Prophages encode phage-defense systems with cognate self-immunity

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In brief
Prophages can encode abortive infection proteins that give their bacterial hosts population-level phage defense. Owen et al. show that some of these proteins contain internal self-immunity systems so that the prophage is not self-targeted by its own phage-defense protein.

Highlights
- BstA is an abortive infection protein found in prophages of Gram-negative bacteria
- *aba*, a short DNA sequence within the *bstA* locus, acts as a self-immunity element
- *aba* gives BstA-encoding prophages immunity to BstA-driven abortive infection
- Variant BstA proteins have distinct and cognate *aba* elements
SUMMARY

Temperate phages are pervasive in bacterial genomes, existing as vertically inherited islands termed prophages. Prophages are vulnerable to predation of their host bacterium by exogenous phages. Here, we identify BstA, a family of prophage-encoded phage-defense proteins in diverse Gram-negative bacteria. BstA localizes to sites of exogenous phage DNA replication and mediates abortive infection, suppressing the competing phage epidemic. During lytic replication, the BstA-encoding prophage is not itself inhibited by BstA due to self-immunity conferred by the anti-BstA (aba) element, a short stretch of DNA within the bstA locus. Inhibition of phage replication by distinct BstA proteins from Salmonella, Klebsiella, and Escherichia prophages is generally interchangeable, but each possesses a cognate aba element. The specificity of the aba element ensures that immunity is exclusive to the replicating prophage, preventing exploitation by variant BstA-encoding phages. The BstA protein allows prophages to defend host cells against exogenous phage attack without sacrificing the ability to replicate lytically.

INTRODUCTION

The eternal battle between bacteria and their viruses (phages) has evolved the evolution of a diverse array of phage-defense systems in bacteria (Bernheim and Sorek, 2020; Hampton et al., 2020; Rostøl and Marraffini, 2019; van Houte et al., 2016). Conversely, it is increasingly recognized that phages have evolved mechanisms to subvert these defense systems (Maxwell, 2017; Samson et al., 2013; Trasanidou et al., 2019).

Although the most intuitive form of phage defense involves the direct rescue of an infected cell, for example, by targeted degradation of phage nucleic acids by CRISPR-Cas or restriction modification systems, many phage-defense systems function solely at the population level. In a mechanism conceptually analogous to the pathogen-stimulated programmed cell death driven by the innate immune systems of higher organisms (Abedon, 2012), phage infection can be prevented from sweeping across populations, at the cost of the lives of infected cells. These population-level phage-defense systems are often grouped together under the umbrella term “abortive infection” (Abi) (Labrie et al., 2010; Lopatina et al., 2020) but actually represent diverse mechanisms to prevent phage replication and induce cell death (Bingham et al., 2000; Cohen et al., 2019; Fineran et al., 2009; Meeske et al., 2019; Pecota and Wood, 1996; Watson et al., 2019). Such mechanistic diversity and the high prevalence of abortive infection systems in nature emphasizes the selective advantage the Abi strategy imparts in the battle against phages (Benler and Koonin, 2020).

However, an important sub-plot in the bacteria-phage conflict is the widespread existence of so-called “temperate” or “lyso- genic” phages within bacterial genomes. Temperate phages exist stably within the bacterial chromosome as latent, vertically inherited islands known as prophages. Crucially, to find new hosts, prophages must escape the bacterial genome and return to the lytic life cycle.

The prophage state imposes unique existential pressures because the fitness of the phage is indefinitely dependent on that of the host bacterium. To favor their own fitness, prophages frequently encode “moron” or “accessory” loci that modulate the biology of their host bacteria (Bondy-Denomy and Davidson, 2014; Cumby et al., 2012; Fortier and Sekulovic,
An important trait conferred by prophages that can significantly increase bacterial fitness is resistance against bacteriophage attack. Indeed, recent work has suggested that prophage accessory genes are an underexplored reservoir of phage-defense systems (Bondy-Denomy and Davidson, 2014; Dedrick et al., 2017; Snyder, 1995).

Here, we report a phage-defense system driven by the BstA protein, which is encoded by prophages of diverse Gram-negative bacteria. When a bacterium harbors a BstA-encoding prophage, the BstA protein confers effective population-level defense against exogenous phage infection via abortive infection. The bstA locus includes an anti-BstA element, which suppresses the activity of the BstA protein to allow the native prophage to switch to a lytic lifestyle. We propose that this self-immunity mechanism has evolved to allow prophages to defend host cells from predatory phages without compromising their own lytic replication cycle.

## RESULTS

### The BstA protein encoded by prophage BTP1 mediates phage defense

*Salmonella enterica* subsp. *enterica* serovar Typhimurium (hereafter *S. Typhimurium*) strain D23580 carries the ~40 kb prophage BTP1 (Owen et al., 2017). An operon within BTP1, the *gtr* locus (*gtrAC<sup>BTP1</sup>), confers resistance against phage P22 by chemically modifying the cellular lipopolysaccharide (LPS), the receptor for phage P22 (Kintz et al., 2015). Therefore, unsurprisingly, deleting the BTP1 prophage from strain D23580 (D23580<sup>D</sup>BTP1) made the strain highly susceptible to infection by phage P22, confirming that resistance to phage P22 is conferred by BTP1 (Figure 1B).

However, inactivation of the *gtr* locus of prophage BTP1 (D23580<sup>D</sup>tsp<sup>-</sup>*gtrAC<sup>BTP1</sup>) did not restore sensitivity to phage P22 to the level of D23580<sup>D</sup>BTP1 (Figure 1B), suggesting the existence of a second BTP1-encoded phage-resistance system.

2013; Howard-Varona et al., 2017), a phenomenon likened to altruism (Shub, 1994).

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Here, we report a phage-defense system driven by the BstA protein, which is encoded by prophages of diverse Gram-negative bacteria. When a bacterium harbors a BstA-encoding prophage, the BstA protein confers effective population-level defense against exogenous phage infection via abortive infection. The bstA locus includes an anti-BstA element, which suppresses the activity of the BstA protein to allow the native prophage to switch to a lytic lifestyle. We propose that this self-immunity mechanism has evolved to allow prophages to defend host cells from predatory phages without compromising their own lytic replication cycle.
Previously, we used transcriptomics to discover that the bstA gene was highly expressed from prophage BTP1 during lysogeny, making it a candidate phage accessory gene (Owen et al., 2020). The bstA gene, encoded downstream of the prophage cl repressor locus, has been implicated phenotypically in both virulence and anti-virulence of Salmonella isolates, but no functional mechanism has been proposed (Herrero-Fresno et al., 2014, 2018; Spiegelhauer et al., 2020), and the BstA protein has not been characterized. We hypothesized that bstA was the second element in the BTP1 prophage that conferred defense against phage P22.

Consistent with this hypothesis, removal of the bstA gene from prophage BTP1 (D23580 ΔbstA) dramatically increased susceptibility to phage P22 (Figure 1B). To confirm that phage resistance was directly mediated by BstA protein, we introduced two stop codons into the beginning of the bstA coding sequence by exchanging 4 nucleotides (D23580 bstASTOP) (Figure 1C). D23580 bstASTOP was highly susceptible to P22 phage, to the same level as D23580 ΔbstA, demonstrating that the BstA protein mediates defense against phage P22. Simultaneous deletion of the gtr locus and inactivation of the BstA protein (D23580 ΔgtrΔgtrACBTP1 bstASTOP) recapitulated the susceptibility to phage P22 achieved by deleting the entire BTP1 prophage (D23580 ΔBTP1), indicating that resistance to phage P22 was solely mediated by the bstA and gtrAC loci in prophage BTP1. These findings were reproduced by assaying the replication of phage P22 on the same strains in liquid culture, quantitatively demonstrating that reduction of plaque formation by BstA truly reflected suppression of phage replication (Figure 1D).

To investigate whether the defense function of the BstA protein depended on other elements from the BTP1 prophage, we constructed an inducible expression system in S. Typhimurium strain LT2, which does not contain the BTP1 prophage. LT2 is the type strain of S. Typhimurium and is natively susceptible to many phages, including P22 (McClelland et al., 2001). Expression of the BstA BTP1 protein in S. Typhimurium LT2 from within a neutral position on the chromosome (LT2 tetR-PbstA-bstA) conferred resistance to P22 and other phages, including ES18 and 9NA (Figures 1E and S1A).

While the induced expression of BstA BTP1 completely eliminated plaque formation of sensitive phages, at very high phage concentrations (109–1010 plaque forming units [PFU]/mL), these phages still produced clearing of the bacterial lawn (Figure S1B), which is consistent with an abortive infection mechanism of phage defense. The expression of the derivative containing two stop codons in the bstA coding sequence (bstASTOP) conferred no phage resistance, demonstrating again that defense is mediated by bstA at the protein level (Figure S1C). However, BstA did not mediate resistance against all of the tested phages. Det7, Felix O1, and notably, phage BTP1 (which encodes the bstA gene) were unaffected by the expression of BstA, both at the level of plaque assay and replication in liquid culture (Figures 1E and S1A). Induction of bstA or bstASTOP expression in the absence of phage infection did not cause a detectable effect on cell growth rate, suggesting that overexpression of BstA BTP1 does not cause toxicity (Figure S1D). We were unable to detect any pattern in the characteristics or gene repertoire of phages that were sensitive or insensitive to BstA protein, which could relate to the mechanistic action of BstA protein.

**BstA represents a family of prophage-encoded phage-defense proteins in diverse Gram-negative bacteria**

Having established that BstA functions as a prophage-encoded phage-defense system, we sought to further characterize the evolutionary conservation of this protein. We identified BstA homologs in the genomes of diverse Gram-negative bacteria (Table S1) and compiled a dataset of 72 homologs representative of phylogenetic diversity. The majority (79%) of BstA homologs co-occurred with phage genes and were designated as putatively prophage associated (Figure 2A). No known phage-associated genes were found in the vicinity of 21% (15 of 72) of BstA homologs, which were defined as putatively prophage independent. A small subset of BstA homologs were plasmid encoded (Figure 2A). Strikingly, in many cases, BstA homologs were located downstream of putative prophage repressor proteins, mirroring the genetic architecture of BstA BTP1 (Figure 2B). We conclude that the BstA protein is highly associated with prophages of Gram-negative bacteria.

While the BstA protein does not exhibit sequence homology to any functionally characterized proteins, remote homology detection methods revealed a KiiA-N (-like) domain in the N-terminal region (residues 32–147 of BstA BTP1) (Figure 2C). Although poorly characterized, the KiiA-N domain is found in proteins from phages and eukaryotic DNA viruses and contains the helix-turn-helix motif characteristic of DNA-binding proteins (Iyer et al., 2002; Medina et al., 2019).

Certain residues in the BstA protein are highly conserved among homologs from diverse members of the alpha, beta, and gamma proteobacteria (Figures 2C and S2A). A small number of BstA homologs only exhibited homology to the N-terminal, KiiA-N (-like) domain. A second small group of homologs were only homologous to the C-terminal region of BstA (shown at the bottom of the alignment in Figure 2C). Such bipartite protein homology suggests that the BstA protein is composed of two functional domains. This conclusion is independently supported by evolutionary covariance analysis (Figure S2B), where the clear depletion of predicted residue contacts between the ranges 1 to ~155 and ~156–307 of BstA BTP1 suggests that there is a domain boundary (Rigden, 2002) around position 155, with the two folded domains making few contacts.

We selected two diverse BstA homologs from Klebsiella pneumoniae (BstAKp, 48.4% amino acid identity to BstA BTP1) and E. coli (BstAEc, 41.7% amino acid identity) to investigate the phage-resistance function of the larger BstA protein family (the native genetic context of these homologs is illustrated in Figure 2B, and their identity to BstA BTP1 is highlighted in the alignment in Figure 2C). We engineered inducible expression systems mirroring the expression construct previously validated for BstA BTP1 (Figures 3A, 3B, and S1C). The expression of BstAKp and BstAEc in S. Typhimurium LT2 conferred resistance to Salmonella phages at a similar level to BstA BTP1, despite these BstA homologs only sharing around 40% identity at the amino acid level (Figures 3A, 3B, and S1E). Unlike BstA BTP1, BstAKp and BstAEc prevented the replication of phage P22 (which encodes bstAPBTP1).
Finally, we tested the function of BstA against well-characterized coliphages. Heterologous expression of BstA BTP1 in E. coli strain MG1655 conferred resistance to phage λ, φ80, P1, and T7 but did not affect phages T4 and T5 (Figure 3C). Surprisingly, we found that BstA Ec was slightly less active against coliphages than BstA BTP1 (Figures 3C and S1E). Replication in liquid culture was a more reliable and reproducible measure of phage susceptibility than plaque assay and frequently revealed stronger resistance phenotypes than by plaque assay (Figures S1F and S1G).

Figure 2. BstA homologs are found in diverse bacterial taxa and are frequently associated with prophages
(A) A dataset of 72 BstA homologs representative of taxonomic diversity were manually curated and analyzed for prophage association based on the co-occurrence of phage-related Pfam domains in the 20 kb either side of each homolog (yielding a total 40 kb window) (Table S1). Homologs without co-occurring phage-related protein domains were assigned to be “putatively prophage independent.” A further subset of the BstA homologs were encoded on plasmids. The top ten most commonly co-occurring Pfam domains with prophage-associated and putatively prophage-independent BstA homologs are shown as bar graphs. (B) Gene maps showing the genetic context of a selection of 6 prophage-associated and 6 putatively prophage-independent BstA homologs (homologs indicated by the gray rectangle). Putative prophage repressor genes are highlighted in red. The top three BstA proteins from BTP1 (BstA BTP1, blue), K. pneumoniae 52.145 (BstA Kp, green), and E. coli NCTC10963 (BstA Ec, orange) are studied experimentally in later stages of this work and therefore are highlighted. Open reading frames associated with functional annotations are shown as solid black arrows, and functional gene names or Pfam domains are annotated.
(C) An alignment of the 72 BstA protein homologs to BstA BTP1, with colors indicating amino acid conservation (Clustal color scheme). Alignment columns containing gaps relative to the reference sequence (BstA BTP1) have been collapsed and are indicated with blue lines and triangles at the base of the alignment (an expanded alignment can be found in Figure S2A). The position of BstA BTP1 and BstA Ec within the alignment is highlighted. The position of the KiA-N (like) domain (BstABTP1 residues 32–147) is indicated by a gray box. Heatmaps on the left of the alignment indicate the prophage and plasmid association of each homolog (lanes 1 and 2) and the taxonomic group each homolog derives from (lane 3). Phage association was split into high and low confidence based on gene co-occurrence criteria (STAR Methods).
We conclude that BstA represents a family of phage-resistance proteins associated with the prophages of diverse Gram-negative bacteria.

**BstA mediates effective population-level phage defense through abortive infection**

Phage-resistance systems operate via diverse functional mechanisms (Hampton et al., 2020; Rostøl and Marraffini, 2019). We used microscopy to dissect BstA-mediated phage resistance. Virulent P22 phages (P22Δc2) were used to infect *Salmonella* cells with and without native BstA BTP1, at high multiplicity of infection (MOI) to ensure that most cells were infected. We were surprised to observe that independent of BstABTP1, all cells lysed within the time course of 3 h (Figure 4A; Video S1), and BstA BTP1 did not appear to confer any direct protection from phage infection at the level of individual infected cells. We conducted the same experiment in liquid culture, measuring phage replication and the fraction of surviving cells post-phage infection. In cells possessing functional BstA (D23580Δtsp-gtrAC), phage P22 Δc2 completely failed to replicate (Figure 4B). In the absence of BstA function (D23580Δtsp-gtrAC bstASTOP), the phage replicated >100-fold. However, despite preventing the replication of phage P22, BstA BTP1 had no effect on cell survival: independent of BstABTP1 function, only 1%–2% of cells survived following P22 infection (Figure 4C). We hypothesized that BstA does not protect single cells and instead mediates phage defense at the population level by sacrificing the life of the infected cell.

To investigate the BstA-protein-mediated population-level phage defense, we conducted a second microscopy experiment, wherein approximately only 1 in every 1,000 cells was infected with phage P22. Unlike culture in liquid media, our microscopy setup involved immobilization of cells on agarose pads, which allows only local movement of phage particles. The spread of infection was tracked as primary infected cells lysed and produced secondary infections in neighboring cells. To visualize these phage epidemics, we used a reporter phage engineered to encode the red fluorescent protein mCherry within the early lytic operon (P22Δc2 P-mCherry); the fluorescence signal indicated phage replication (Figure 4D).

In the population lacking functional BstA BTP1 (D23580Δtsp-gtrAC bstASTOP), primary infected cells lysed after around 30 min (Figure 4D; Video S2). Subsequently, the red fluorescence signal was observed in neighboring cells, revealing secondary infection, followed by cell lysis, a cycle that repeated until all cells in the radius of the primary infected cell had lysed, reminiscent of plaque formation (Figure 4D). The impact of the epidemic of phage infection upon bacterial cells lacking BstA BTP1 can be visualized in Video S2.

In contrast, in the D23580Δtsp-gtrAC population, no secondary infections were observed in the neighboring cells following the lysis of the primary infected cells. Instead, cells continued to divide normally, eventually forming a confluent lawn (Figure 4D; Video S2). The lack of subsequent rounds of infection after the primary cell lysis events shows that few or no infectious phage particles were generated.

Taken together, these experiments demonstrate that the BstA protein inhibits successful phage replication, but it does not prevent the death of the infected cell. Therefore, BstA provides phage defense at the population level and prevents the spread of phage epidemics. Accordingly, we propose that BstA is an abortive infection system.
BstA protein responds dynamically to phage infection and colocalizes with phage DNA

To explore the molecular activity of BstA during phage infection, we first constructed a functional translational fusion of the BstABTP1 protein to superfolder green fluorescent protein (sfGFP) (Figure S3A). We then used time-lapse fluorescence microscopy to observe the dynamics of the BstA protein inside individual cells during infection with two BstA-sensitive phages, P22 and 9NA. In the absence of phage infection, the BstA protein was distributed diffusely within the cytoplasm of the cells, suggesting no particular subcellular localization (Figures S3B and S3C; Video S3). However, approximately 20 min after infection with phages P22 and 9NA, we consistently observed BstA protein aggregating into discrete foci toward the center of infected cells (Figure S3C; Video S3). Cell lysis occurred approximately 40 min after the formation of BstA foci.

We speculated that the dynamic establishment of foci by BstA in response to phage infection was likely to reflect the mechanistic activity of the protein. We noticed that the foci dynamics of BstA proteins during phage infection resembled phage replication foci (Cenens et al., 2013; Trinh et al., 2017). Therefore, we speculated that the focus of the BstA protein in phage-infected cells might correspond to the replicating DNA.

Figure 4. BstA mediates population-level phage defense through abortive infection

BstA protein does not protect individual cells from phage infection

(A) Cells natively expressing BstA (D23580 Δtap-grtAC, JH4287) or possessing a mutated BstA locus (D23580 Δtap-grtAC bstASTOP, SNW431) were infected with the obligately virulent P22-derivate phage, P22 Δc2, at an MOI of 5 to increase the likelihood of infecting all cells. Infected cells were imaged on agarose pads and the images represent a time series. Regardless of BstA function, almost all cells were observed to lyse (indicated by loss of defined cell shape and phase contrast). Videos of the time series are presented in Video S1. A scale bar representing 10 μm is shown in the first image for each series.

(B) A phage replication assay showed that P22 Δc2 phage failed to replicate after 3-h growth on the BstA+ strain (D23580 Δtap-grtAC) but replicated ~136-fold when BstA was inactivated (D23580 Δtap-grtAC bstASTOP).

(C) Survival assay of the same strains after infection by phage P22 Δc2, at an MOI of 5. Consistent with the microscopy data in (A), BstA function did not affect cell survival from phage infection. D23580 Δc2 (P22) (SSO-128), a phage P22 lysogen (and therefore natively resistant), was used as a negative control. Data in (B) and (C) are presented as the mean of biological triplicates ± SD.

(D) A fluorescent reporter module for phage replication was added to P22 Δc2 (P22 Δc2 P-mCherry) so that phage replication yielded red fluorescence (mCherry was inserted downstream of the replicative genes of the phage). A similar experiment to (A) was conducted, but P22 Δc2 P-mCherry infected cells were mixed 1:1,999 with uninfected cells. In the BstA+ cells, primary infected cells lysed but did not stimulate secondary infections of neighboring cells, and they eventually formed a confluent lawn. In BstA cells, primary lysis events caused secondary infections (neighboring cells showing red fluorescence and subsequent lysis), causing an epidemic of phage infection reminiscent of plaque formation. Cartoons schematize the outcomes of these experiments in the two strain backgrounds. Representative micrographs of each time series are shown. Videos of the time series are presented in Video S2. All experiments were carried out in liquid or solid M9 Glu+ media. A scale bar representing 30 μm is shown in the first image for each series of micrographs.
In summary, our data are consistent with a model that involves the movement of BstA protein to sites of phage DNA replication inside infected cells, followed by prevention of phage replication.

**BstA phage-resistance systems contain anti-BstA elements (aba) that suppress the activity of BstA**

When characterizing the sensitivity of different phages to the activity of BstA<sub>BP1</sub> (Figure 1E), we observed that phage BTP1 (which itself encodes the BstA<sub>BP1</sub> gene) was not affected by heterologous expression of BstA<sub>BP1</sub>, while the replication of phage P22 was inhibited. cbr-(B) Schematic of the BTP1-derived phages used and the corresponding effect on sensitivity to BstA<sub>BP1</sub> expression (plaque assay).

In trans. Prophage induction was measured in strain D23580<sup>ΔTn</sup>21(Ap<sup>S</sup>) lysogenized with BTP1 WT (aba<sup>WT</sup>) or BTP1 abamu<sup>1</sup> strain (SNW576 for (B) and (C) and strains JH4400 (BstA<sup>BTP1++</sup>), JH4404 (BstA<sup>Kp++</sup>), or JH4408 (BstA<sup>Ec++</sup>) for (F).

(F) Each bstA locus encodes a homolog-specific anti-BstA element (aba) that suppresses BstA-mediated phage defense. Transfer of each bstA locus to phage P22 only confers immunity against the cognate BstA protein. Plaque assays were carried out with the indicated phages, applied on mock-induced (BstA<sup>/C0</sup>) or AHT-induced (BstA<sup>++</sup>) lawns of the indicated strain: SNW576 for (B) and (C) and strains JH4400 (BstA<sub>BP1</sub>+), JH4404 (BstA<sub>Kp</sub>+), or JH4408 (BstA<sub>Ec</sub>+)+) for (F).
To identify the genetic basis of BstA self-immunity, we constructed a series of BTP1 mutant phages, carrying truncations of different lengths from the 3’ end of the bstA locus (Figure S4A) and screened these phages for the ability to replicate in the presence of BstA\textsuperscript{BTP1} expression. Self-immunity (i.e., insensitivity to BstA\textsuperscript{BTP1} expression) was preserved in all mutant phages except the mutant with the longest bstA truncation (BstA\textsuperscript{BTP1} bstA\textsuperscript{134}) in which just the first 24 bp of the bstA reading frame were intact (Figure S4A). A similar truncation mutant containing just the first 34 bp of bstA (BTP1 bstA\textsuperscript{134}) retained immunity to BstA, suggesting that the first 34 bp of the bstA gene are essential for the activity of the anti-BstA determinant. The transfer of bstA\textsuperscript{134} (the first 34 bp of bstA, along with the upstream sequence) to phage P22 (P22 bstA\textsuperscript{134}) conferred BstA immunity (Figure S4B). To identify the minimal sequence required for BstA self-immunity, we further constructed P22 bstA\textsuperscript{334}-derived phages, successively truncating the transferred sequence from the 5’ end (P22 bstA\textsuperscript{334-2} bstA\textsuperscript{334V}, Figure S4B). We discovered that a 63 bp sequence (GCCGGCCACATTTTAAAGGAAAT CAAATGGTTATCGAATAAGGCTATCCATACCCGGCC) spanning 29 bp of the upstream region and the first 34 bp of the bstA coding sequence (start codon underlined) was necessary and sufficient to confer the self-immunity (Figure 5C). We designated this element “aba,” for anti-BstA. Supplying the 63 bp aba sequence on the high-copy-number pUC18 plasmid (pUC18-aba) rescued P22 phage replication in the presence of BstA protein, demonstrating that the self-immunity effect of aba is retained in trans (when aba is not carried by the targeted phage but is supplied on another replicative element) (Figure S5A). The intracellular localization of the BstA protein following phage infection was unaffected by the presence of the pUC18-aba plasmid (Figure S6B).

The aba element appears to be DNA based

In the native BTP1 prophage, the aba sequence overlaps the start of the bstA gene, preventing mutational disruption of the aba element without modification of the BstA protein sequence. Therefore, we used the plasmid trans-complementation system (wherein the BstA protein and the aba sequence are independently encoded) to probe the function of the aba sequence (Figure S5A). A notable feature of the aba sequence is the presence of a direct “CCCGCC” repeat at the terminal ends, which we hypothesized was functionally important. Single-nucleotide exchange of the CCCGCC → CCCTCC in the first and second repeat (aba\textsuperscript{mut1} and aba\textsuperscript{mut2}, respectively) abolished the self-immunity function of the aba element, both when located on a phage (Figure 5B) and from a plasmid in trans (Figure S5C), showing that the aba terminal direct repeats are required for aba function. Plasmid-borne expression of BstA efficiently suppressed plaque formation of P22 and BTP1 phages lacking a functional aba sequence (P22 WT, BTP1 bstA, or BTP1 aba\textsuperscript{mut2}) but had no effect on BTP1 WT, which natively encodes aba (Figure S5D).

The aba plasmid trans-complementation system additionally allowed us to interrogate the genetic nature of the aba element, which we hypothesized was either DNA, RNA, or peptide based. Although three short open reading frames exist within the aba sequence, nonsynonymous mutation of the reading frames did not ablate aba function (Figure S6A), suggesting the aba-driven immunity is not mediated by a small peptide.

Second, we investigated whether the aba element is DNA or RNA based by assessing whether transcription of aba is necessary for suppression of BstA. The aba sequence was cloned into the high-copy pUC18 vector with no promoter and flanked by terminators to abrogate transcription (Figure S6B). This created a scenario with high-copy aba DNA and minimal aba transcription. In parallel, we inserted the aba sequence into the Salmonella chromosome downstream of the arabinose-inducible $P_{BAD}$ promoter (2D3580 $\Delta b$ tetR-bstA\textsuperscript{BTP1} $P_{BAD}$-aba-gfp; Figure S6B). In this scenario, aba exists as a single copy of DNA but is highly transcribed. In both plasmid and chromosomal constructs, a gfp gene was transcriptionally fused to the aba sequence to report the level of transcription. Our chromosomal $P_{BAD}$-aba-gfp construct generated a high level of green fluorescence in our assay conditions, whereas fluorescence was barely detectable for our plasmid-based aba constructs (Figure S6B), demonstrating that much more aba RNA is transcribed from the single-copy chromosomal construct than the high-copy plasmid construct. We assessed the activity of BstA in both scenarios by challenging the cells against phages P22 and 9NA. aba only functioned to suppress the activity of BstA (i.e., allow plaquing of P22 and 9NA) in the high-copy DNA, low-transcription scenario, suggesting that the aba element is DNA based. However, a single chromosomal copy of aba did not confer self-immunity (Figure S6B), suggesting that aba DNA can only suppress BstA when supplied on high-copy replicative elements. Further mutational disruption of the aba sequence revealed that the self-immunity function was sensitive to mutation at multiple sites in the 63 bp sequence (Figure S6C).

Collectively, our data suggest that aba-driven suppression of BstA is neither peptide nor transcript mediated, and supports a model where BstA suppression is mediated by aba DNA.

The aba element prevents the bstA-encoding prophage from aborting its own lytic replication

Unlike most mechanistically characterized abortive infection systems, a unique feature of the BstA system is its frequent occurrence on prophages (Figure 2A). Prophages must be able to switch to lytic replication, or else the prophage state becomes an evolutionary dead end for the phage.

We hypothesized that the primary biological role of the aba element is to allow the endogenous bstA-encoding phage to escape BstA-mediated inhibition upon induction from the prophage state. To test this, we measured the level of induction of prophage P22 in the presence of heterologously expressed BstA\textsuperscript{BTP1} protein (Figure 5D). In the absence of BstA\textsuperscript{BTP1} expression, the P22 prophage generated a titer of $4 \times 10^9$ PFU/mL after 5-h growth with an inducing agent (Mitomycin C, MitC). However, with BstA\textsuperscript{BTP1} expression, the MitC-induced titer of P22 dropped $>300$-fold to $\sim 1 \times 10^5$ PFU/mL, showing that BstA inhibited P22 phage replication. The transfer of the aba sequence to prophage P22 (P22 aba) significantly increased the induced titer in the presence of BstA\textsuperscript{BTP1} to $\sim 1.5 \times 10^7$, restoring it to the level seen in the absence of BstA and showing that the aba element rescues prophage induction via suppression of BstA.

Finally, we validated the importance of the aba element in the context of native BTP1 prophage induction. The presence of additional copies of the aba sequence in trans on the pUC18 plasmid...
Finally, we determined whether the elements Distinct BstA proteins are associated with cognate is required for the bstAaba element. When the Cell Host & Microbe and therefore that variant BstA proteins have cognate bacteria. We challenged the P22 could suppress the activity of variant BstA proteins of other elements (Figure 5 F). To test this hypothesis, we engineered P22 BstA-respective upstream sequence). Consistent with a cognate ment, where the infecting phage integrates into the genome of development, where the infecting phage integrates into the genome of the bacterium, was affected by BstA expression. We used an antibiotic-tagged derivative of P22 (P22mid in the presence of BstA to BstA-negative levels (Figure 6D). (pP22-aba
 element that is inactive (BTP1aba
 tetR-PtetA-bstABTP1), we first tested the replication of the BstA-sensitive virulent phage, 9NA. In the absence of BstA expression, the level of phage 9NA DNA gradually increased over a 50-min infection time course, reflecting successful phage replication (Figure 6B). However, no accumulation of phage 9NA DNA was observed in the presence of BstA
, suggesting that BstA protein strongly inhibited the replication of phage DNA.

Consistent with the self-immunity function of aba, BTP1 phage DNA replication was not affected by the expression of BstA
, unless the aba element was nonfunctional (BTP1 aba
 tetR-PtetA-bstABTP1) (Figure 6C). Likewise, successful replication of phage P22 DNA in the presence of BstA
 only occurred when the phage possessed a functional aba element (Figure 5B).

To confirm that BstA protein inhibits DNA replication, we constructed small phage-derived plasmids ("phagemids") based on the phage P22 replication module (pP22) (Figure 6D) and a P22 phagemid that included the 63 bp aba sequence (pP22-aba).

Salmonella cells were transformed with the phagemids in the presence or absence of BstA
 protein expression. In the absence of BstA, the stable replication of both P22 phagemids in Salmonella cells generated >10^6 transformants/ng phagemid. However, the expression of BstA
 reduced the transformation efficiency of P22 (lacking the aba sequence) to around 10^6 transformants/ng. The addition of the aba sequence to the phagemid (pP22-aba) restored the transformation efficiency of the phagemid in the presence of BstA to BstA-negative levels (Figure 6D).

We conclude that phage DNA replication is strongly suppressed by BstA, but replication can be rescued by the aba element, presumably by suppression of BstA protein activity. As replicated phage DNA is an essential substrate for packaging into phage capsids, the inhibition of DNA replication is likely to prevent the production of infectious progeny phages, consistent with the observation that infectious phages are not released from BstA-expressing cells following cell lysis (Figure 4). We propose that BstA protein mediates abortive infection by suppressing phage DNA replication, a process that can be circumvented by the native prophage carrying the aba self-immunity element.

**DISCUSSION**

Here, we have discovered a family of prophage-encoded abortive infection proteins (BstA), which efficiently defend bacterial did not affect the titer of BTP1 phage generated after 5-h growth with an inducing agent (MitC), suggesting that native levels of the BstA protein do not constrain BTP1 prophage induction in the presence of the native, functional aba element (Figure 5E). However, when the aba
 mutation (exchange of a single functionally important nucleotide in the terminal direct repeat) was introduced into the BTP1 prophage, the MitC-induced titer of phage BTP1 was reduced ~40-fold in the presence of native BstA expression. This reduction was almost entirely rescued when the aba sequence was supplied in trans on the pUC18 plasmid, confirming that the aba
 mutation ablates the function of the aba element. When the aba
 mutation was introduced into the BTP1 prophage in the absence of native BstA protein expression (D23580 ΔΔφ [BTP1 aba
 bstABTP1]) there was no effect on prophage induction (Figure S6D), confirming that the effect of the aba
 mutation is dependent on the presence of BstA.

These experiments demonstrate that a functional aba element is required for the bstA-encoding prophage to switch from a lysogenic to lytic lifestyle. In the absence of aba, the bstA-encoding prophage suffers replication inhibition by endogenous BstA protein (self-targeting), presumably by the same abortive infection mechanism that inhibits exogenous phage infection.

**Distinct BstA proteins are associated with cognate aba elements**

Finally, we determined whether the aba sequence from bstABTP1 could suppress the activity of variant BstA proteins of other bacteria. We challenged the P22 bstABTP1 phage (immune to expression of BstA
 due to the presence of aba
) against expression of BstA Ec or BstA Kp. The bstABTP1 locus did not protect P22 from the variant BstA proteins, suggesting that the aba element from bstABTP1 only confers immunity against BstA Ec and therefore that variant BstA proteins have cognate aba elements (Figure 5F). To test this hypothesis, we engineered P22 phages to encode either bstA Ec or bstA Kp loci (including the respective upstream sequence). Consistent with a cognate BstA-aba interaction, P22 bstA Ec became specifically immune to expression of BstA Ec, and P22 bstA Kp gained specific immunity to BstA Kp expression (Figure 5F).

We conclude that while BstA proteins are broadly functionally interchangeable in terms of their phage-defense activity, each bstA locus contains a cognate aba element that is inactive against variant BstA proteins. The specificity of the aba self-immunity element means that phages encoding bstA variants are unable to bypass all BstA-mediated abortive infection, making aba-mediated suppression of BstA exclusive to the corresponding induced bstA-encoding prophage.

**BstA protein does not affect phage lysogenic development but inhibits DNA replication during lytic development**

To interrogate how the BstA protein interacts with infecting bacteriophages, we determined whether lysogenic phage development, where the infecting phage integrates into the genome of the bacterium, was affected by BstA expression. We used an antibiotic-tagged derivative of P22 (P22 Apic::aph) to determine the frequency of lysogeny with and without BstA expression. We found that the frequency of lysogeny was approximately 6% (Figure 6A) regardless of the presence of BstA, suggesting that BstA expression does not affect phage lysogenic development. This finding suggests that BstA activity is triggered by, or targets, an aspect of phage lytic replication not shared by lysogenic development. Further, it implies that BstA has no effect on the initial stages of phage infection that occur prior to lysogenic development, i.e., adsorption and DNA translocation.

The sequence-based analysis of BstA protein homologs suggested that the N-terminal domain may bind DNA (Figure 2C), and fluorescence microscopy showed BstA protein colocalizing with phage DNA (Figure 3D). The replication of DNA is crucial for phage morphogenesis, as a new copy of the phage chromosome is required for packaging into the capsid of each new virion. To test whether BstA protein inhibits phage DNA replication during lytic development in a manner that can be suppressed by aba, we conducted Southern blot experiments to monitor levels of phage DNA during infection. Using our prophage-negative, inducible BstA-expression strain (D23580 ΔΔφ tetR-Faba-bstABTP1), we first tested the replication of the BstA-sensitive virulent phage, 9NA. In the absence of BstA expression, the level of phage 9NA DNA gradually increased over a 50-min infection time course, reflecting successful phage replication (Figure 6B). However, no accumulation of phage 9NA DNA was observed in the presence of BstA
, suggesting that BstA protein strongly inhibited the replication of phage DNA.

Here, we have discovered a family of prophage-encoded abortive infection proteins (BstA), which efficiently defend bacterial
populations from phage epidemics. The BstA protein is constitutively expressed inside cells that carry the prophage and provides effective population-level phage defense through abortive infection, inhibiting phage replication at the cost of the viability of individual infected cells. Possession of such innate phage-defense systems by active prophages imposes the following challenge: the prophage must avoid self-targeting by its own defense system when switching to lytic replication.

The BstA system solves this problem with the aba element (anti-BstA), a co-encoded short DNA sequence that specifically suppresses the activity of BstA protein upon prophage induction, giving the induced prophage self-immunity against endogenous BstA protein. Theoretically, such a system might leave BstA-expressing cells vulnerable to infection by other BstA-encoding phages, which could use their own aba elements to bypass native BstA. This problem is avoided by cognate BstA-aba pairs, as each BstA protein is suppressed only by the cognate, co-encoded aba element, ensuring that BstA suppression is specific to the native BstA-encoding prophage.

Although we present a high-level overview of the BstA phage-defense system and the corresponding anti-BstA-aba element, we are left with two major questions regarding the activity of the BstA protein. First, what are the phage determinants for BstA sensitivity? Although BstA was active against approximately 50% of the phages tested, we did not detect similarities between BstA-targeted and non-targeted phages that could reflect the molecular determinants of sensitivity. It is possible that rather than responding to a physical phage stimulus, such as phage DNA or protein, BstA protein responds to a cellular stimulus produced by the infection of specific types of phages, for example, the recruitment of DNA replication machinery.

Second, what is the molecular mechanism by which BstA protein inhibits phage DNA replication? Our data suggest that phage DNA does not replicate in the presence of BstA. Although numerous Abi systems in Lactococcus have been proposed to interfere with phage DNA replicative functions (Chopin et al., 2005), the molecular mechanisms have not been well characterized. The existence of a putative DNA-binding domain in BstA proteins and the microscopic observation of BstA colocalization with phage DNA make it tempting to speculate that BstA interacts physically with phage DNA to prevent replication, for example, by occlusion of a replication initiation site.
Alongside the mechanistic details of the BstA protein that remain to be established, little is known about the interaction of BstA with the aba element. Our data show that aba interacts with BstA in DNA form, but the mechanism by which aba DNA suppresses the BstA protein is unclear. Our findings indicate that multiple copies of the aba element are required to suppress BstA protein in trans. However, copy number cannot be the only factor affecting aba functionality because a prophage is evidently able to suppress the BstA protein right from the initial stages of prophage induction, when aba is present as just a single copy on the chromosome. While we did not observe a loss of BstA focus formation during suppression with high-copy aba DNA, we are cautious to interpret this as evidence against a direct interaction between aba DNA and BstA protein. It remains possible that the phage-DNA colocalization behavior and abortive activity of BstA proteins are mechanistically uncoupled (indeed, BstA is predicted to contain two domains; Figure 2) or that reduction in focus formation is beyond the sensitivity of our microscopy methods. Further study of the BstA-aba system is required to resolve the precise molecular mechanisms by which BstA-encoding prophages, such as BTP1, achieve self-immunity.

We consistently observed that phage-infected cells that contained BstA protein underwent lysis, probably in the absence of infectious progeny phage release. However, we cannot be certain whether the BstA protein acts actively or passively to cause cell lysis. Abi systems have frequently been termed “altruistic suicide” systems, which mediate “programmed cell death” in response to phage infection (Abedon, 2012; Shub, 1994). While perhaps a useful conceptual analogy for the strictly population-level effect of Abi systems, this narrative implies that Abi systems actively cause cell death. Although this may often be the case, such as in the CBASS system (Cohen et al., 2019), Abi can also be achieved by simple disruption of the phage replication pathway. Because phage lysis is generally a temporarily programmed event that occurs independently of successful virion morphogenesis (Cahill and Young, 2019), phage-mediated cell lysis can occur in the absence of virion assembly. For example, many Lactococcus Abi systems target aspects of phage replication, such as AbiZ, which is thought to interact with phage holin proteins, to stimulate premature cell lysis before virion assembly is completed (Durmaz and Klaenhammer, 2007).

It is possible that the BstA protein simply inhibits viable phage particle formation, while allowing the phage lytic pathway to proceed unperturbed to cell lysis. However, inhibition of phage DNA replication would dramatically reduce substrates for transcription and translation of phage lysis gene products; yet, we did not observe a difference in the timing of cell lysis for phage-infected cells in the presence or absence of BstA during microscopy studies. The exact mechanism of cell lysis during BstA Abi activity will require further study.

An intriguing feature of the BstA phage-defense system is its tight association with prophages, and specifically, with the prophage repressor locus. Although we found homologs in diverse Gram-negative bacteria, the genetic architecture of the bstA locus (i.e., lying downstream of and presumably sharing the promoter of the prophage repressor) was strikingly conserved. The region between the repressor (cl) and c gene of lambdoid prophages has previously been identified as a hotspot of mosaic diversity (Degnan et al., 2007). In fact, the corresponding site in phage Lambda harbors the rexAB genes, perhaps the most widely studied prophage-encoded abortive infection system (Snyder, 1995). Despite >60 years of research, the molecular mechanisms of RexAB activity are poorly understood. RexB is reported to be an ion channel, which triggers the loss of cell membrane potential upon activation by the intracellular sensor RexA (Labrie et al., 2010; Snyder, 1995). While not mechanistically comparable to BstA, perhaps the shared synteny of the BstA and RexAB abortive infection systems points to a functional significance of this genomic region, as the cl repressor gene is one of the most highly transcribed prophage promoters during lysogeny.

Although somewhat functionally analogous to toxin-antitoxin systems, to the best of our knowledge, no other examples of self-immunity mechanisms have been described within prophage-encoded abortive infection systems. However, some evidence supports the widespread existence of such mechanisms. For example, the activity of Lambda RexB protein can be suppressed by the overexpression of the rexB gene relative to rexA. It has been speculated, but not shown experimentally, that high levels of RexB might allow phage Lambda to replicate lyrically in the presence of RexAB (Parma et al., 1992), i.e., giving the Lambda prophage self-immunity against its own Abi proteins.

In conclusion, the discovery of the BstA-aba system opens unexplored avenues of research into the mechanisms used by prophages to suppress their own phage-defense activities. We anticipate that similar strategies may be widespread and commonplace, perhaps existing within known prophage-encoded phage-defense systems. Given the huge mosaic diversity of temperate phages and high prevalence of uncharacterized accessory genes, the reservoir of prophage-encoded phage-defense and self-immunity systems is likely to be vast and largely unexplored.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chom.2021.09.002.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.V.O., N.W., and J.C.D.H.; methodology, S.V.O., N.W., C.L.D., N.Q.-O., and D.J.R.; investigation, S.V.O., N.W., C.L.D., E.V.R., A.B.-B., N.Q.-O., and D.J.R.; resources, E.J.R. and E.C.G.; writing – original draft, S.V.O. and N.W.; writing – review & editing, S.V.O., N.W., C.L.D., M.B., and J.C.D.H.; visualization, S.V.O., N.W., C.L.D., D.J.R., and M.B.; supervision, E.J.R., E.C.G., M.B., and J.C.D.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-Digoxigenin-AP, Fab fragments (Roche) | MilliporeSigma | Cat# 11093274910; RRID: AB_2734716 |
| Bacterial and virus strains |        |            |
| All the bacterial strains and bacteriophages are listed and described in Table S2 | N/A | N/A |
| Chemicals, peptides, and recombinant proteins |        |            |
| L-(+)-arabinose | Melford | Cat#A51000-100.0 |
| Betaine | MilliporeSigma | Cat#B2629 |
| Bacto Agar (BD) | Appleton Woods | Cat#MN663 |
| Bacto Casamino Acids Technical (BD) | Appleton Woods | Cat#223110 |
| EGTA | MilliporeSigma | Cat#E3889 |
| M9 Salts 5X | MilliporeSigma | Cat#M6030 |
| Maltose | MilliporeSigma | Cat#M5885 |
| m-toluic acid | MilliporeSigma | Cat#T36609 |
| Tryptone (BD) | Appleton Woods | Cat#MN649 |
| Yeast Extract (BD) | Appleton Woods | Cat#DM832 |
| Sodium Chloride | MilliporeSigma | Cat#S3014 |
| Ampicillin Sodium | Melford | Cat#A40040-25.0 |
| Anhydrotetracycline hydrochloride (AHT) | MilliporeSigma | Cat#37919 |
| Chloramphenicol | MilliporeSigma | Cat#C0378 |
| Gentamicin sulfate | Melford | Cat#G38000-25.0 |
| Kanamycin monosulfate | Melford | Cat#K22000-25.0 |
| Mitomycin C | MilliporeSigma | Cat#M0503 |
| Tetracycline hydrochloride | MilliporeSigma | Cat#T7660 |
| EcoRI | ThermoFisher Scientific | Cat#ER0271 |
| BamHI | ThermoFisher Scientific | Cat#ER0051 |
| DpnI | New England Biolabs | Cat#R0176S |
| KpnI-HF | New England Biolabs | Cat#R3142S |
| Bsai-HF | New England Biolabs | Cat#R3535S |
| Smal | ThermoFisher Scientific | Cat#ER0661 |
| Sali | ThermoFisher Scientific | Cat#ER0641 |
| XbaI | New England Biolabs | Cat#R0145S |
| T4 Polynucleotide Kinase | ThermoFisher Scientific | Cat#EK0031 |
| T4 DNA ligase | ThermoFisher Scientific | Cat#EL0014 |
| CutSmart buffer | New England Biolabs | Cat#B7204S |
| Tango DNA ligase 10X | ThermoFisher Scientific | Cat#BY5 |
| T4 DNA ligase Reaction Buffer | New England Biolabs | Cat#B0202S |
| Phusion High Fidelity DNA polymerase | New England Biolabs | Cat#M0530S |
| MyTaq Red PCR mix 2X | Bioline | Cat#BIO-25043 |
| Taq DNA polymerase | Bioline | Cat#BIO-21105 |
| NEBuilder HiFi DNA Assembly Cloning Kit | New England Biolabs | Cat#E5520S |
| D/Nase I | MilliporeSigma | Cat#DN25 |
| RNase A | MilliporeSigma | Cat#R6513 |
| Proteinase K | Bioline | Cat#BIO-37037 |
| dNTP mix | Bioline | Cat#BIO-39025 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sián Owen (sianvictoriaowen@gmail.com).

Materials availability
All unique bacterial, phage strains, and plasmids generated in this study are available from the lead contact without restriction.

Data and code availability
- All raw data from assays and microscopy reported in this paper will be shared by the lead contact upon request. This paper analyses existing, publicly available data. These accession numbers for the datasets are listed in Table S1.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacteria and bacteriophages
The full list of bacterial strains used and constructed is available in Table S2. All the Salmonella strains were derived from the African S. Typhimurium ST313 strain D23580 (GenBank: FN424405.1) (Kingsley et al., 2009) or the model S. Typhimurium strain LT2

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### Table: REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Datp                | Bioline | Cat#BIO-39036 |
| Dgtp                | Bioline | Cat#BIO-39037 |
| dCTP                | Bioline | Cat#BIO-39038 |
| Dttp                | Bioline | Cat#BIO-39039 |
| DIG-11-dUTP, alkali-stable (Roche) | MilliporeSigma | Cat#11093088910 |
| Midori Green DNA/RNA staining | Nippon Genetics | Cat#MG06 |
| Electroporation cuvettes | Geneflow | Cat#E6-0060 |
| Nylon membrane, positively charged (Roche) | MilliporeSigma | Cat#11417240001 |
| Blocking Reagent (Roche) | MilliporeSigma | Cat#11096176001 |
| DIG Easy Hyb Granules (Roche) | MilliporeSigma | Cat#11796895001 |
| Critical commercial assays | | |
| ISOLATE II Plasmid Mini Kit | Bioline | Cat#BIO-52057 |
| ISOLATE II PCR and Gel Kit | Bioline | Cat#BIO-52060 |
| Norgen Phage DNA Isolation Kit (46850) | GeneFlow | Cat#P4-0134 |
| Quick-DNA Universal Kit | Zymo | Cat#D4069 |
| Invitrogen Qubit dsDNA HS Assay Kit | ThermoFisher Scientific | Cat#Q32851 |
| SYTOX Orange Nucleic Acid Stain – 5 mM in DMSO | ThermoFisher Scientific | Cat#S11368 |
| Amicon Ultra-15 centrifugal filter units | MilliporeSigma | Cat#UFC910024 |
| Oligonucleotides | | |
| All the DNA oligonucleotides | N/A | N/A |
| Recombinant DNA | | |
| All the plasmids | N/A | N/A |
| Software and algorithms | | |
| GraphPad Prism 8.4.1 | N/A | N/A |
| HMMER webserver | N/A | https://www.ebi.ac.uk/Tools/hmmer/ |
| Prokka 1.13 | N/A | N/A |
| HHPred webserver | N/A | https://toolkit.tuebingen.mpg.de/tools/hhpred |
| EMBOSS Needle webserver | N/A | https://www.ebi.ac.uk/Tools/psa/emboss_needle/ |
All the *Escherichia coli* strains constructed were derived from *E. coli* strain K-12 substrain MG1655 (GenBank: NC_000913.3) (Riley et al., 2006). The bstA homolog genes were cloned from *E. coli* NCTC10963 (GenBank: NZ_CAADJH01000002.1) or from *K. pneumoniae* Kps2.145 (GenBank: FO834906.1) (Bialek-Davenet et al., 2014). Bacteriophages (phages), including the temperate phages P22 (GenBank: NC_002371.2) (Pedulla et al., 2003) and BTP1 (GenBank: NC_042346.1) (Owen et al., 2017) and their derivatives, are described in Table S2. The genomic coordinates and gene identifiers indicated below refer to the GenBank accession numbers mentioned above.

**METHOD DETAILS**

**Growth conditions and transformation**

All suppliers of chemical and reagents are specified in the key resources table. Unless stated otherwise, bacteria were grown at 37°C in autoclaved Lennox Broth (LB): 10 g/L Bacto Tryptone, 5 g/L Bacto Yeast Extract, 5 g/L NaCl with aeration (shaking 220 rpm) or on LB agar plates, solidified with 1.5% Agar. The salt-free LBO medium contained 10 g/L Bacto Tryptone, 5 g/L Bacto Yeast Extract. Pre-cultures were inoculated with isolated colonies from agar plates and grown to stationary phase (for at least 6 hours) in 5 mL LB in 30 mL universal glass tubes or in 50 mL plastic tubes (Greiner).

Cultures were typically prepared by diluting the pre-cultures (1:100) or (1:1000) in LB, and bacteria were grown in conical flasks containing 10% of their capacity of medium (i.e. 25 mL LB in a 250 mL conical flask) with aeration. For fluorescent microscopy experiments, bacteria were grown in M9 minimal medium (Sambrook and Russell, 2001) supplemented with 0.4% glucose and 0.1% Bacto Casamino Acids Technical (M9 Glu").

When required, antibiotics were added to the media: 50 μg/mL kanamycin monosulfate (Km), 100 μg/mL Ampicillin sodium (Ap), 25 μg/mL tetracycline hydrochloride (Tc), 20 μg/mL gentamicin sulfate (Gm), 20 μg/mL chloramphenicol (Cm). Bacteria carrying inducible constructs with genes under the control of the P<sub>BAD</sub> or P<sub>m</sub> promoters were induced by adding 0.2 % (w/v) L-(+)-arabinose or 1 mM m-toluate, respectively. For the strains carrying <i>tetR</i>-<i>P<sub>total</sub></i> modules, <i>P<sub>total</sub></i> induction was triggered by adding 500 ng/mL of anhydrotetracycline hydrochloride (AHT, stock solubilized in methanol). For these constructs, the same volume of methanol was added to the non-induced cultures (mock treatment). Chemically-competent *E. coli* were prepared with RbCl-based solutions and were transformed by heat shock (Green and Rogers, 2014).

For the preparation of electro- competent cells, bacteria were grown in the salt-free medium LBO to an Optical Density at 600 nm (OD<sub>600</sub>) of 0.4-0.5. The bacteria were washed twice with cold sterile Milli-Q water (same volume as the culture volume) and were concentrated 100 times in cold 10% glycerol, prior to storage at -80°C. When ultra-competent Salmonella cells were required, the bacteria were grown in LBO at 45°C to OD<sub>600</sub> 0.4-0.5, because growth at high temperature inactivates the Salmonella restriction systems (Edwards et al., 1999). Competent cells (10-50 μL) were mixed with 10-5000 ng of DNA in electroporation cuvettes (2 mm gap) and the reactions were electroporated (2.5 kV) using a MicroPulser electroporator (Bio-Rad). Bacteria were re-suspended in 0.5-1 mL LB and incubated for recovery at 37°C (30°C for temperature sensitive plasmids) with aeration, for at least one hour. Finally, the transformed bacteria were spread on selective LB agar plates and transformant colonies were obtained after at least 12 hours incubation at 30-37°C.

For assessment of strain growth kinetics with BstA expression, a FLUOstar Omega plate reader (BMG LABTECH) was used as follows: bacteria were inoculated at an initial OD<sub>600</sub> of 0.01 (six replicates) in 200 μL of LB or LB + AHT in 96-well plates (Greiner). Bacteria were grown at 37°C with aeration (500 rpm, orbital shaking) and the OD<sub>600</sub> was monitored every 15 min for 15 hours. Uninoculated LB medium was used as blank.

**Cloning procedures**

All the plasmids and DNA oligonucleotides (primers) are listed in Table S2. DNA manipulation and cloning procedures were carried out according to the enzyme and kit supplier recommendations and to standard procedures (Sambrook and Russell, 2001). DNA purity and concentration were measured with a DeNovix DS-11 FX spectrophotometer/fluorometer and using the Qubit dsDNA HS assay Kit.

For all the cloning procedures, Polymerase Chain Reactions (PCRs) were performed with the Phusion High Fidelity DNA polymerase, purified template DNA and primers in the presence of 3 % Dimethyl Sulfoxide and 1 M betaine, when required. Prior to Sanger sequencing of the constructs, PCR reactions were carried out directly from bacteria or phages with MyTaq Red Mix 2X. PCR fragments were analysed by electrophoresis, purified and finally sequenced with the appropriate primers (Lightrun service, Eurofins Genomics) (Table S2).

All the plasmids were constructed as detailed in the Table S2 and were verified by Sanger sequencing. Insertions of DNA fragments into plasmids were performed by digestion/ligation procedures, using restrictions enzymes and the T4 DNA ligase. In addition, PCR-driven restriction-free cloning techniques were used: overlap extension PCRs (Heckman and Pease, 2007) and plasmid assembly by PCR cloning (Van Den Ent and Löwe, 2006) were performed with chimeric primers, purified template DNA and Phusion DNA polymerase, as described previously (Owen et al., 2020). Cloning reactions were transformed by heat shock into *E. coli* Top10 (Invitrogen) or S17-1 <i>pir</i> (Simon et al., 1983). New template plasmids were constructed to insert fluorescent protein encoding genes into *Salmonella* or *E. coli* chromosomes, as reported previously (Geriac et al., 2007). These plasmids carry the oriR6K γ origin of replication of pEMG, the <i>ft</i>-<i>aph</i>-<i>f</i><i>rt</i> (Km<sup>β</sup>) module of pKD4 linked to gfp<sup>+</sup> (pNAW52), μ<sub>g</sub><i>fp</i> (encoding for superfolder GFP, pNAW62) or <i>m</i><i>cherry</i> (pNAW73), amplified respectively from plasmids pZEP09, pXG10-SF (Corcoran et al., 2012) and pFCcGi (Figueira et al., 2013). A
similar template plasmid, carrying the $frt$-$frt$-$tetR$-$P_{bstA}$ module (pNAW55) was constructed and was used to insert the $tetR$ repressor and the AHT-inducible promoter $P_{tot}$ upstream of genes of interest, as reported earlier (Schulte et al., 2019). For the construction of gentamicin resistant plasmids, the aacC1 resistance gene was obtained from plasmid pME4510 (Rist and Kertesz, 1998).

The high copy number plasmid pUC16 was used to clone the different versions of the anti-bstA (aba) fragment: the aba fragments (aba1-aba14 alleles) were amplified by PCR, digested with EcoRI and BamHI and ligated into the corresponding sites of pUC18. For cloning of the aba fragments fused to $gfp$ and flanked by terminators, 20nt overlapping DNA fragments were amplified with Q5 high fidelity polymerase, pooled and digested with DpnI prior to four piece isothermal assembly using the NEBuilder HiFi DNA Assembly Cloning Kit.

Phagemids based on the phage P22 replication module were constructed by EcoRI/KpnI digestion and ligation, as follows: the $P_{fr}$ promoter and the $cro$-c1-orf48-O-P genes of P22 (coordinates 31648-34683) were amplified and circularized by ligation with the $aph$ KmR cassette of pKD4 or with the aba-aph modules, amplified from strain SNW617. The ligation reactions were purified and electroverted into ultra-competent SNW555, a prophage-free and plasmid-free derivative of S. Typhimurium D23580. The resulting phagemids pNAW229 (pP22-aph), pNAW230 (pP22-aba-aph) were obtained after selection on Km medium.

Phage DNA was extracted from high titer lysates in LBO: nine volume of the phage lysates were mixed with one volume of 10 X DNase buffer (100 mM Tris-HCl, 25 mM MgCl$_2$, pH 7.5) supplemented with RNase A (40 $\mu$g/ml final) and DNase I (400 $\mu$g/ml final). After 1 hour incubation at 37°C, DNase I was heat-inactivated at 75°C for 10 min and phage DNA was extracted from 500 $\mu$L of the nuclease-treated lysates with the Norgen Phage DNA Isolation after Proteinase K treatment, as specified by the manufacturer.

**Genome editing techniques**

Strain constructions are detailed in Table S2. For chromosomal insertions and deletions, $\lambda$ red recombination was carried out with the arabinose-inducible plasmid pKD46 (for E. coli) or with the heat inducible plasmid pSIM5-tet (for Salmonella), both expressing the $\lambda$ red genes. Bacteria were grown to exponential phase in LBO, according to the resistance and induction condition of the respective $\lambda$ red plasmid (Datjenko and Wanner, 2000; Hammarlöf et al., 2011) and electro-competent cells were prepared as mentioned above. PCR fragments carrying a resistance cassette were constructed by overlap extension PCR or were directly obtained by PCR from the appropriate plasmid or strain. Electro-competent cells (40-50 $\mu$L) were transformed with 500-5000 ng of the PCR fragments and the recombinants were selected on selective LB agar plates.

Mutations or insertions linked to selective markers were transduced into Salmonella strains using the P22 HT 105/1 int-201 (P22 HT) transducing phage (Owen et al., 2017; Schmierer, 1972). For E. coli, the transducing phage P1 vir was used (Ikeda and Tomizawa, 1965; Tiruvadi Krishnan et al., 2015). Transductants were grown on selective LB agar plates supplemented with 10 mM EGTA. After two passages, clearance of the transducing phages was confirmed by diagnostic PCR using primer pairs NW_62/NW_63 for P22 HT or NW_392/NW_393 for P1 vir and by a passage on Green Agar medium (Maloy, 1990). To remove the antibiotic cassettes, flanked by FLP recognition target sites (frt), the FLP recombinase expressing plasmids pCP20, pCP20-TcR and pCP20-Gm were used, as previously reported (Cherepanov and Wackernagel, 1995; Doublet et al., 2008; Hammarlöf et al., 2018; Kintz et al., 2015). The inducible $tetR$-$P_{bstA}$ modules were constructed by fusing the $frt$-aph-$frt$-$tetR$-$P_{bstA}$ module of pNAW55 to the bstA gene of D23580 (bstA$^{RPTP}$, STMMW_03537), E. coli NCTC10963 (bstA$^{C}$, E4V89_RS07420) or K. pneumoniae Kp52.145 (bstA$^{I}$, BN49_1470). Each construct carries the native bstA ribosome binding site and Rho-independent terminator. The $tetR$-$P_{bstA}$-bstA modules were inserted by $\lambda$ red recombination into the STMM1553 pseudogene of S. Typhimurium LT2 (between coordinates 1629109-1629311), corresponding to STMMW_15481 in D23580 (coordinates 1621832-3). Previously we have shown that the STMM153 and STMMW_15481 genes are not expressed at the transcriptional level (Canals et al., 2019).

In E. coli MG1655, the bstA modules were inserted into the glmS-pstS intergenic region (coordinates 3911773-4). To generate Ap and Cm sensitive D23580 strains, the pSLT-BT plasmid-encoded Tn21-like element, that carries the resistance genes (Kingsley et al., 2009), was replaced by the KmR cassette of pKD4 by $\lambda$ red recombination (deletion coordinates 34307 to 57061, GenBank: NC_013437.1). The resulting large single-copy plasmid pSLT-BT (coordinates 31648-34683) was extracted (Heringa et al., 2007) and electroverted into the strains of interest. After selection on Km medium, the Ap and Cm sensitivity was confirmed and the KmR cassette was flipped out using pCP20-Gm. For scarless genome editing, the pEMG plasmid-based allelic exchange system was used (Martinez-Garcia and de Lorenzo, 2011). The pEMG derivative suicide plasmids were constructed as specified in Table S2 and were replicated in E. coli MG1655, STMMW_15481 genes are not expressed at the transcriptional level (Canals et al., 2019).

In E. coli MG1655, the bstA modules were inserted into the glmS-pstS intergenic region (coordinates 3911773-4). To generate Ap and Cm sensitive D23580 strains, the pSLT-BT plasmid-encoded Tn21-like element, that carries the resistance genes (Kingsley et al., 2009), was replaced by the KmR cassette of pKD4 by $\lambda$ red recombination (deletion coordinates 34307 to 57061, GenBank: NC_013437.1). The resulting large single-copy plasmid pSLT-BT (coordinates 31648-34683) was extracted (Heringa et al., 2007) and electroverted into the strains of interest. After selection on Km medium, the Ap and Cm sensitivity was confirmed and the KmR cassette was flipped out using pCP20-Gm. For scarless genome editing, the pEMG plasmid-based allelic exchange system was used (Martinez-Garcia and de Lorenzo, 2011). The pEMG derivative suicide plasmids were constructed as specified in Table S2 and were replicated in E. coli MG1655, STMMW_15481 genes are not expressed at the transcriptional level (Canals et al., 2019).

**Plasmid deletion in S. Typhimurium D23580**

The pSLT-BT, pBT1, pBT2 and pBT3 plasmids (Kingsley et al., 2009) were cured from strain D23580, using the CRISPR-Cas9-based methodology (Lauritsen et al., 2017). A CRISPR-Cas9 Km resistant plasmid (pNAW136) was obtained by ligating the CRISPR-Cas9 module of plasmid pCas9 (Jiang et al., 2013) with the unstable origin of replication oriRK2, the $trfA$ replication gene and the $aph$ KmR gene. Anti-plasmid protospacers (30 bp) were generated by the annealing of 5’-phosphorylated primer pairs that targeted the pSLT-BT, pBT1, pBT2 and pBT3 plasmids, designed according to the Marraffini Lab protocol (Jiang et al., 2013). The protospacers were ligated into Bsal-digested pNAW136 with T4 DNA ligase and the resulting plasmids were checked by Sanger sequencing, using primer NW_658.
The resulting plasmids pNAW168 (anti-pSLT-BT) and pNAW169 (anti-pBT1), pNAW139 (anti-pBT2) and pNAW191 (anti-pBT3) were electroporated into D23580-derived strains and transformants were selected on Km plates. After two passages on Km, the loss of the pSLT-BT, pBT1, pBT2 or pBT3 plasmids was confirmed by diagnostic PCR. The absence of the unstable pNAW136-derived plasmids was confirmed by the Km sensitive phenotype of colonies after two passages on non-selective medium.

**Phage stock preparation and plaque assays**

All phage stocks were prepared in LB or LBO. For *Salmonella* phages, the prophage-free strain S. Typhimurium D23580 Δφ (JH3949) was used as host (Owen et al., 2017). Exponential phase cultures of D23580 Δφ were infected with ~10^6 Plaque Forming Units (PFU) and infected cultures were incubated for at least 3 hours at 37°C (with aeration). Phage lysates were spun down (4,000 × g, 15 min) and supernatants were filter-sterilized (0.22 μm, StarLab syringe filters). The resulting phage lysates were stored at 4°C in the presence 1% chloroform to prevent bacterial contamination.

Coliphage lysates were prepared similarly with *E. coli* MG1655 as host. When required, maltose (0.2%), CaCl_2 (10 mM) and MgSO_4 (10 mM) were added during the infection (λ, P1 vir and φ80pSU3*). For φ80-derived phages, the infection temperature was reduced to 30°C (Rotman et al., 2010).

Phage lysates were serial-diluted (decimal dilutions) with LB and virion enumeration was performed by double-layer overlay plaque assay (Kropinski et al., 2009), as follows. Bacterial lawns were prepared with stationary phase cultures of the reporter strains, diluted 40 times with warm Top Agar (0.5% agar in LB, 50°C). The seeded Top Agar was poured on LB 1.5% agar bottom layer: 4 mL for 8.6 cm diameter petri dishes or 8 mL for 12 × 12 cm square plates.

When inducible P_βgal or P_BAD constructs were present in the reporter bacteria, 500 ng/mL of AHT or 0.2% arabinose were added in the Top Agar. When required, antibiotics were included in the Top Agar layer. The bacterial lawns were incubated for 30 min at room temperature with the appropriate inducer, to allow solidification and the expression of the inducible genes. Finally, phage suspensions (5-20 μL) were applied on the Top Agar surface and pictures of the resulting plaques were taken with an ImageQuant LAS 4000 imager (GE Healthcare) after 16-20 hours incubation at 30 or 37°C.

**Construction of P22 virulent phages**

For the generation of obligately virulent P22 phages, a 633 bp in-frame deletion (coordinates 31028-31660) was introduced in the tsp-gtrAC repressor gene by λ red recombination in a P22 lysogen as follows. Two fragments of ~500 bp, flanking c2, were amplified with primers pairs NW_818 / NW_819 and NW_820 / NW_821. The two amplicons were fused by overlap extension PCR and 1000-3000 ng of the resulting Δc2 fragment were electroporated into P22 lysogens (in the prophage-free D23580 Δφ background) carrying the λ red recombination plasmid pSIM5-tet, as described above. The transformation reactions were re-suspended in 5 mL LB and incubated for 2 hours at 37°C with aeration. The resulting plasmids pNAW168 (anti-pSLT-BT) and pNAW169 (anti-pBT1), pNAW139 (anti-pBT2) and pNAW191 (anti-pBT3) were electroporated into D23580-derived strains and transformants were selected on Km plates. After two passages on Km, the loss of the pSLT-BT, pBT1, pBT2 or pBT3 plasmids was confirmed by diagnostic PCR. The absence of the unstable pNAW136-derived plasmids was confirmed by the Km sensitive phenotype of colonies after two passages on non-selective medium.

**Use of the Δtsp-gtrAC genetic background**

Where possible, experiments were carried out with native BstA expression (from its natural locus within the BTP1 prophage), to best recapitulate the natural biological activity of the protein. However, as the gtr locus of phage BTP1 blocks attachment of many phages including P22 and BTP1, to achieve efficient phage infections we consistently used a strain background where the gtr locus has been inactivated (Δtsp-gtrAC). The BTP1 prophage spontaneously induces to a titer of ~10^9 PFU/mL in liquid culture (Owen et al., 2017), and in the absence of gtr activity in surrounding cells, free BTP1 phages mediate cleavage of the O-antigen via the putative enzymatic activity of the tailspike protein (Kintz et al., 2015). Consequently, to avoid an unnatural, short LPS phenotype as a result of the putative enzymatic activity of the tailspike protein, we additionally inactivated the upstream gene encoding the BTP1 tailspike (gtr activity in surrounding cells, free BTP1 phages mediate cleavage of the O-antigen via the putative enzymatic activity of the tailspike protein (Kintz et al., 2015)).

**Phage replication assay**

Stationary phase cultures of the reporter bacteria were diluted to OD_600 0.4 with LB. Aliquots (0.2 mL) were prepared in 1.5 mL tubes and phage stock suspensions were added to a final phage titer of 100-1000 PFU/mL. The infections were carried out at 37°C (30°C for φ80pSU3*) with shaking for 2-4 hours and were stopped by the addition of 20 μL of chloroform. After a 10 sec vortex, the lysates were centrifuged (20,000 X g, 5 min) and serial diluted. When M9 Glu + was used, *Salmonella* strains were grown to OD_600 ~ 0.5 in this medium prior to phage infection.

Phage titer was determined by plaque assay: 10 μL of the dilutions were applied to bacterial lawns of the appropriate reporter strain in technical triplicates. Plaques were enumerated after 16-20 hours of incubation and phage titers (PFU/mL) were calculated for each lysate. To measure the phage input at time 0 (T0), the same volume of stock phage suspension was added to 0.2 mL of bacteria-free LB and the titer was determined as described above. The fold-replication for each phage was calculated as the phage titer of the lysate post infection divided by the input phage titer at T0. When the phage titer in the lysate was lower than the phage input, the replication was considered to be null (<1-fold). When AHT inducible tetR-P_βgal-bstA strains were used, AHT (500 ng/mL) or methanol (mock) were added to the diluted bacterial suspension and phages were added after 15 min of incubation at 37°C with aeration.
For replication assays of the coliphages λ, P1 vir and φ80pSU3⁺. E. coli strains were grown to exponential phase (OD₆₀₀ 0.4) in LB and phages were added as mentioned above. To stimulate infection by these phages, maltose (0.2%), CaCl₂ (10 mM) and MgSO₄ (10 mM) were added during the infection and in the lawns of the reporter E. coli MG1655. All the phage replication experiments presented were carried out at least twice with biological triplicates.

**Induction of P22 and BTP1 prophages**

D23580 Δφ-derived lysogens that carried the different versions of P22 and BTP1 were constructed as detailed in the Table S2. For complementation with the pUC18-derived plasmids (Ap⁺), Ap sensitive lysogens were constructed by the inactivation of the Tn21-like element, as described above. The resulting lysogens were grown to stationary phase in LB and the pre-cultures were diluted 100-1000 times in fresh LB and grown to OD₆₀₀ 0.4-0.5, prior addition of Mitomycin C (MitC, 2 µg/mL). The induced cultures were incubated for 3-5 hours at 37°C with aeration and cultures were filter sterilized and serial diluted. The phage titer was measured by plaque assay on the appropriate host strain lawn with technical replicates, as described above. All the prophage induction experiments were carried out at least twice with biological triplicates.

**Survival assays**

For the survival assays, D23580 Δtsp-gtrAC (JH4287), D23580 Δtsp-gtrAC bstA₃⁰ (SNW431) or D23580 Δφ [P22] (SSO-128) were grown in M9 Glu⁺ to OD₆₀₀ ~0.5 and two 0.5 mL subcultures were prepared for each culture. The use of D23580 Δφ [P22] in these experiments controlled for the effect of lysis from without due to use of high multiplicity of infection (MOI). The strain is a lysogen for WT P22 phage, and therefore is highly resistant to infection by P22-derived phages. P22 Δc2 was added at an MOI of 5. The same volume of LB was added to the two remaining subcultures (non-infected controls). Samples were incubated for 15 min at 37°C to allow phage attachment. To stop phage development, the cultures were chilled on ice and bacteria were washed with 0.5 mL of cold PBS. All the samples were serial-diluted in PBS to 10⁻⁶ and kept on ice. For the measure of survival post-infection, 10 µL of diluted infected or non-infected cultures were applied in technical triplicates on LB agar supplemented with 10 mM EGTA (EGTA was used to minimize secondary infection by free phages). Colony forming Units (CFU) were enumerated and the survival rate, was calculated as the ratio of CFUs in infected cultures divided by the CFUs obtained from non-infected cultures (in %). All the survival experiments were carried out at least twice with biological triplicates.

**Frequency of lysogeny assays**

For the frequency of lysogeny assays, a derivate of phage P22 was used that has the pid locus replaced with an aph cassette yielding kanamycin resistant lysogens (P22 Δpid::aph, SNW490). The pid locus has previously been shown to be non-essential in phage P22 and does not establishment of lysogeny (Cenens et al., 2013). D23580 Δφ tetR-PtetA-bstA (SNW576) cells were grown in 3 mL of LB to OD₆₀₀ ~0.35. Methanol (mock) or AHT (500 ng/mL, inducer) were added to the cultures and bacteria were incubated to induce BstA for 1 hour at 37°C. 200 µL samples of the bacteria were mixed in triplicate with P22 Δpid::aph phage to achieve a MOI of 0.1, and incubated at 37°C for 20 minutes to allow adsorption and ejection of nucleic acids. Cells were pelleted and resuspended in LB media supplemented with 10 mM EGTA to minimize secondary infection by any free phages (along with methanol or AHT) and incubated at 37°C for a further 20 minutes to allow integration and expression of the kanamycin resistance determinant. CFU were enumerated on LB kanamycin. Frequency of lysogeny was determined as the kanamycin resistant CFU/mL divided by the PFU/mL of input phage.

**Phage DNA detection by Southern Blotting**

D23580 Δφ tetR-PtetA-bstA (SNW576) was grown in 50 mL LB to OD₆₀₀ ~0.35. The culture was split in two 20 mL sub-cultures and methanol (mock) or AHT (500 ng/mL, inducer) were added to each subculture. Bacteria were incubated to induce BstA for 20 min at 37°C and the phage of interest was added at an MOI of 5. Infections were carried out at 37°C with aeration and total DNA was extracted (Quick-DNA Universal Kit Zymo) from 1.5 mL of culture at 0, 10, 20, 30, 35, 40 and 50 minutes post Infection. Total DNA (100 ng, according to QuBit quantification) was size-separated (2 hours at 100 V in TAE 1X) on a 0.8 % agarose-TAE gel containing Midori Green DNA staining (4 µL for 100 mL gel). One hundred nanograms of non-infected D23580 Δφ tetR-PtetA-bstA genomic DNA were used as a negative control. DNA was fragmented by exposing the agarose gel to UV light for 5 min on a UV-transilluminator. DNA was denatured by soaking the gel in the Denaturation Solution (0.5 M NaOH, 1.5 M NaCl) for 30 min and then in the Neutralization Solution (1.5 M NaCl, 1 M Tris-HCl, pH 7.6) for 30 min. DNA was transferred on a positively-charged Nylon membrane using the capillary blotting method. Phage DNA was detected with DIG labelled dsDNA probes generated by PCR amplification with MyTaq DNA polymerase (Bioline), buffer, phage DNA and primers (0.4 µM each), in the presence of 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.13 mM dTTP and 0.07 mM DIG-11-dUTP. For the 9NA probe a 588 bp PCR fragment was generated with primer pair NW_602 / NW_603 and for the P22/BTP1 probe a 725 bp PCR fragment was generated with primer pair SO-128 / SO-129. The DNA probes were heat-denatured at 95°C for 15 min and the DNA-DNA hybridizations were carried out at 45°C for 16 hours in DIG-Easy Hyb buffer. The washing and immunodetection procedures were carried out, as specified in the DIG Application Manual for Filter Hybridization (Roche) and the chemiluminescence signal was detected using an ImageQuant LAS 4000 imager (GE Healthcare). Prior to DNA transfer onto the membrane, the Midori green-stained DNA was visualized under UV and the resulting image was used as a loading control.
Phagemid efficiency of transformation

To avoid a reduction in transformation caused by interspecies DNA modification/restriction interference between *E. coli* and *Salmonella*, all the P22-derived phagemids were first replicated and extracted from *S. Typhimurium SNW555* before efficiency of transformation assays.

*Salmonella* strains carrying the tetR-PtrtA-bstA module were grown in 50 mL LBO culture. When OD$_{600}$ ~0.4 was reached, each culture was split into two 25 mL sub-cultures and methanol (mock) or AHT (inducer) were added to each subculture. Bacteria were incubated for BstA induction during 15 min at 37°C. The cultures were incubated on ice for 5 min and bacteria were washed twice with cold water (25 mL) and were concentrated in 0.1 mL of ice-cold sterile 10% glycerol. The OD$_{600}$ of each electro-competent cell sample was measured by diluting 10 μL of competent cells with 990 μL of 10% glycerol. Cell concentration was adjusted with 10% glycerol for each sample, according to the sample with the lowest OD$_{600}$. The competent cells (20 μL) were mixed with 10 ng (estimated by Qubit) of the P22 phagemids, pP22 (pNAW229) or pP22-aba (pNAW230) and the mixture was incubated on ice until electroporation (2.5 kV). Transformation reactions were re-suspended in 1 mL LB or 1 mL LB + AHT (for the bstA-induced bacteria) and were incubated for 60 min at 37°C, for recovery. The transformations were diluted (decimal dilution to 10$^{-3}$) in LB or LB+AHT and 100 μL of each dilution (including the non-diluted sample) were spread on LB agar Km or LB agar Km+AHT plates. After incubation at 37°C, the number of Km$^+$ transformants was enumerated for each transformation and efficiency of transformation was defined as the number of transformants obtained per ng of phagemid. This experiment was performed with biological triplicates and was repeated twice with LT2 tetR-PtrtA-bstA (SNW389) and once with D23580 ΔtetRtetA-bstA (SNW576), giving similar results.

**Microscopy- general**

For all imaging experiments, bacteria were sub-cultured in liquid M9 Glu$^+$ media. All images were collected with a wide field Nikon Eclipse Ti-E inverted microscope equipped with an Okolab Cage Incubator warmed to 37°C with Cargille Type 37 immersion oil. A Nikon CFI Plan Apo DM Lambda 100X 1.45 NA Oil objective and a Nikon CFI Plan Apo DM Lambda 20X.75 NA objective were used with Perfect Focus System for maintenance of focus over time. Superfolder GFP, mCherry and SYTOX Orange Nucleic Acid Stain (ThermoFisher) were excited with a Luminenc Spectra X light engine with Chroma FITC (470/24) and mCherry (575/25) filter sets, respectively and collected with a Spectra Sedat Quad filter cube ET435/26M-25 ET515/30M-25 ET595/40M-25 ET705/72M-25 and a Spectra CFP/YFP/mCherry filter cube ET475/20M-25 ET540/21M-25 ET632/60M-25. Images were acquired with an Andor Zyla 4.2 sCMOS controlled with NIS Elements software. For time-lapse experiments, images were collected every 3 minutes (unless specified otherwise) via ND acquisition using an exposure time of 100 ms and 50% or 100% illumination power for fluorescence. Multiple stage positions (fields) were collected using the default engine Ti Z. Fields best representing the overall experimental trend with the least technical artefacts were chosen for publication. Gamma, brightness, and contrast were adjusted (identically for compared image sets) using FIJI (Schindelin et al., 2012). The FIJI plug-ins Stack Contrast (Capek et al., 2006) and StackReg (Thévenaz et al., 1998) were used for brightness matching and registering image stacks.

**Microscopy- agarose pads**

Agarose pads were prepared with 2% agarose and M9 Glu$^+$ media, and mounted on MatTek dishes (No. 1.5 coverslip, 50 mm, 30 mm glass diameter, uncoated). Cells (D23580Δtsp-gtrAC (JH4287) or D23580Δtsp-gtrAC bstASTOP (SNW431)) were grown to log phase (OD$_{600}$ ~ 0.4) in M9 Glu$^+$ at 37°C with shaking (220 RPM), and where required, diluted in fresh M9 Glu$^+$ to achieve the desired cell density on the agarose pad. For experiments where all cells were infected (Figure 4A), phage P22 Δc2 was added at an MOI of 5. Phage adsorption and initial infection was facilitated by incubation at 37°C with shaking for 10 minutes. Subsequently, infected cells were pelleted at 5000 x g and resuspended in ice-cold PBS to pause phage development. Two microliters of chilled, infected cells were spotted onto opposite sides of an agarose pad (two strains were imaged on the same pad) and inverted onto the MatTek imaging dish. Experimental MOIs were immediately confirmed by CFU and PFU/mL measurement of the cell and phage preparations. Phase-contrast images using the 100X objective were collected every 3 minutes for 3 hours.

Procedures for experiments involving a subset of infected cells (Figure 4C) were identical, except cells infected with P22 Δc2 *m-cherry* were washed an additional 4 times in ice-cold PBS to reduce the concentration of un-adsorbed, free phage. In parallel, uninfected cells were washed once in ice-cold PBS. Infected cells were mixed at a ratio of 1:1000 with uninfected cells of the same genotype before being spotted onto the agarose pad. This ratio of uninfected to infected cells was optimized such that in randomly chosen microscopy fields (without prior knowledge of which cells in the field were infected) there was likely to be at least 1 infected cell. Infected cells were retrospectively identified during image analysis by their synchronized lysis within a 10-minute window at the beginning of the microscopy timelapse. For these experiments, phase-contrast and fluorescence images (mCherry) using the 20X objective were collected every 3 minutes for 3 hours.

**Microscopy- microfluidic infection**

The CellASIC ONIX2 system from EMD Millipore with B04A plates was used for microfluidic imaging experiments (Figure S3). Phages used in microfluidic infection experiments shown in Figure S5B (P22 HT or 9NA) were stained with SYTOX Orange Nucleic Acid Stain according to the protocol previously described (Valen et al., 2012). Stained phages washed 4 times in 15 mL M9 Glu$^+$ media using Amicon Ultra-15 centrifugal filter units. After staining, the titer and viability of phages were immediately assessed by plaque assay, and once stained, phages were used for no longer than 2 weeks. For use in the microfluidic experiments, SYTOX Orange stained phages were normalized to a titer of approximately 10$^{10}$ PFU/mL. Cells (D23580 bstA<sub>GFP</sub>, SNW403) were grown to early
exponential phase (OD_{600} \sim 0.1) in M9 Glu^+ at 37°C with shaking (220 RPM) before being loaded into CellASIC B04A plates using the pressure-driven method according to the manufacturer protocol for bacterial cells. The slanted chamber of the plate immobilizes the cells, but allows media to flow continuously. Firstly, cells were equilibrated with constant M9 Glu^+ media flow for approximately 30 minutes. Secondly, stained phages suspended in M9 Glu^+ media were flowed over the cells until the majority of cells were infected (typically 10-30 minutes). In the case of P22 HT phage (which exhibits inefficient adsorption to D23580 bstA-sfgfp due to the gtr locus of prophage BTP), phages were continuously flowed. Finally, M9 Glu^+ media was flowed over the cells for the duration of the experiment. Microfluorometric experiments typically lasted 5 hours, after which time uninfected cells outgrew the chamber. Phase-contrast and fluorescence images were collected every 1.5 minutes for the experiments in Figure S3C.

For the microfluidic imaging experiments shown in Figure S3D, strain SVO251 (S. Typhimurium D23580 Δψ STM1553::(P_{teta}− bstA-sfgfp-frt) ΔpSLT-BT ΔpBT1 pAW61 (P_{BAD}-parB-mcherry) was used. This strain contains the bstA-sfgfp fusion construct under the control of the P_{teta} promoter. However, this strain lacks the tetaT gene, and therefore expression of bstA-sfgfp is constitutive (not inducible). Additionally, this strain is cured of two natural plasmids that contain native partitioning systems (pSLT-BT and pBT1), and therefore might interfere with the correct function of the ParB-parS system used for phage DNA localization. The ParB-mCherry fusion protein is expressed from the pAW61 plasmid (Ap^+) under the control of the P_{BAD} promoter (induced by L-arabinose). Strain SVO251 was grown in M9 Glu^+ supplemented with 100 μg/mL ampicillin to maintain the pAW61 plasmid and 0.2% L-arabinose to induce expression of ParB-mCherry. The same supplemented media was used in the microfluidic chamber. Cells were grown to OD_{600} 0.1 before loading into the CellASIC B04A plate as described above. After 15 minutes growth, phage P22 Δpid::(parS-aph) (which contains one parS site along with a kanamycin resistance locus, aph, in place of the non-essential pid locus [Cenens et al., 2013]) diluted to a concentration of 10^8 PFU/mL (in M9 Glu^+ amp100 0.2% L-ara) was flowed into the chamber. Phase contrast and red and green fluorescence images were collected every 2 minutes for 4 hours.

**BstA protein homolog analysis**

BstA protein homologs were identified using tblastn (database: non-redundant nucleotide collection) and the HMMER webserver (Potter et al., 2018) (database: Reference Proteomes). The dataset of BstA protein homologs was manually curated to reflect the diversity of taxonomic background harbouring homologs. Evolutionary covariance analysis was done using DeepMetaPSICOV (Buchan and Jones, 2019) at the PSI-PRED server (Kandathil et al., 2019). To analyse the genetic context of BstA homologs, the sequence region 20 kb either side of the homolog (40 kb total) was extracted (BstA 40 kb neighbourhoods). To produce homogenous and comparable annotations, each region was re-annotated using Prokka 1.13 (Seemann, 2014). Additionally, the resulting annotated amino acid sequences were queried against our custom BstA profile-hmm and the Pfam 31.0 database (El-Gebali et al., 2019) with hmmerscan (Eddy, 1998), and the highest scoring significant hit per ORF was considered for the results shown in Figure 2. All the code is available in https://github.com/baymLab/2020_Owen-BstA.

Pairwise identity of homologs in Figure 2B to BstA^{BTP1} was computed using the EMBOSS Needle webserver (Needleman and Wunsch, 1970). BstA homologs were designated “putatively-prophage associated” if annotated genes in the 40 kb neighborhood contained any instance or the word “phage” or “terminase”. For categorization in Figure 2C, homologs were classed as having “high confidence association” if instances of gene annotations including the aforementioned key words occurred both before, and after, the BstA gene within the 40 kb neighborhood (i.e., to account for the possibility that a prophage-independent homolog could co-occur next to a prophage region by chance). Homologs classed as having “low confidence association” had at least one instance of genes whose annotations included “phage” or “terminase” either in the upstream or downstream 20 kb, but not both. Plasmid status was determined from information in the sequence records. The HHpred webserver was used to annotate the putative KilA-N domain (Zimmermann et al., 2018). All homolog neighbourhoods, homolog alignments and sequences is available to download https://github.com/baymLab/2020_Owen-BstA.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The phage replication, survival rate, efficiencies of transformation and of lysogeny were calculated as mentioned above. The numerical data were plotted and analyzed using GraphPad Prism 8.4.1. Unless stated otherwise in the Figure legends, data are presented as the mean of biological triplicates ± standard deviation. The unpaired t-test was used to compare the groups and statistical significance is indicated on the figures. P values are reported using the following criteria: < 0.0001 = ****, 0.0001 to 0.001 = ***, 0.001 to 0.01 = **, 0.01 to 0.05 = *, ≥ 0.05 = ns.