. The first examples,

388 like the T7 5.5 protein or Mip [74, 75] encoded by a *Pseudomonas* phage, demonstrate that counteracting

389 XS represents an important aspect of lytic infection. As illustrated by the examples of Lsr2-like proteins

390 encoded by various actinophages, the function of XS proteins apparently has been adopted by phages as

391 well. Nevertheless, many gaps remain in the prokaryotic and phage sequence space as for the majority of

392 prokaryotic phyla, no XS protein has been identified so far. Furthermore, it is striking that while homologs

393 of H-NS, MvaT, Rok and Lsr2 are encoded by plasmids, only the function of Lsr2-like proteins has been

394 employed by phages. Considering the high sequence variability of phage-encoded silencers, these proteins

395 most likely perform many different functions depending on the particular lifestyle of the phage, which

396 needs to be addressed in future studies.

397

398

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633

634 **Tables**

635 **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	[56]
	S. onediensis MR-1	45.9	CP4So (cryptic)	36	43	[59]
MvaT	*P. aeruginosa* PAO1	66.6	Filamentous phage Pf4	15.7	58.7	[54, 55]
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*)	66.2 (62.5*)	[38]
			Prophage region 2, Rv2645-2664, (Rv2658-2659c)	12.3 (1.5*)	66.2 (63.5*)	

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

636

637 **Table 2: Counteracting xenogeneic silencing 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* (enteropathogenic 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. aerigunosa* 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.a.* C600)	H-NS	Phage T7 (virulent)	[78, 99]
ArdA	The crystallization of the ArdA dimer led to the assumption that ArdA is a DNA mimic proteins. Further, 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, 100]

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Figures and figure legends
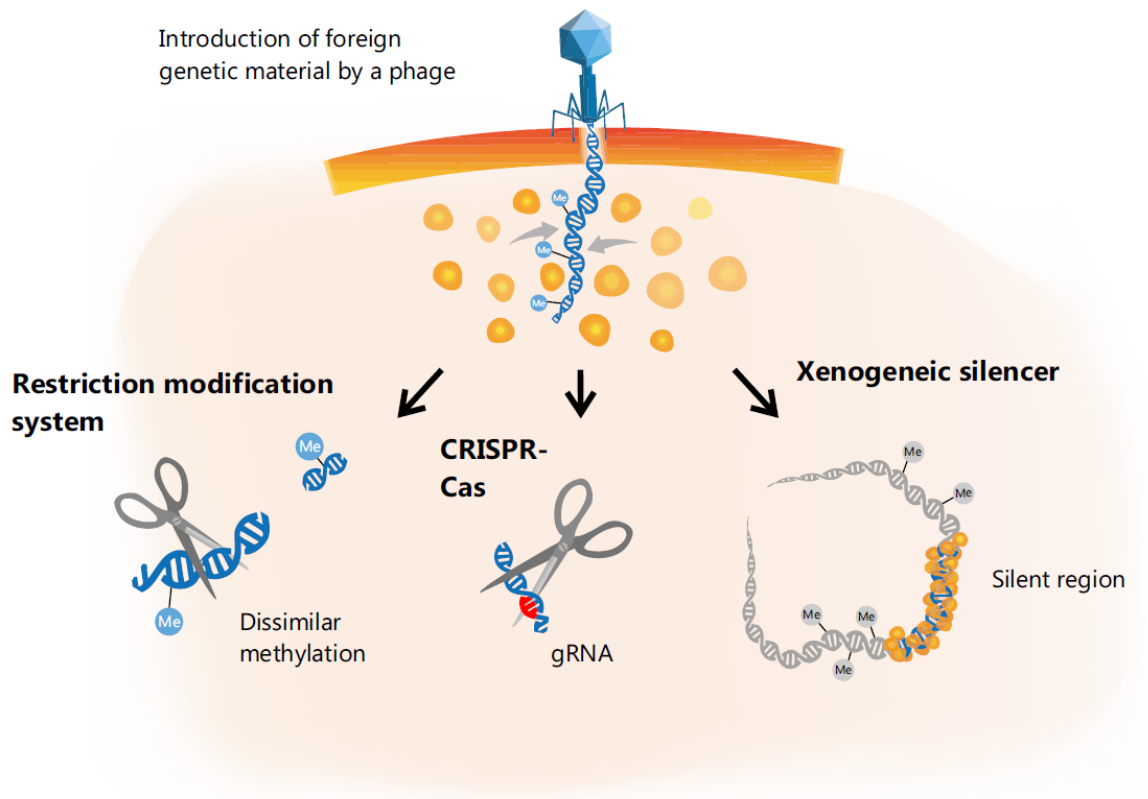
Figure 1: Xenogeneic silencing of foreign DNA. Microbial cells have evolved a variety of different defense mechanisms to deal with viral DNA and to counteract potential detrimental effects. Schematically included examples are the restriction modification (RM) systems and CRISPR-Cas. In contrast, xenogeneic silencing 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].
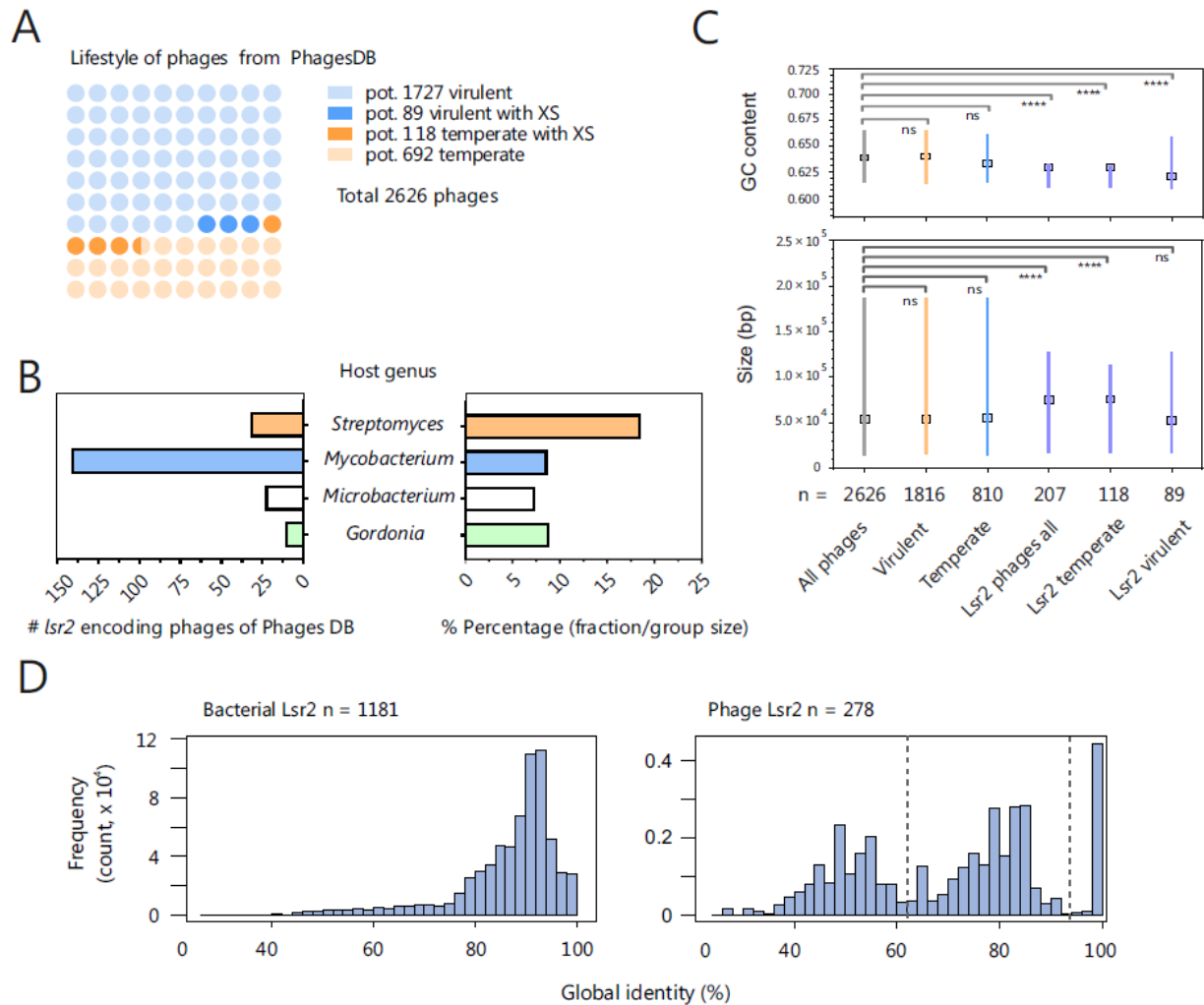
Figure 2. Lsr2-like proteins are encoded on actinobacteriophage genomes. A. Distribution of Lsr2-encoding phages is shown among temperate and virulent actinophages. Based on the genomes downloaded from the actinobacteriophage database PhagesDB [83], coding sequences were predicted by prodigal [101] and the lifestyles of phages were predicted using PHACTS [85]. 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 and 118 temperate phages (orange balls). Phages containing more than one gene

660   encoding an Lsr2-like protein were counted only once; hits found in draft genomes were excluded. **B**.

661   Overview of the host genus of Lsr2-encoding phages. On the left side, the absolute numbers are indicated.

662   The right side of plot shows the proportion of Lsr2-encoding phages among all phages for the respective

663   host genus**. C** GC contents and sizes of temperate, virulent and silencer encoding phages were compared

664   with reference group (all phages) by the Kruskal-Wallis test. The medians are indicated with boxes. In the

665   GC-content plot, the lines represent the interquartile ranges, whereas in the size plot, the lines indicate

666   the range of the minimal and maximal values. **D.** Global pairwise secondary structure identity between

667   Lsr2 sequences encoded in bacterial (left side) and phage genomes (right side). The identity of Lsr2

668   structure within bacterial genomes is relative high (mean ~87%) compared to the phage encoded Lsr2

669   sequences (mean ~70%). Furthermore, the distribution of the phage encoded Lsr2 structure identity

670   evinces a higher diversity by pointing to the existence of particular pairs with high identity to each other

671   and low to the rest of the data set.

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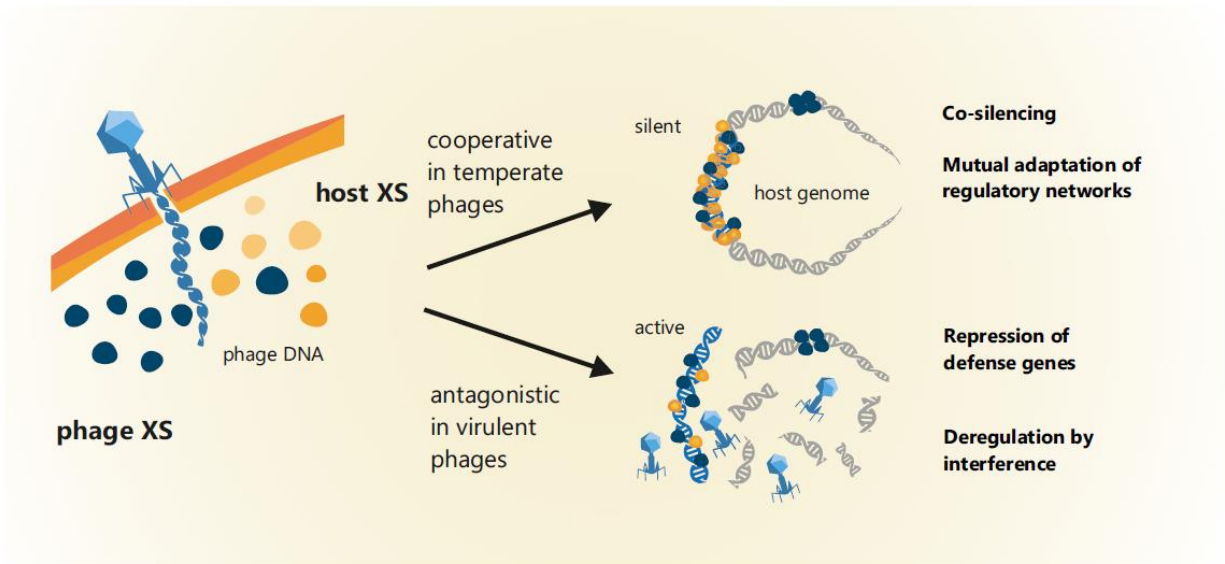
Figure 3. Model for the functions of phage-encoded silencers depending on the phage lifestyle. Examples like the Lsr2-like silencer CgpS demonstrated that XS proteins may play an important role in maintaining the lysogenic state. Depending on the XS repertoire or the particular host strain, (pro-)phage-encoded XS proteins may also cooperate with the host-encoded protein 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.

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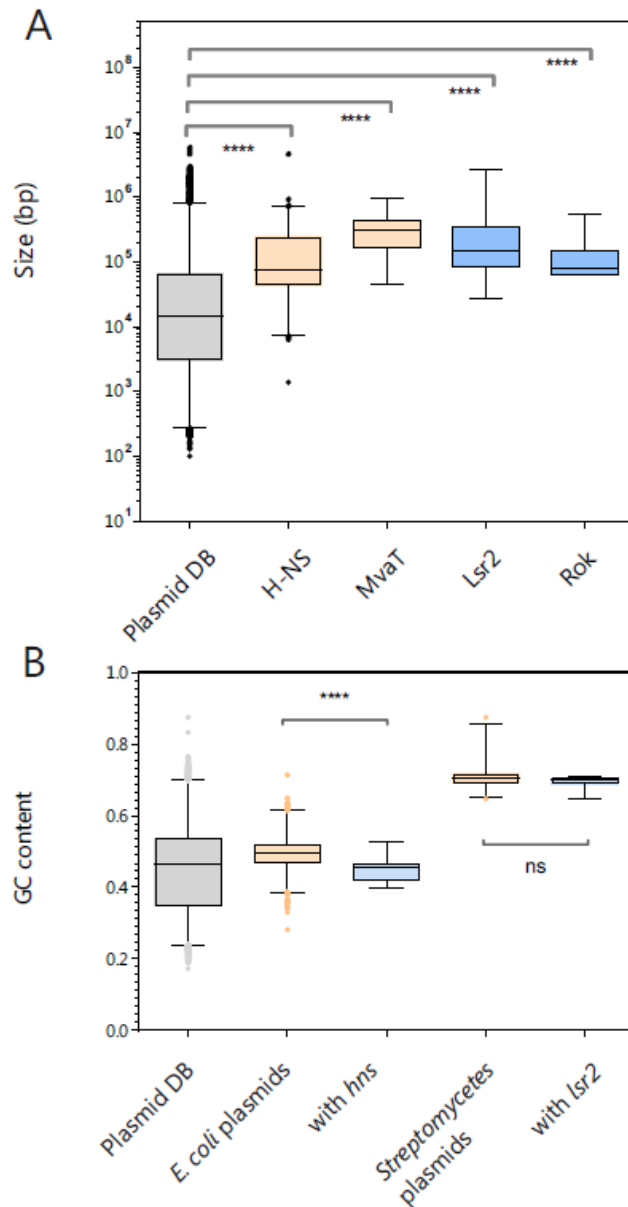
Figure 4: Distribution of silencer-encoding genes on plasmids. A. Overall, 24197 sequences for plasmids were retrieved from the NCBI nucleotide database using RefSeq as the source data base (Filter criteria: Bacteria, genomic DNA, Plasmid, RefSeq on 29.10.2018). Via a local blastp search (e-value < 0.005) 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 1-99% 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*

694   plasmids (n=147) with *lsr2* (13) were compared in a boxplot (range 1-99%, evaluated by Kruskal-Wallis

695   tests).

696 **Supplementary files**

697 Table S1: Lsr2-encoding phages among 2626 temperate and virulent actinophages from PhagesDB.

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