A dual-function phage regulator controls the response of cohabiting phage elements via regulation of the bacterial SOS response

**Highlights**

- *Listeria monocytogenes* strain 10403S harbors two phage elements in its chromosome
- The lytic response of the phage elements is synchronized under SOS conditions
- AriS, a dual-function phage regulator, fine-tunes the elements’ response under SOS
- AriS regulates both its encoding phage and the bacterial SOS response

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**In brief**

Azulay et al. describe a phage factor that regulates cohabiting phage elements by controlling the bacterial SOS response.
A dual-function phage regulator controls the response of cohabiting phage elements via regulation of the bacterial SOS response

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SUMMARY

Listeria monocytogenes strain 10403S harbors two phage elements in its chromosome; one produces infective virions and the other tailocins. It was previously demonstrated that induction of the two elements is coordinated, as they are regulated by the same anti-repressor. In this study, we identified AriS as another phage regulator that controls the two elements, bearing the capacity to inhibit their lytic induction under SOS conditions. AriS is a two-domain protein that possesses two distinct activities, one regulating the genes of its encoding phage and the other downregulating the bacterial SOS response. While the first activity associates with the AriS N-terminal AntA/AntB domain, the second associates with its C-terminal ANT/KilAC domain. The ANT/KilAC domain is conserved in many AriS-like proteins of listerial and non-listerial prophages, suggesting that temperate phages acquired such dual-function regulators to align their response with the other phage elements that cohabit the genome.

INTRODUCTION

Most bacterial pathogens, and bacteria in general, are lysogens, namely, they carry prophages within their genome; in many cases, they carry more than one (Burns et al., 2015; Casjens, 2003). Under stress conditions, the prophages can switch into the lytic cycle, producing infective virions that are released via bacterial lysis, a process referred to as phage induction (Oppenheim et al., 2005). In λ phage, it was shown that this induction is achieved by inactivation of the phage main repressor (the CI repressor), a process that is linked to the bacterial SOS response (Ptashne, 2004). The SOS response is triggered upon severe DNA damage caused, for example, by ultraviolet (UV) irradiation or chemical reagents (e.g., mitomycin C) and is regulated by RecA and LexA (Kreuzer, 2013; Ptashne, 2004; Sutton et al., 2000). RecA binds to single-stranded DNA (ssDNA) at the site of the lesion, forming active RecA-ssDNA complexes (RecA*) that then trigger the autocleavage of LexA, the main repressor of the SOS genes, thereby activating the SOS response (including, for example, umuC, umaD, uvrA, and lexA and recA themselves) (Little, 1991). Notably, RecA* complexes were also shown to trigger the autocleavage of phage repressors, particularly those that share some structural homology with LexA, such as the CI repressor of λ phage, thus triggering phage induction under SOS conditions (Ptashne, 2004; Casjens and Hendrix, 2015). While this mechanism of repressor inactivation was originally identified in lambdoid phages, it is now evident that additional mechanisms exist in lambdoid and non-lambdoid phages that involve anti-repressor proteins that either cleave the main phage repressor, inhibit it via direct binding, or compete with its binding to DNA (Argov et al., 2019; Kim et al., 2016; Lemos Rocha and Blokesch, 2020; Mardanov and Ravin, 2007; Silpe et al., 2020). Importantly, these anti-repressors were still shown to be linked to the SOS response, indicating an intimate interaction between temperate phages and the bacterial SOS system.

The role of the SOS system in phage induction is even more intriguing when considering polylysogenic strains, whose genomes carry multiple prophages and phage-derived elements that can independently trigger bacterial lysis (Burns et al., 2015). In such strains, the SOS system essentially induces all the phage elements that inhabit the genome, activating their lytic cycles and, hence, bacterial killing (Burns et al., 2015). While this scenario may trigger a direct competition between neighboring phage elements (i.e., for the production of progeny), a competition that can lead to the loss of some of them, it can also lead to the development of inter-phage cross-regulatory interactions that support their coexistence. In this regard, previous studies from our group identified such an inter-phage cross-regulatory interaction in the human bacterial pathogen Listeria monocytogenes (Lm) (Argov et al., 2019; Pasechnek et al., 2020; Rabino-vich et al., 2012).

Lm is a saprophyte and a facultative intracellular pathogen that invades a wide array of mammalian cells, including immune cells (Freitag et al., 2009). It resides in the soil and on food products
and infects humans via the consumption of contaminated food. Upon traveling to the gut, the bacteria can colonize the intestine and translocate across the epithelial barrier into the lamina propria and invade phagocytic cells such as macrophages (Cossart, 2011). Upon invasion, the bacteria are initially found in phagosomes, from which they escape into the host cell cytosol in order to replicate (Portnoy et al., 2002). Notably, most Lm strains carry prophages and phage-derived elements in their genome, yet the interaction between these elements and their impact on Lm survival in the mammalian environment, are not well understood. Previous studies from our group discovered that Lm strain 10403S carries two lytic phage elements in its chromosome, which are perfectly adapted to the pathogenic lifestyle of this bacterium (Argov et al., 2019; Pasechnik et al., 2020; Rabinovich et al., 2012). The first element is an infective prophage of the Siphoviridae family (φ10403S) that is integrated within the comK gene, and the other is a highly conserved cryptic phage element that encodes phage tail-like bacteriocins (tailocins), named monocins (Argov et al., 2017b; Lee et al., 2016; Rabinovich et al., 2012; Zink et al., 1995). Under SOS conditions, the two phage elements are simultaneously activated, producing virions and monocins that are released via bacterial lysis, independently driven by each phage element (Argov et al., 2019). Interestingly, upon Lm infection of macrophage cells, the two phage elements are also activated, although their lytic pathway is arrested halfway (i.e., their late genes encoding structural and lysis proteins are not expressed), thereby avoiding the production of virions and monocins, as well as bacterial lysis in the intracellular niche (Argov et al., 2019; Pasechnik et al., 2020). This partial response of the phage elements was shown to promote the excision of φ10403S from the comK gene, yielding an intact comK gene that produces a functional ComK protein (Pasechnik et al., 2020; Rabinovich et al., 2012). In Bacillus subtilis, ComK functions as the activator of the competence system (the com genes) (Dubnau, 1999), yet it was considered non-functional in Lm owing to the phage insertion. Further experiments uncovered that upon Lm invasion into macrophage cells, the phage excises its genome, resulting in the expression of ComK, which in turn activates the expression of the com genes, some of which were found to promote Lm escape from the macrophage phagosome, to the cytosol (i.e., comG and comEC) (Rabinovich et al., 2012). During Lm replication in the macrophage cytosol, the phage DNA, which is maintained as an episome, was found to integrate back into comK, thereby shutting off its transcription in the cytosolic niche (Pasechnik et al., 2020). While these findings revealed an intriguing bacteriophage adaptive behavior that supports the survival of the bacterial host in the mammalian environment, they further demonstrated that prophages can serve as regulatory switches of bacterial genes via a mechanism of excision and re-integration, a phenomenon named active lysogeny (Argov et al., 2017a; Feiner et al., 2015).

It was recently demonstrated by our group that φ10403S is tightly linked to the monocin (mon, in short) element (Argov et al., 2019). Bioinformatic and experimental data uncovered that φ10403S lost its main anti-repressor and became fully dependent on the anti-repressor of the mon element, a metallo-protease named MpaR. MpaR was shown to simultaneously cleave the CI-like repressors of both elements, thereby synchronizing their lytic induction under SOS conditions and during Lm infection of macrophage cells. While the evolutionary forces that drove this inter- phage cross-regulatory interaction remain unclear, this finding implied an intimate interaction between φ10403S and the mon element that requires their coordination. In light of this premise, we hypothesized that the two phage elements further “communicate” via ancillary interactions that balance or align their lytic pathways. To address this hypothesis, we searched for φ10403S genes that concomitantly affect the lytic pathway of the two phage elements under SOS conditions. Using this approach, we identified a phage regulator, named here ArIS, that has the capacity to control the phage elements via regulation of the bacterial SOS response.

RESULTS

φ10403S encodes a protein that blocks the production of virions and monocins

In a search for φ10403S genes that concomitantly affect the lytic pathway of φ10403S and mon, we ectopically expressed specific phage genes, mostly early genes with unknown function, and examined their impact on the elements’ lytic response under SOS. To this end, each gene was cloned into the integrative pPL2 plasmid, under the control of a Tet repressor (TetR)-regulated promoter (PtetR), and introduced into Lm strain 10403S (wild-type [WT] Lm). Bacteria expressing each gene were grown in rich brain heart infusion (BHI) medium and subjected to mitomycin C (MC) treatment to trigger phage and mon induction. It was previously demonstrated that under MC treatment, the bacteria undergo lysis, which is independently driven by each phage element (Argov et al., 2017b, 2019). When monitoring for bacterial lysis under MC treatment, it was noted that a strain overexpressing the early gene LMRG_02920 (annotated in public databases as a putative phage anti-repressor) failed to undergo bacterial lysis like WT Lm (Figure 1A). To examine whether LMRG_02920 expression indeed inhibits bacterial lysis that is driven by each phage element independently, the gene was expressed in bacteria under MC treatment was then evaluated. Ectopic expression of LMRG_02920 effectively prevented bacterial lysis that was driven by each phage element in an independent manner (Figures 1B and 1C). In accordance, expression of LMRG_02920 was associated with reduced virion and monocin release, as determined using a plaque-forming assay and a monocin killing assay, respectively (Figures 1D and 1E). In these experiments, a mutant deleted of φ10403S integrase gene (Jint, which fails to produce infective virions) and a mutant deleted of the mon element (Jmon) were used as controls. Taken together, these findings demonstrated that φ10403S encodes an early protein that has the capacity to concomitantly block the lytic pathway of the two phage elements.

LMRG_02920 inhibits the induction of the two phage elements under SOS

Having discovered this role of LMRG_02920, we tried to relate it to previous data published by our group, characterizing a mutant
deleted of this gene (\textit{ΔLMRG_02920}) (Pasechnik et al., 2020). \textit{ΔLMRG_02920} was shown to exhibit reduced production of virions (\textit{ΔLD} \textit{C24} 60\% less) and a differential transcription profile of the phage compared with WT \textit{Lm}. More specifically, late into the lytic pathway (i.e., 4–5 h post induction), it demonstrated a high transcription of the early genes and a low transcription of the late genes, suggesting that it coordinates the transcription of the phage in the course of the lytic pathway (i.e., tuning down the early genes while upregulating the late genes). While the low production of virions accorded with the low transcription of the late genes, the mechanism by which \textit{LMRG_02920} differentially regulated the phage early- and late-lytic gene modules remained unclear. To gain a better understanding of \textit{LMRG_02920} activity, we next aimed to decipher how it inhibits the lytic pathway of the two phage elements. We first investigated whether \textit{LMRG_02920} interferes with early stages of induction. Quantitative real-time PCR analyses indicated that ectopic expression of \textit{LMRG_02920} largely prevented phage excision, as determined by quantification of the formation of intact \textit{comK} genes (the phage \textit{attB} site), demonstrating that it holds the capacity to attenuate the induction of the phage (Figure 2A). To examine whether this is also the case with the \textit{mon} element (\textit{Δmon}), as a control. A diluted overnight culture of the indicator \textit{Lm} strain ScottA was supplemented with serial dilutions of filtered supernatants containing monocins and grown at 30\% C. Optical density (OD) \textit{600} was measured after 10 h. The error bars represent the standard deviation of three independent experiments.

\textbf{Figure 1. Ectopic expression of \textit{LMRG_02920} inhibits the production of virions and monocins}

(A–C) Growth analysis of WT \textit{L. monocytogenes (Lm)} (A) and mutants harboring deletions of the \textit{\textit{\alpha}10403S-prophage (\textit{Δp})} (B) or of the structuralis module of the monocin element (\textit{\textit{\alpha}LMRG_02368-0278, \textit{ΔStruc-lys}}) (C) with or without ectopic expression of \textit{LMRG_02920} from pPL2-plasmid (pPL2-2920). Bacteria were grown in brain heart infusion (BHI) medium in the presence (+) or absence (-) of MC at 30\% C. The error bars represent the standard deviation of three independent experiments and are sometimes hidden by the symbols.

(D) A plaque-forming assay (PFU) of WT \textit{Lm}, WT \textit{Lm} overexpressing \textit{LMRG_02920} (pPL2-2920), and a mutant deleted of the \textit{\alpha}10403S integrase gene (\textit{LMRG_01511, \textit{ΔIntegrase}}) as a control. Virions obtained from MC-treated bacterial cultures (6 h after MC treatment) were tested on the indicator \textit{Lm} strain Mack861 for plaque formation (numeroned PFUs). The error bars represent the standard deviation of three independent experiments.

(E) A monocin killing assay performed with monocins obtained from MC-treated bacterial cultures (6 h after MC treatment) of WT \textit{Lm}, \textit{Lm} over-expressing \textit{LMRG_02920} (pPL2-2920) and a mutant lacking the \textit{mon} element (\textit{Δmon}), as a control. A diluted overnight culture of the indicator \textit{Lm} strain ScottA was supplemented with serial dilutions of filtered supernatants containing monocins and grown at 30\% C. Optical density (OD) \textit{600} was measured after 10 h. The error bars represent the standard deviation of three independent experiments.
introduced into this strain using the pPL1 integrative plasmid (pPL1-2920), and cleavage of the mon CI-like repressor was monitored during bacterial growth in the presence of MC. The full-length repressor and its cleavage product were detected and quantified using anti-GFP antibodies on western blots. Comparison of the cleaved fraction of the mon CI-like repressor in bacteria expressing versus not expressing LMRG_02920 indicated that LMRG_02920 inhibits the cleavage of the mon CI-like repressor (Figure 2B, right panel). Of note, the western blots also demonstrated that the full-length CI-like repressor accumulates in LMRG_02920-expressing bacteria, indicating that LMRG_02920 somehow stabilizes the mon CI-like repressor (Figure 2B, left panel). To corroborate these findings, we analyzed the transcription level of representative phage and mon genes (e.g., genes encoding capsid, tail, and lysis proteins) under MC treatment in bacteria expressing or not expressing LMRG_02920. In line with the observation that ectopic expression of LMRG_02920 prevented the induction of the two phage elements, their corresponding genes were not expressed (Figure 2C). These results demonstrated that LMRG_02920 holds the capacity to inhibit the induction of the two phage elements.

**LMRG_02920 inhibits the bacterial SOS response**

Given the observation that overexpression of LMRG_02920 inhibits the induction of the two phage elements, we hypothesized that it either interferes with MpaR activity, or operates upstream of MpaR, by inhibiting the bacterial SOS response. To differentiate between these two possibilities, we examined whether LMRG_02920 has any effect on the induction of SOS response. The transcription levels of key SOS genes, i.e., *lexA*, *recA*, and...
Figure 3. Overexpression of LMRG_02920 inhibits the bacterial SOS response

(A) Quantitative real-time PCR analysis of representative SOS genes (recA, lexA, and uvrX/LMRG_02221) in WT Lm and Lm bacteria expressing LMRG_02920 (using pPL2-2920). Indicated strains were grown in BHI medium with MC treatment at 30°C for 45 min. mRNA levels are presented as RQ relative to their levels in WT bacteria. The error bars represent the standard deviation of three independent experiments.

(B) Quantitative real-time PCR analysis of representative SOS genes (recA, lexA, and uvrX/LMRG_02221) in ΔΦ/Δmon and ΔΦ/Δmon bacteria expressing LMRG_02920 (using pPL2-2920). Indicated strains were grown in BHI with MC treatment at 30°C for 45 min. mRNA levels are presented as RQ relative to their levels in WT bacteria. The error bars represent the standard deviation of three independent experiments.

(C) Quantitative real-time PCR analysis of representative SOS genes (lexA and uvrX/LMRG_02221) in WT Lm and lexA3 bacteria expressing or not expressing recA from pPL2 plasmid (pPL2-recA). Indicated strains were grown in BHI medium with MC treatment at 30°C for 45 min. mRNA levels are presented as RQ relative to their levels in WT bacteria. The error bars represent the standard deviation of three independent experiments.

(legend continued on next page)
uvrX, were analyzed under MC treatment in bacteria expressing or not expressing LMRG_02920. As shown in Figure 3A, the transcription level of the SOS genes was significantly reduced (by >80%) in bacteria expressing LMRG_02920, demonstrating that this protein downregulates the SOS response. Notably, a similar downregulation of the SOS genes was observed in Δφl/Lmon bacteria expressing LMRG_02920 (Figure 3B), indicating that this activity of LMRG_02920 is independent of the phage elements. In light of these observations, we speculated that LMRG_02920 targets RecA or LexA, thereby preventing the induction of the SOS response and the induction of the phage elements. To address this hypothesis, we first investigated the role of LexA and RecA in the induction of the phage elements under MC treatment. For this purpose, we used a lexA3 mutant that encodes a non-cleavable variant of LexA (i.e., insensitive to RecA) (Argov et al., 2019; Lin and Little, 1988) and a plasmid ectopically expressing RecA under the regulation of the P\text{ uncomfortable} promoter (pPL2-recA). Transcription of SOS genes and φ10403S virion production were analyzed under MC treatment in lexA3 bacteria expressing or not expressing RecA compared with WT Lm. As expected, lexA3 bacteria exhibited low transcription of SOS genes (shown on uvrX and lexA itself) and produced less virions compared with WT Lm (~80% less) (Figures 3C and 3D). However, when these bacteria ectopically expressed RecA, virion production was restored to nearly WT levels without further activating the SOS genes (Figures 3C and 3D). These results indicated that RecA is the SOS determinant that is responsible for the induction of φ10403S, acting upstream to MpaR. We next investigated whether ectopic expression of RecA can also rescue the inhibition of the phage and the SOS response caused by LMRG_02920. For this purpose, we conjugated WT bacteria with pPL2 plasmids expressing either recA, LMRG_02920, or both genes (under the regulation of the P\text{ uncomfortable} promoter, pPL2-recA, pPL2-2920, and pPL2-recA-2920) and monitored for virion production and SOS gene transcription under MC. Ectopic expression of RecA alleviated all the inhibitory effects of LMRG_02920, bringing phage induction (shown by plaque-forming units [PFUs]) and SOS gene transcription (shown on uvrX and lexA) to WT levels (Figures 3E and 3F). Of note, ectopic expression of RecA in WT bacteria (not expressing LMRG_02920) did not lead to higher virion production or SOS gene transcription, implying that the WT level of RecA protein is sufficient to trigger their full induction. These findings suggested that LMRG_02920 interferes with RecA activity, thereby affecting both the phage elements and the bacterial SOS response. Furthermore, the observation that RecA expression failed to restore the induction of the SOS genes in lexA3 bacteria, yet fully recovered their induction in bacteria expressing LMRG_02920 (Figures 3D and 3F), supported the premise that LMRG_02920 acts as a RecA inhibitor rather than as a LexA corepressor.

To confirm that LMRG_02920 prevents virion production by inhibiting the SOS system rather than by interfering with the phage lytic pathway per se, we compared virion production upon φ10403S infection of Lm bacteria expressing versus not expressing LMRG_02920 using free phage particles, a process that does not require the SOS system. To avoid possible phage immunity or lysogenization, we infected a strain that was cured of the phage and lacked the comK gene (Δφl/comK). In this model, LMRG_02920 expression did not interfere with the phage lytic pathway, as a comparable number of virions were produced in bacteria expressing or not expressing LMRG_02920 (Figure 3G). In contrast, in a control experiment, no virions were detected upon infection of bacteria expressing the phage CI-like repressor (LMRG_01514) instead of LMRG_02920 (using pPL2-1514) (Figure 3G). These results supported the conclusion that LMRG_02920 is a phage protein that targets the bacterial SOS system.

The ANT/KiiAC domain of LMRG_02920 is responsible for inhibition of the SOS response

As mentioned, LMRG_02920 is annotated as a putative phage anti-repressor. It possesses two distinct structural domains (based on the Pfam database), one at the N terminus, belonging to the AntA/AntB family (Pfam database: pfam08346), and the other at the C terminus, belonging to the ANT/KiiAC family (Pfam database: pfam03374) (Figure 4A). While both of these domains are associated with known phage anti-repressors (e.g., Ant1 and Ant2 of E. coli phages 1 and 7 [P1 and P7]) (Hansen, 1989; Iyer et al., 2002; Riedel et al., 1993), their exact function is still unclear. Since our data indicated a novel function for LMRG_02920, i.e., inhibition of the bacterial SOS response, hereafter it is referred to as AriS, for anti-regulator inhibitor of the SOS response. To determine whether AriS is conserved in other listerial comK prophages, we examined all the available complete Listeria genomes that contain a comK prophage (~132 out of 356 complete Listeria genomes that were available) and found that all of them encode an AriS homolog that is located at a similar position in the early-lytic gene module (examples are shown in Figures 4A and S1). Interestingly, based on
Figure 4. Domain architecture in AriS homologous proteins of different phages

(A) Examples of AriS homologous proteins of different Listeria comK-associated prophages.
- AriS (LMRG_02920), Lm 10403S φ10403S
- Gp42, Lm WSCL1118 comK-prophage
- Lmo2324, Lm EGDe comK-prophage
- Lin2418, L. innocua 11262 comK-prophage

(B) Examples of AriS homologous proteins of Listeria non-comK-associated phages.
- Gp37, Listeria phage A500
- SE25_043, Listeria phage vB_LmoS_193
- SE24_035, Listeria phage vB_LmoS_188
- Gp36, Listeria phage A006
- Gp72, Listeria phage B054

(C) Examples of AriS homologous proteins encoded by phages of other Firmicutes species.
- Javan553_0038, Streptococcus phage Javan553
- PHIEF11_0038, Enterococcus phage phiEF11
- Gp35, Clostridium perfringens phage φCP26F
- 55ORF018, Staphylococcus aureus phage φ55
- Javan527_0066, Streptococcus phage Javan527
- Gp07, Staphylococcus aureus phage Phi11
- BJD82_gp39, Staphylococcus phage CNPx

The N- and C-terminal domains shown here are not to scale.
amino-acid-sequence similarity and gene location, we found AriS homologs also in other lyster phages (i.e., non-comK associated) such as A500 (GenBank: NC_009810.1), A006 (GenBank: DQ003642.1), and B054 (GenBank: NC_009813.1) (examples are shown in Figure 4B), as well as in phages of other bacteria of the Firmicutes phylum, e.g., phage Javan553 of Streptococcus suis (GenBank: MK448804.1), phage Ef11 of Enterococcus faecalis (GenBank: NC_013696.1), phage CP26F of Clostridium perfringens (GenBank: NC_019496.1), and phage 11 of Staphylococcus aureus (GenBank: NC_004615.1) (Das and Biswas, 2019) (Figure 4C). Comparing these AriS-like proteins, we noticed that they all possessed a C-terminal ANT/KilAC domain, while their N-terminal domain varied between three different structural domains, AntA/AntB (Pfam database: pfam08346), Bro-N (SMART and COG databases: either smart01040 or COG3617), and pRha (Pfam and COG databases: either pfam09669 or COG3646), all of which are putative regulatory domains that associate with phage and viral regulators (Figures 4A–4C) (Iyer et al., 2002). Intrigued by this finding, we analyzed the distribution of these N-terminal domains in comK prophages and found that the AntA/AntB domain was the most prevalent, found in ~87% of the AriS-like proteins, while Bro-N was found in ~11% of the AriS-like proteins and pRha in only few prophages. To determine which of the two structural domains of AriS drives the SOS-inhibitory activity, we cloned each domain alone, i.e., the AntA/AntB domain (residues 1 to 144) and the ANT/KilAC domain (residues 144 to 259) in pPL2 plasmid, under the regulation of the P_{vir} promoter (pPL2-antA/B and pPL2-kilAC, respectively), and evaluated the ability of each domain to prevent bacterial lysis and to inhibit the SOS response under MC treatment. The results indicated that the ANT/KilAC domain is responsible for the inhibition of the SOS response, as its overexpression effectively prevented bacterial lysis and SOS gene transcription similarly to the AriS protein, whereas overexpression of the AntA/AntB domain failed to do so (Figures 5A and 5B). To examine whether this activity of ANT/KilAC is conserved in other AriS-like proteins, Gp42 of Lm strain WSCL1118 comK phage (sharing 77.48% sequence identity with AriS) (Loessner et al., 2000), comprising AntA/AntB and ANT/KilAC domains, and Lin2418 of L. innocua strain CLIP 11262 comK phage (sharing 30.45% sequence identity with AriS), comprising Bro-N and ANT/KilAC domains (Figures 4A and S1A–S1C), were cloned in the pPL2 plasmid (pPL2-gp42 and pPL2-lin2418, respectively) and expressed in Lm strain 10403S. As shown in Figures 5C and 5D, both proteins effectively prevented bacterial lysis under MC treatment, similar to AriS of φ10403S, therefore demonstrating that the ANT/KilAC SOS-inhibitory activity is conserved. These findings establish a new family of phage regulators that control the bacterial SOS response.

The AriS N-terminal AntA/AntB domain regulates the phage and not the mon
domains
Since the bioinformatic analysis indicated that the N-terminal domains of AriS varies between prophages, we speculated that it holds a distinct activity that is specific to the encoding phage. To assess this hypothesis, we overexpressed the N-terminal AntA/AntB domain of AriS in WT bacteria (using pPL2-antA/B) and examined its impact on the transcription of phage and mon genes under MC treatment. We also examined its influence on phage excision, phage DNA replication, and virion production. Overexpression of AntA/AntB had no effect on the transcription of the mon genes (shown on two representative genes, lmAC and LMRG_02371) or on φ10403S excision (as shown by quantification of intact comK genes/attB sites), indicating that this domain does not affect the induction of the phage elements in contrast to the ANT/KilAC domain (Figures 6A and 6B). However, bacteria expressing this domain exhibited reduced transcription of the phage genes (~50% less), as shown for the early gene LMRG_01516 and the late gene LMRG_01534 encoding a capsid protein (Figure 6C). In accordance, these bacteria demonstrated reduced phage DNA replication (~80% less, shown by quantification of the phage DNA attP site) and ~10-fold less virion production compared with WT bacteria (Figures 6D and 6E). These findings implied that the AntA/AntB domain acts specifically on the phage and not on the mon, regulating its lytic genes post induction.

To examine the impact of each domain on the phage and the mon elements, mutants deleted of each one of AriS domains were generated (∆antA/B and ∆kilAC). The mutants were tested for virion and monocin production and SOS gene transcription under MC treatment compared with ∆ariS and WT Lm. As shown in Figure 7, ∆antA/B and ∆kilAC behaved similarly to ∆ariS, producing fewer virions and a WT level of monocins (Figures 7A and 7B). Moreover, the mutants exhibited a WT level of SOS gene transcription, indicating that AriS or the ANT/KilAC domain fail to inhibit the SOS response under these conditions (shown on uvrX; Figure 7C). Nevertheless, these data demonstrated that under these conditions, the native level of AriS promotes the lytic pathway of the phage. Taken together, these findings characterized AriS as a dual-function phage regulator that holds the capacity to regulate both phage and bacterial genes, which likely evolved to fine tune the response of the phage in the course of the lytic pathway, aligning it with the other phage elements that inhabit the genome.

DISCUSSION
Coordination between cohabiting phage elements in polylysogenic strains is a prerequisite not only for the survival of the phage elements but also for the survival of the bacteria, as it minimizes the bacterial lysis events and maximizes the chance that the elements will produce virions/particles before the host cells lyse. Such coordination requires both bacteria-phage and phage-phage cross-regulatory interactions that collectively align the response of the phage elements with that of the host. In this study, we uncovered yet another example of a cross-regulatory interaction between Lm strain 10403S and its resident phage elements and identified a phage protein that controls the bacterial SOS response, hence holding the capacity to regulate all the phage elements that inhabit the genome. Previous studies from our group indicated that the two phage elements of Lm strain 10403S are evolutionarily interlinked. They are both induced by the same anti-repressor (MpaR) and simultaneously trigger bacterial lysis, hence coconcomitantly releasing virions and monocins to the environment (Argov et al., 2019). While these
findings demonstrated that the lytic cycles of the two phage elements are well coordinated (from start to finish), they raised the hypothesis that the elements further “communicate” via auxiliary cross-regulatory interactions that support their coexistence. With this in mind, we set out to search for phage factors that affect the lytic pathway of the two elements, which led to the discovery of AriS, a conserved phage protein that regulates both phage and bacterial genes.

AriS was identified based on its ability to inhibit the induction of the two phage elements under MC treatment when overexpressed. It was further found to inhibit the bacterial SOS response, independently of the phage elements, indicating that it functions upstream of MpaR, by inhibiting one of the key regulators of the SOS system (e.g., RecA or LexA). Examining the role of RecA and LexA in the induction of the phage elements, we found that RecA, and not LexA, is directly involved in their induction. Further experiments suggested that AriS inhibits RecA (directly or indirectly) thereby preventing induction of the phage elements and the SOS response. In these experiments, ectopic expression of RecA rescued the induction of the phage in lexA3

Figure 5. The ANT/KilAC domain is responsible for the SOS inhibitory activity of AriS
(A) Growth analysis of WT Lm and Lm bacteria overexpressing LMRG_02920 (Lm pPL2-ariS) [the same plasmid as pPL2-2920], the N-terminal AntA/B domain (Lm pPL2-antA/B), or the C-terminal ANT/KilAC domain (Lm pPL2-kilAC). Bacteria were grown in BHI medium in the presence of anhydrotetracycline (AT) to induce the Ptet promoter and in the presence (+) or absence (-) of MC at 30°C. The error bars represent the standard deviation of three independent experiments and are sometimes hidden by the symbols.
(B) Quantitative real-time PCR analysis of representative SOS genes: recA, lexA, and uvrX (LMRG_02221) in ΔΔmon and ΔΔmon bacteria expressing each domain encoded by pPL2-antA/B and pPL2-kilAC plasmids. Indicated strains were grown in BHI medium with MC treatment at 30°C for 45 min. mRNA levels are presented as RQ relative to their levels in WT bacteria. The error bars represent the standard deviation of three independent experiments.
(C) Growth analysis of WT Lm with or without overexpression of Gp42 of the comK-phage of Lm WSLC1118 (Lm pPL2-gp42) in the presence (+) or absence (-) of MC at 30°C. Overexpression of arIS (Lm pPL2-ariS) was used as a control. The experiment was performed three times, and the figure shows a representative result. The error bars represent the standard deviation of three independent experiments and are sometimes hidden by the symbols.
(D) Growth analysis of WT Lm with or without overexpression of Lin2418 of the comK phage of Listeria innocua Clip 11262 (Lm pPL2-lin2418) in the presence (+) or absence (-) of MC at 30°C. Overexpression of arIS (Lm pPL2-ariS) was used as a control. The error bars represent the standard deviation of three independent experiments and are sometimes hidden by the symbols.
bacteria without triggering the SOS response, yet it rescued both the phage induction and the SOS response in bacteria expressing AriS. These findings, together with the observation that RecA is not limited in bacteria not expressing AriS (i.e., in WT bacteria), supported the premise that AriS most likely inhibits RecA rather than acting as a LexA corepressor. Interestingly, this SOS inhibitory activity of AriS was associated with its C-terminal ANT-KilAC domain, a domain that was found to be conserved in many AriS-like proteins of listerial and non-listerial prophages. Examining the AriS-like proteins of Lm strain WSLC1118 and L. innocua

**Figure 6. The N-terminal AntA/AntB domain regulates the phage genes and not the mon genes**

(A) Quantitative real-time PCR analysis of representative mon genes, *ImaC* and *LMRG_02371*, in WT Lm and Lm bacteria expressing each domain separately via pPL2-antA/B and pPL2-kilAC. Indicated strains were grown in BHI medium with MC treatment at 30°C for 3 h. mRNA levels are presented as RQ relative to their levels in WT bacteria. The error bars represent the standard deviation of three independent experiments.

(B) Quantitative real-time PCR analysis of *10403S attB* site (representing the intact comK gene) in WT Lm and in Lm bacteria expressing each domain separately via pPL2-antA/B and pPL2-kilAC. Indicated strains were grown in BHI with MC treatment at 30°C for 3 h. The data are presented as RQ relative to the levels in WT bacteria. The error bars represent the standard deviation of three independent experiments.

(C) Quantitative real-time PCR analysis of representative phage genes: the early gene *LMRG_01516* and the late gene *LMRG_01534* encoding a capsid in WT Lm, and Lm bacteria expressing each domain separately using pPL2-antA/B and pPL2-kilAC. Indicated strains were grown in BHI medium with MC treatment at 30°C for 3 h. mRNA levels are presented as RQ relative to their levels in WT bacteria. The error bars represent the standard deviation of three independent experiments.

(D) Quantitative real-time PCR analysis of the *10403S attP* site in WT Lm and in Lm bacteria expressing each domain separately via pPL2-antA/B and pPL2-kilAC. Indicated strains were grown in BHI medium with MC treatment at 30°C for 3 h. The data are presented as RQ relative to the levels in WT bacteria. The error bars represent the standard deviation of three independent experiments.

(E) A plaque-forming assay of WT Lm and Lm bacteria expressing each domain separately via pPL2-antA/B and pPL2-kilAC. Virions obtained from MC-treated bacterial cultures (6 h after MC treatment) were tested on an indicator strain for plaque formation (numerated as PFUs). The experiment was performed three times, and the error bars represent the standard deviation of three independent experiments. Asterisks represent p values (p < 0.05).
CLIP 11262 comK phages, we demonstrated that both the ANT-KilAC domain and its SOS inhibitory activity are conserved, indicating that inhibition of the SOS response is a common strategy of temperate phages. Moreover, we found the AriS-like proteins to possess a distinct structural domain at their N terminus, which alternates between a set of three phage-related structural domains, AntA/AntB, Bro-N, and pRha. Investigating the N-terminal AntA/AntB domain of AriS, we found that it holds a distinct activity that specifically regulates the phage and not the mon element.

Interestingly, all of these structural domains were suggested to be DNA-binding domains like those that are commonly found in multidomain proteins of large DNA viruses (Iyer et al., 2001, 2002). Comparative studies suggested that these phage-related domains originated from eukaryotic DNA viruses, which carry transcription regulators with a similar domain architecture (Iyer et al., 2002). The phenomenon of combinatorial domain shuffling was also observed in these studies, demonstrating limited sets of domains that combine in regulatory proteins of bacterial and eukaryotic viruses, increasing their diversity and repertoire of activities (Iyer et al., 2002). In line with these reports, our discovery that the alterable N-terminal domain of AriS is the one that specifically regulates the phage (and not the mon or the SOS response) supports the hypothesis that its modularity evolved to fit the encoding phage and not the host, providing a molecular insight into the evolution of the AriS-like proteins. Taken together, the characterization of the two activities of AriS and their corresponding structural domains uncovered yet another mechanism by which prophages can directly and indirectly modulate their lytic response, taking into account the other phage elements that inhabit the genome. In this regard, we hypothesize that AriS did not evolve to completely block the host SOS response under conditions of DNA damage, as it wouldn’t benefit the bacteria or the phage, but rather it evolved to manipulate it post induction, providing the phage with other means to control its lytic pathway.

Figure 7. AriS affects only the phage and not the mon under MC treatment.

(A) A plaque-forming assay of +10403S. Virions were obtained from MC-treated cultures (6 h after the addition of MC) of WT Lm, ΔariS, and bacteria deleted of each AriS domain separately: ΔantA/B and ΔkilAC. Virions were used to infect an indicator strain and are enumerated as PFUs. Error bars represent the standard deviation of three independent experiments.

(B) A monocin killing assay performed with monocins obtained from MC-treated bacterial cultures (6 h after MC treatment) of WT Lm, ΔariS, ΔantA/B, and ΔkilAC. A diluted overnight culture of the indicator Lm strain ScottA was supplemented with serial dilutions of filtered supernatants containing monocins and was grown at 30°C. OD$_{600}$ was measured after 10 h. The error bars represent the standard deviation of three independent experiments.

(C) Quantitative real-time PCR analysis of uvrX in WT Lm and indicated mutants. The strains were grown in BHI medium with MC treatment at 30°C for 45 min. mRNA levels are presented as RQ relative to their levels in WT bacteria. The error bars represent the standard deviation of three independent experiments.
This may explain why the SOS inhibitory activity of AriS was observed only when overexpressed, as the native level of AriS does not have the capacity to prevent the robust induction of the SOS response.

As mentioned, a mutant deleted of the ariS gene was previously generated by our group and investigated for its behavior under SOS conditions (Pasechnik et al., 2020). This mutant exhibited impaired virion production and differential phage gene transcription, demonstrating high transcription of early genes and low transcription of late genes compared with WT bacteria. Interestingly, this differential transcriptional response was detected late into the lytic pathway (i.e., 4–5 h post induction), suggesting that AriS acts as a temporal regulator that fine tunes the phage lytic response (i.e., downregulating the early genes and upregulating the late genes) (Pasechnik et al., 2020). While the mechanism by which AriS executes this activity is still unclear, the results presented here support this hypothesis, as they demonstrate that AriS holds both direct and indirect means of regulating the phage transcriptional response. For example, downregulation of the early genes can be mediated by the ANT/KiIC domain, which attenuates RecA. Unfortunately, we could not differentiate between the two activities of AriS using deletion mutants of each domain as they exhibited phenotypes that were similar to ΔariS. It remains to be determined when and where AriS executes each one of its activities and how they are regulated in the course of the lytic pathway. In this regard, the regulation of the two activities may be even more complex. A previous study identified a tyrosine residue (Tyr98) that was phosphorylated in the AriS-like protein of Lm strain EGD-e-comK phage (sharing 83.40% amino-acid-sequence identity with AriS; Figure S1D) (Misra et al., 2011). In an attempt to examine if this phosphorylation affects AriS activities, we substituted the corresponding tyrosine in the AriS of φ10403S with alanine (AriS-Y99A). Yet, this substitution yielded a non-functional protein that failed to inhibit the SOS response, reminiscent of ΔariS (Figure S2). While this result made it difficult to decipher the effect of this tyrosine on AriS activities, it is still possible that AriS is regulated by protein phosphorylation, a hypothesis that requires further investigation.

Several reports demonstrated phage-mediated manipulation of the SOS system. For example, in Salmonella enterica, it was shown that infection with lytic mutants of P22 and SE1 phages results in the activation of the SOS response, which, in turn, triggers the induction of resident prophages (Campoy et al., 2006). This phenotype was linked to the kil gene, which is carried by both phages. Overexpression of this gene was shown to inhibit bacterial cell division. However, since finding that it activates the SOS response, this phenotype was suggested to relate to the expression of SulA, a known SOS-regulated cell-division inhibitor (Schoemaker et al., 1984). While the biological benefit of SOS induction upon phage infection remains unclear, it was proposed to serve as a fail-proof mechanism to prevent the loss of prophages in the evolutionary arms race with lytic phages (Campoy et al., 2006). Another example of a viral protein that interacts with the SOS system is gp7 of Bacillus thuringiensis temperate phage GIL01. Protein gp7 was shown to directly interact with LexA and act as its corepressor, hence preventing induction of the SOS genes. Notably, in this case, it was shown that GIL01 is missing a CI-like repressor and that it uses the bacterial LexA as its main repressor, albeit only in the presence of gp7. Further, it was demonstrated that gp7 forms a stable complex with LexA, enhancing its binding to the phage operator sites in a way that interferes with its autocleavage by RecA (Caveney et al., 2019; Fornelos et al., 2015). Such a mechanism appears to also exist in other phages, e.g., in CTXφ of Vibrio cholerae, where its XRE-family repressor RstR similarly interacts with LexA (Quinones et al., 2005). As described, our data favor the possibility that AriS inhibits the activity of RecA rather than acting as a LexA corepressor. Although, RecA plays a critical role in the induction of phages, rendering it a perfect target for phage manipulation, we did not find in the literature examples of phage proteins that interfere with RecA activity. Notwithstanding, we found one report on a protein encoded by a conjugative plasmid (PsiB) that inhibits RecA activity, hence enabling recipient cells to accept ssDNA by conjugation without triggering the SOS response, which can be deleterious (Petrova et al., 2009). In light of this example, we surmise that temperate phages also acquired mechanisms to manipulate RecA for their own benefit. The examples presented here suggest that regulation of the SOS system by temperate phages and other mobile genetic elements is a common strategy driving their interaction with the host. In this respect, AriS represents yet another player in the intricate interaction between bacteria and temperate phages.

**Limitations of the study**

In Figure 2B, we observed an accumulation of the mon CI-like repressor in bacteria overexpressing AriS. It appears that AriS stabilizes the mon repressor, and this can be done directly or indirectly. It is possible that there is another interaction between AriS and the mon CI-repressor. It is important to note that the system used in this experiment is, in a way, artificial, as MpaR, AriS, and the mon CI-like repressor are overexpressed in a strain that is deleted of the two phage elements. The purpose of using this system was to examine whether AriS has any effect on the cleavage of the mon CI-repressor, and after calculating the cleaved fraction of the CI-like repressor, the answer was yes. In addition, in this study, we do not provide the precise mechanism by which AriS inhibits RecA and the SOS response. Several attempts to find the direct interactors(s) of AriS using various pull-down techniques have failed. Moreover, the main observations in this study are based on overexpression of AriS and its related domains.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.110723.

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AUTHOR CONTRIBUTIONS

G.A. and A.A.H. designed the study. G.A., A.P., O.S., A.M.F., and N.S. performed the experiments. N.S. validated the results. I.B. performed bioinformatic analysis, and A.A.H. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure diversity in experimental samples through the selection of the genomic datasets. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

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10-2577.
STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| anti-6His tag antibody | Abcam   | ab9108; RRID:AB_307016 |
| Anti-GFP antibody   | BioLegend | 902601 (MMS-118P); RRID:AB_2565021 |
| Bacterial and virus strains | Prof. Daniel Portnoy (University of California, Berkley) | N/A |
| Chemicals, peptides, and recombinant proteins | Sigma | M4287 |
| Bacterial and virus strains | Sigma | 53286-5000G |
| Critical commercial assays | Quantabio | 95047-025 |
| Deposited data | This study | Mendeley Data, V1, https://doi.org/10.17632/63pm8ctx22.1 |
| Software and algorithms | Applied Biosystems | N/A |
| Other | BioTek | N/A |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anat Herskovits (anathe@tauex.tau.ac.il).

Materials availability
This study did not generate new unique reagents. The plasmids and strains used in this study will be made available on request, but we may require a payment and/or a completed Materials Transfer Agreement if there is potential for commercial application.

Data and code availability
- The accession number for the underlying data (source file) of this paper is Mendeley Data doi: https://doi.org/10.17632/63pm8ctx22.1.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains
Listeria monocytogenes (Lm) strain 10403S was obtained from Prof. Daniel Portnoy (University of California, Berkley) and used as the WT strain. Lm 10403S strain cured of DPL-4056 phage was generated by Prof. Richard Calendar (University of California, Berkley) by biological curing. E. coli XL-1 Blue (Stratagene) was utilized for vector propagation. E. coli SM-10 was utilized for conjugative plasmid delivery to Lm bacteria. Lm strains were grown in brain heart infusion (BHI) (Merck) medium at 37°C or 30°C as specified, and E. coli strains were grown in Luria-Bertani (LB) (Acumedia) medium at 37°C.
**METHOD DETAILS**

**Bacterial strains, plasmids, and growth conditions**

*L. monocytogenes* strain 10403S was used as a WT strain and as a parental strain for the generation of all mutants in this study, unless otherwise indicated. *E. coli* XL-1 Blue (Stratagene, Agilent) was utilized for genetic manipulation. *E. coli* SM-10 was utilized for conjugative plasmid delivery to *L. monocytogenes* bacteria. *Listeria* strains were grown in BHI (Merck) medium, at 37°C or 30°C, and *E. coli* strains were grown in Luria-Bertani (LB) (Acumedia) medium at 37°C. Phusion DNA polymerase was used for all cloning purposes and Taq polymerase for verification of the different plasmids and strains by PCR. Antibiotics were used as follows: chloramphenicol (Cm), 10 μg/mL; streptomycin (Strep), 100 μg/mL; kanamycin (Km), 30 μg/mL; and mitomycin C (MC) (Sigma), 1.5 μg/mL. All restriction enzymes were purchased from New England BioLabs. Strain, plasmids and primers used in this study are described in Table S1.

**Bacterial lysis under MC treatment**

Bacteria were grown overnight (O.N.) at 37°C, with agitation, in BHI broth, and then diluted to an OD at 600 nm (OD600) of 0.15 and pipetted in triplicates into a 96-well plate with or without MC (1.5 μg/mL). The plates were incubated at 30°C, in a Synergy HT BioTek plate reader, and the OD600 was measured every 15 min after 2 min of shaking. All experiments were repeated at least three times.

**Plaque-forming assay**

Bacteria were grown O.N. at 37°C, with agitation, in BHI broth, then diluted by a factor of 10 in fresh BHI broth, incubated without agitation at 30°C, to reach an OD600 of 0.4, then diluted to an OD600 of 0.15, and the lytic cycle was induced by the addition of MC (1.5 μg/mL) and incubation at 30°C for 6 h, without agitation. Bacterial cultures were then passed through 0.22 μm filters that do not allow the passage of bacteria. Dilutions of the filtered supernatants (100 μL) were added to 3 mL melted LB-0.7% agar medium at 56°C, supplemented with 10 mM CaCl2, and 300 μL of an O.N. culture of *L. monocytogenes* Mack861 or Δp/ΔcomK (used as indicator strains), and quickly overlaid on BHI-agar plates. Plates were incubated for 3–4 days at room temperature to allow plaques to form.

**Monocin killing assay**

10403S *Lm* bacteria were grown O.N. at 37°C, with agitation, in BHI broth, then diluted 1:10 in BHI broth, incubated without agitation at 30°C to reach an OD600 of 0.4, and diluted again to an OD600 of 0.15. Thereafter, the lytic cycle was induced by the addition of MC (1.5 μg/mL) and bacteria were incubated for 6 h. Bacterial cultures were then passed through 0.22 μm filters. An O.N. culture of *Lm* strain ScottA was inoculated 1:100 in BHI medium supplemented with indicated dilutions of the monocins filtrate obtained after lytic induction of *Lm* strain 10403S. Technical duplicates were pipetted into 96-well plates and were incubated at 30°C in a Synergy HT BioTek plate reader. OD600 was measured every 15 min after 2 min of shaking. The OD of the cultures after 10 h of incubation was plotted against the monocin concentration. All experiments were repeated at least three times.

**Generation of gene deletion mutants, complementation and overexpression strains**

To prepare gene deletion mutants, upstream and downstream regions of the target gene were amplified using Phusion DNA polymerase, and cloned into the pBHE vector (qKSV-ornt). Cloned plasmids were verified by PCR and their PCR-amplified inserts were sequenced. The plasmids were then conjugated to *L. monocytogenes* using the *E. coli* SM-10 strain. Transconjugants were selected on BHI agar plates supplemented with chloramphenicol and streptomycin and transferred to BHI supplemented with chloramphenicol. Of note, once pPL2 is integrated in the bacterial chromosome, sensitive colonies were validated for gene deletion by PCR. For overexpression strains, the gene was cloned into the pPL2 integrative vector under regulation of the PTetR promoter (Pasechnik et al., 2020). Of note, once pPL2 is integrated in the bacterial chromosome, no antibiotic selection is used. The PCR-amplified insert was sequenced, and the plasmid was conjugated to *L. monocytogenes* strain 10403S. Transconjugants were selected on BHI plates and on BHI plates supplemented with chloramphenicol. The sensitive colonies were validated for gene deletion by PCR. For overexpression strains, the gene was cloned into the pPL2 integrative vector under regulation of the PTetR promoter (Pasechnik et al., 2020). Of note, once pPL2 is integrated in the bacterial chromosome, no antibiotic selection is used. The PCR-amplified insert was sequenced, and the plasmid was conjugated to *L. monocytogenes* strain ScottA was inoculated 1:100 in BHI medium supplemented with indicated dilutions of the monocins filtrate obtained after lytic induction of *Lm* strain 10403S. Technical duplicates were pipetted into 96-well plates and were incubated at 30°C in a Synergy HT BioTek plate reader. OD600 was measured every 15 min after 2 min of shaking. The OD of the cultures after 10 h of incubation was plotted against the monocin concentration. All experiments were repeated at least three times.

**Quantitative real-time PCR analysis**

Bacteria were grown at 37°C O.N., with agitation, in BHI broth, then diluted 1:10 in BHI broth, incubated, without agitation, at 30°C to reach an OD600 of 0.4, and then diluted to an OD600 of 0.15. Thereafter, a lytic cycle was induced by addition of MC (1.5 μg/mL). Bacteria were harvested by centrifugation at indicated time points and snap-frozen in liquid nitrogen. Total nucleic acids were isolated using standard phenol-chloroform extraction methods. For analysis of attB levels by RT-qPCR, 0.04 ng total nucleic acids were used, while the *Lm* 16S rRNA gene was used as a reference for sample normalization. For gene expression analysis, the samples were treated with DnaseI, and 1 μg RNA was reverse-transcribed to cDNA using a qScript (Quanta) kit. RT-qPCR was performed on 10 ng cDNA. The relative expression of bacterial genes was determined by comparing their transcript levels with those of the *Lm* 16S rRNA or rpoD gene, which served as a reference. All RT-qPCR analyses were performed using the PerfeCTa SYBR Green
FastMix (Quanta) on the StepOnePlus RT-PCR system (Applied Biosystems), as per the standard ΔΔCt method. Statistical analysis was performed using StepOne V2.1 software. Error bars represent the 95% confidence interval.

**Analyzing mon CI-like repressor cleavage by western blot**

The mon-Cl-like repressor was tagged by translational fusion of GFP to the C-terminus of the protein under the regulation of a constitutive promoter (Argov et al., 2019). The tagged CI-like repressor was cloned on the integrative pPL2 plasmid harboring the MpaR protease, under the regulation of the inducible PtetR Promoter. The resulting plasmid (pPL2-mon-Cl-gfp-mpaR) was delivered by conjugation into ΔφλΔmon L. monocytogenes bacteria. For co-expression of LMRG_02920, the gene under the regulation of the inducible PtetR promoter was cloned on the integrative pPL1 plasmid harboring a kanamycin-resistance gene for selection, and the resulting plasmid (pPL1-LMRG_02,920) was delivered by conjugation into ΔφλΔmon Lm + pPL2-mon-Cl-gfp-mpaR. Bacteria were grown at 30°C, in 100 mL BHI broth, to OD600 of 0.3. The cultures were then supplemented with MC (1.5 µg/mL) and anhydro-tetracycline (AT) (100 ng/mL), and grown for an additional 2.5 h, harvested, washed with Buffer A (20 mM Tris-HCl pH = 8, 0.5 M NaCl, and 1 mM EDTA), resuspended in 1 mL Buffer A supplemented with 1 mM PMSF, and lysed by ultra-sonication. Total protein content was quantified using a modified Lowry assay, and samples with equal amounts of total proteins were separated on 15% SDS-polyacrylamide gels, and then trans-blotted onto nitrocellulose membranes. Proteins were probed with rabbit anti-GFP antibodies (BioLegend 902601) at a 1:50,000 dilution, for the detection of mon-Cl-GFP, followed by HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, USA) at a 1:20,000 dilution. Western blots were developed using homemade enhanced chemiluminescence (ECL) detection reagents. Images were obtained using an Amersham Imager 600 (GE Healthcare Life Sciences).

**Genome analysis of Listeria comK-associated prophages**

A number of complete Listeria genus and individual Listeria species genomes were retrieved from the NCBI Genomes database (https://www.ncbi.nlm.nih.gov/genome accessed on November 1, 2021). Particularly, the website Genome Assembly and Annotation report (https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/159/accessed on November 1, 2021) provides a list of both complete genomes and incomplete genome assemblies of Listeria monocytogenes strains. The above-mentioned website also provides a filter option by which, one can analyze exclusively complete genomes of L. monocytogenes (for that “Chromosome” and “Complete” should be selected); this approach revealed a number of complete genomes of other Listeria species (e.g. L. innocua, L. seeligeri, L. ivanovii). To determine the number of complete Listeria genomes that carry intact comK-associated prophages, we developed an approach that is based on a comK-phase-specific query (the integrate gene sequence of the Listeria phage A118) and the customized Nucleotide BLAST machine setup at the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on November 1, 2021), using the following filters: i) Nucleotide collection (nr/nt) database (consists of GenBank + EMBL + DDBJ sequences, excluding WGS data); ii) Max target sequences (500); iii) Expect threshold (either 10 or 100). The result of this search yielded a list of complete genomes of Listeria species as well as the two known Listeria phages, A118 and PSU-VKH-LP019, that possess an entire sequence of the integrate gene. A similar approach was used to verify a number of complete Listeria genomes using the nucleotide sequence of either dnaA or dnaN gene of the reference L. monocytogenes strain 10403S. As for May 2021, 356 complete Listeria genomes were identified, of which 132 were found to contain a prophage in the comK gene. Having these genomes the sequences of the comK-associated prophages were extracted and further analyzed.

**Bioinformatic analysis of AriS homologs**

The different functional domains of AriS and its homologues were identified using the Pfam 34.0 protein family database (http://pfam.xfam.org/accessed on November 1, 2021), the integrated resource of Protein Domains (InterPro) (http://www.ebi.ac.uk/interpro/accessed on November 1, 2021), the database of protein families and domains PROSITE (https://prosite.expasy.org/accessed on November 1, 2021), and the SMART database (http://smart.embl-heidelberg.de/accessed on November 1, 2021). BLAST analyses of protein amino acid sequences were performed using the NCBI server (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on November 1, 2021) (Altschul et al., 1990). BlastP search for AriS homologues distinguished in their N-terminal sequences was conducted by using the following Reference Sequences as queries: WP_014601097.1 (AнтA/AнтB/pfam08346 and KилAК/pfam03374), WP_031644323.1 (Bro-N/COG3617 and KилAК/COG3645), WP_010991177.1 (COG3617 and KилAК/COG3645), WP_050018552.1 (COG3561 and KилAК/pfam03374), and WP_003731503.1 (Phage_pRha/pfam09669 and KилAК/COG3645).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All data are presented as mean of three biological repeats (n = 3) ± 1 standard deviation, unless indicated otherwise. Statistical significance was calculated using Student’s t test. For western blotting analysis a representative experiment is shown, and additional biological repeats can be found in the provided raw data file. Details of statistical analysis can be found in the Figure Legends.
A dual-function phage regulator controls
the response of cohabiting phage elements
via regulation of the bacterial SOS response

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Figure S1. Sequence alignments of AriS homologs of different *comK*-associated phages. A. Clustal W alignment of deduced amino acid sequences of AriS (LMRG_02920; *comK*-prophage, *L. monocytogenes* strain 10403S), Gp42 (*Listeria* phage A118), and Lin2418 (*comK*-prophage, *L. innocua* strain CLIP 11262). Alignment length: 264 residues. Identical amino acids (28.03%) are shown in red and marked with asterisks, highly similar residues (25.00%) in green and marked with colons, and weakly similar residues (9.09%) in blue and marked with dots; different residues (37.88%) in black and unmarked. Tyrosine residues of AriS and Gp42 (Y99 and Y102, respectively) that were predicted to be phosphorylated are shown in bold and underlined. The glutamine Q144 of AriS (in bold and yellow highlighted) was the last residue of the recombinant amino-terminal domain expressed by pPL2-antA/B. The sequence alignment was performed using the public server of the PRABI Rhone-Alpes Bioinformatics Center (France) running the Clustal W program (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html), and then manually curated.

B. Clustal W alignment of deduced amino acid sequences of AriS (LMRG_02920; *comK*-prophage, *L. monocytogenes* strain 10403S) and Gp42 (*Listeria comK* phage A118). Alignment length: 262 residues. Identical amino acids (77.48%) are shown in red and marked with asterisks, highly similar residues (11.45%) in green and marked with colons, and weakly similar residues (3.82%) in blue and marked with dots; different residues (7.25%) in black and unmarked. Tyrosine residues of AriS and Gp42 (Y99 and Y102, respectively) that were predicted to be phosphorylated are shown in bold and underlined. The glutamine Q144 of AriS (in bold and yellow highlighted) is the last residue of the recombinant amino-terminal domain expressed in pPL2-antA/B. The basic sequence alignment was performed using the public server of the PRABI Rhone-Alpes Bioinformatics Center (France) running the Clustal W program (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html), and then manually curated.
C. Clustal W alignment of deduced amino acid sequences of AriS (LMRG_02920; comK-prophage, L. monocytogenes strain 10403S) and Lin2418 (comK-prophage, L. innocua strain CLIP 11262). Alignment length: 266 residues. Identical amino acids (30.45%) are shown in red and marked with asterisks, highly similar residues (24.81%) in green and marked with colons, and weakly similar residues (12.03%) in blue and marked with dots; different residues (32.71%) in black and unmarked. A tyrosine residue (Y99) of AriS that was predicted to be phosphorylated is shown in bold and underlined. The glutamine Q144 of AriS (in bold and yellow highlighted) is the last residue of the recombinant amino-terminal domain expressed by pPL2-antA/B. The basic sequence alignment was performed using the public server of the PRABI Rhone-Alpes Bioinformatics Center (France) running the Clustal W program (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html), and then manually curated.

D. Clustal W alignment of deduced amino acid sequences of AriS (LMRG_02920; comK-prophage, L. monocytogenes strain 10403S) and Lmo2324 (comK-prophage, L. monocytogenes strain EGD-e). Alignment length: 259 residues. Identical amino acids (83.40%) are shown in red and marked with asterisks, highly similar residues (7.34%) in green and marked with colons, and weakly similar residues (3.86%) in blue and marked with dots; different residues (5.41%) in black and unmarked. Tyrosine residues of AriS and Lmo2324 (Y99 and Y98, respectively) that were predicted to be phosphorylated are shown in bold and underlined. The glutamine Q144 of AriS (in bold and yellow highlighted) is the last residue of the recombinant amino-terminal domain expressed by pPL2-antA/B. The basic sequence alignment was performed using the public server of the PRABI Rhone-Alpes Bioinformatics Center (France) running the Clustal W program (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html), and then manually curated. Related to Figure 4.
Figure S2

**A.** RT-qPCR analysis of representative SOS genes (recA, lexA and uvrX) in ΔΦ/Δmon and ΔΦ/Δmon bacteria expressing AriS-Y99A (using pPL2-ariS-Y99A). Indicated strains were grown in BHI medium with MC treatment, at 30 °C, for 45 min. mRNA levels are presented as relative quantity (RQ), relative to their levels in WT bacteria. The error bars represent the standard deviation of three independent experiments.

**B.** Growth analysis of WT Lm with and without overexpression of AriS-Y99A (Lm pPL2-ariS-Y99A) in the presence (+) or absence (−) of MC, at 30 °C. Overexpression of AriS (Lm pPL2-ariS) was used as a control. The experiment was performed three times and the figure shows a representative result. The error bars represent the standard deviation of three independent experiments, and are sometimes hidden by the symbols.

**C.** Virions obtained from MC-treated cultures (6 h after MC treatment) of WT Lm, a deletion mutant of the AriS gene (ΔariS) or Lm bacteria possessing the AriS-Y99A mutation in the prophage genome (i.e., in the Lm genome) were tested on an indicator strain for plaque formation (numerated as plaque-forming units, PFUs). The error bars represent the standard deviation of three independent experiments.

**D.** Western blot analysis comparing the protein levels of His-tagged AriS and AriS-Y99A proteins expressed using the pPL2 plasmid. Bacteria were grown in BHI medium for 6 h. Equal amounts of total proteins were separated on 15% SDS-PAGE, blotted and probed with anti-His tag antibody. Related to Figure 7.