Systematic Discovery of Salmonella Phage-Host Interactions via High-Throughput Genome-Wide Screens

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Abstract

Salmonella species comprise a chronic threat to human health, with over 1.35 million infections and 420 deaths occurring in the US per year due to non-typhoidal, foodborne *Salmonella* infection. With the rise of antimicrobial resistant (AMR) infections, it is imperative to develop alternative prevention and treatment strategies, such as utilization of lytic bacteriophage as pathogen countermeasures. However, phage-host interactions remain poorly understood, impeding widespread practice. Here, we employed high-throughput, functional analyses to discover the genetic determinants of phage-host interactions between a model enteric *Salmonella* species, *Salmonella enterica serovar* Typhimurium (*S. typhimurium*) and its phages. To rapidly assess genetic contributions to phage sensitivity, we created a 66,996 member randomly barcoded transposon (RB-TnSeq) library in *S. typhimurium* MS1868. Using this library, we compared fitness across eleven diverse lytic *Salmonella* phages. Consistent with other loss of function studies against bacteriophage predation, this approach efficiently identified receptors essential to adsorption as well as their regulation. While many of the tested phages bound directly to the lipopolysaccharide (LPS) layer, we report a highly resolved view of differential structural LPS layer requirements for diverse *Salmonella* phages, including novel adsorption strategies. We also uncover unique routes to phage resistance, including phage-specific metabolic requirements, ion flow, and global transcription factor interplay, difficult to find through traditional approaches. constitutes a major challenge and opportunity. We highlight several examples of how the scale of barcoded screens allowed systems-level hypotheses to be efficiently formulated. These include discovery of a number of cases of cross-resistance and collateral sensitivity among the diverse phage

tested. We anticipate that the phage-resistance landscape presented here will faciliate better design of phage-based biocontrol treatments and phage-antibiotic combination therapies.

Introduction

Nontyphoidal *Salmonella enterica* infections comprise a chronic burden to human health. In the United States, the CDC estimates 1.35 million infections, 26,500 hospitalizations, and 420 deaths from nontyphoidal *Salmonella* sp. per year [1]. Most infections are spread through consumption of contaminated food products including poultry, plant, and waterborne routes of infection [2,3]. With increasing frequency, *Salmonella* infections display increased resistance to antibiotics, increased virulence, and multidrug-resistance-associated mortality, driving the need for antibiotic alternatives as both preventative and treatment options [1,2,4].

Globally, phage therapy is undergoing a renaissance and emerging as a viable antibiotic alternative against many pathogens. An increasing number of high-profile compassionate use cases in the clinic have highlighted the efficacy and potential of carefully administered bacteriophage treatment against numerous pathogens [5–7]. Additionally, direct bacteriophage application to food products and meat manufacturing pipelines serves as a preventative measure against foodborne pathogens such as *Salmonella* while limiting the use of traditional antibiotics and subsequent spread of antibiotic resistance [8].

Akin to antibiotic treatments, bacterial resistance to any individual phage inevitably emerges after phage application, typically located to phage-receptor genes [9]. However, by leveraging phage that target different receptors, combinations of phage (or phage cocktails) can be rationally chosen to both extend host range and limit the rate of resistance emergence. Evolutionarily, there are few documented single-mutation routes to resistance to phages that utilize different receptors and thus single-mutation routes to cross-resistance are generally minimized [9–12]. Thus, characterization of host-side requirements such as receptors are critical for phage application as a therapeutic or product [13].

Historically, *S. typhimurium* played a critical role in the identification of phage receptors, yielding critical insights into the genetics of complex receptors such as LPS and O-antigen and their structural composition [14–19]. However, mapping many phage-resistant mutations was a laborious process for a single phage, nonetheless for multiple diverse phages. Thus many of these mutants remain unmapped [9,16]. Many therapeutically employed *Salmonella* phages do not have identified receptors. For instance, phage cocktails from the ELIAVA institute in Tbilisi, Georgia are generated through successive enrichment of phage lysates on carefully chosen strains. Metagenomics and isolation studies from these complex samples have identified numerous Salmonella phages, but they often lack substantial characterization [13,20–22]. Metabolic, stress-response, and regulatory host-factor requirements beyond receptors are known for individual phages [23–26], but have not yet entered the conversation for therapeutic design.

Recent papers from our group and others demonstrated substantial increases in both the pace and resolution that we can infer bacteriophage-host interactions by use of pooled genetic screens (such as randomly barcoded transposon sequencing (RB-TnSeq)). Because these screens rely on sequencing of short barcodes as a proxy for genetic variants, they can be efficiently and economically scaled to assess the fitness benefits of thousands of genes across hundreds of conditions including bacteriophage predation [26–29]. However, the scale of these screens has not yet been applied to many phages in model pathogens such as *S. typhimurium*.

Here, we extend RB-TnSeq to *S. typhimurium* MS1868 to investigate its interactions with 11 of its bacteriophages, of which only 4 had substantial host-side genetic investigation prior to this work. We uncover putative receptor requirements for all 11 phages tested. We also identify unanticipated, complex host requirements more readily discovered using an unbiased genetic screen. These genotypes identify previously uncharacterized host-side requirements for phage predation, thereby providing insights into new routes to phage resistance.

Results

Assessing Fitness Against Diverse Bacteriophages using RB-TnSeq

We constructed a randomly barcoded mutant library in *Salmonella enterica* serovar Typhimurium MS1868 (MS1868) using methods previously reported for other RB-TnSeq transposon libraries (Methods). As a LT2-derived strain, MS1868 benefits from a long history of *Salmonella* phage-host genetic interaction studies and a well-characterized genome. After transposon mutagenesis, we obtained a 66,996 member pooled library consisting of transposon-mediated disruptions across 3,759 of 4,610 genes, with an average of 14.8 disruptions per gene (median 12) (Figure 1A). After comparing to a non-barcoded reported transposon mutant library in *S. typhimurium* 14028s [30], we suspect 434 of the 851 unmutated genes are likely essential, and 380 likely nonessential. We abstain from interpreting essentiality of 37 additional genes due to inability to uniquely map insertions or due to gene content differences between the two libraries. Additional details for the composition of the MS1868 library can be found in Supplementary Table 1 and Supplementary Dataset 1.

To assess host-side requirements for phage infection, we challenged the MS1868 mutant library with a diverse panel of dsDNA lytic phages (Table 1) at multiplicities of infection ≥2, where nearly each cell was exposed to an infective unit of phage at time of exposure. An overview of the experimental workflow is described in Figure 1B. For each phage, we challenged our mutant library in liquid culture, where fitness scores reflected competition between mutant strains. Akin to Mutalik et al [26], we also performed non-competitive experiments where we plated the library shortly after phage exposure (thus non-competitively reflecting strain fitness against phage). For each gDNA sample (collected before and at the end of each experiment), we performed deep sequencing on the 20 base pair barcodes (ie BarSeq) associated with each transposon mutant. We then assessed relative fitness defined as relative log2-fold-change of barcodes before versus after phage selection (Figure 1B). Thus, in this study, a high, positive fitness score

indicates loss-of-function mutants in which phage are unable to efficiently infect MS1868. In other words, a gene denoted with a high-fitness score is a host-side gene critical for phage infection. As observed in our earlier work [26], we observed very strong phage selection pressures during our experiments, so we limited analysis to positive fitness scores.

Using this experimental and analytical approach, we explored host-side fitness requirements for 11 lytic, dsDNA *Salmonella* phages that are currently employed as treatments and diagnostics. These phages are diverse, representing 3 Myoviridae, 5 Podoviridae, and 3 Siphoviridae. Only 4 of the phages investigated here have well-studied host-requirement characterization: Chi, FelixO1, P22Lys (a clear-plaque, obligately lytic mutant of P22), and S16 [15,16,18,28,31,32]. An additional 3 have suspected, but not extensively studied, host-factor requirements: Br60, Ffm, and SP6 [19,32,33]. We additionally isolated and subsequently investigated 4 bacteriophages from a commercial Intesti bacteriophage preparation in Georgia (see Methods): Aji_GE_EIP16 (Aji_GE), Reaper_GE_8C2 (Reaper_GE), Savina_GE_6H2 (Savina_GE), and Shishito_GE_6F2 (Shishito_GE). In aggregate, we report the results from 42 genome-wide loss of function assays across liquid and solid growth formats and discover 301 genes to be important for phage infection across the 11 phages studied (Supplementary Table 2, Supplementary Dataset 4). Here, we detail a comprehensive picture of the fitness landscape of critical factors for phage infection in *S. typhimurium*.

LPS and O-antigen are Critical Adsorption Elements for Salmonella Phages

In *Salmonella* species, LPS is well-characterized and highly diversified at O-antigen residues. Generally, LPS structure consists of 4 regions: KDO sugars covalently bonded to lipid A, the inner core, outer core, and O-antigen repeat (Figure 2A). In wild-type *S. typhimurium* LT2 (parental strain for MS1868), the O-antigen is made up of 200 hexose monomers per polymer [34]. These regions vary phylogenetically, with KDO and inner core structures being conserved across many genera and outer core structures not conserved between species. O-antigen structures in particular exhibit high degrees of variability – approximately 46 O-antigen structures have been discovered in *Salmonella spp.* [35]. Within the same strain, O-antigen structures can exhibit variability; through a variety of regulatory mechanisms, O-antigen can manifest as capsular polysaccharide, vary in repeat number, and vary in sugar modifications [34,36–38].

Ecologically, LPS-O-antigen structure and composition play a critical role in the lifestyle of *Salmonella*. Truncation, loss, or reduction of LPS and O-antigen in *Salmonella* is associated with decreased virulence; *in vitro* and *in vivo* studies indicate O-antigen-deficient strains have increased susceptibility to antibiotics, decreased swarming motility, decreased colonization, and decreased fitness [39–41]. However, it is well-established that intact LPS and O-antigen have a myriad of effects on phage predation. Depending on the phage, intact O-antigen can occlude, assist, or be the receptor for phage adsorption [18,32,42,43]. In essence, LPS and O-antigen can be considered as a series of layers that can insulate *Salmonella* from its environment, but potentially serve as the receptor to phage in its environment. All 11 phages tested against our library had some degree of LPS and O-antigen specificity.

The MS1868 library included mutants in 25 genes responsible for LPS biosynthesis and transport. 15 of these genes are involved in core LPS biosynthesis (*rfaH*, *rfaC*, *rfaZ*, *yaeD*, *rfaD*, *rfaP*, *rfaE*, *rfaF*, *rfaQ*, *rfaY*, *rfaG*, *rfaB*, *galE*, *rfaI*, and *rfaK*) and 9 genes are involved in O-antigen biosynthesis (*rfaL, wzzB*, *rfbP*, *rfbK*, *rfbC*, *rfbD*, *rfbB*, *pgm*, *galE*, and *oafA*). GalE synthesizes a precursor used in both LPS core and O-antigen biosynthesis (Figure 2A). In general, a loss of function mutation in a biosynthetically upstream gene disrupts remaining LPS and O-antigen biosynthesis. However, there are some notable exceptions. (1) *rfaZ*, *rfaY*, and *rfaQ* are not required for O-antigen maturation [44], (2) *rfaB* mutants yield strains with a heterogenous LPS O-antigen phenotype [45], (3) *yaeD* mutants in *E. coli* can form heptose-less LPS [46], and (4) *oafA* mutants do not otherwise affect O-antigen maturation [47]. In general, LPS requirements for each phage were found to be consistent between fitness data and an established chemotype-defined LPS mutant panel in *S. typhimurium*, representing 14 distinct LPS chemotypes (Salmonella Genetic Stock Center (SGSC), Supplementary Figures 1-10).

Four of the phages tested (P22Lys, SP6, Reaper_GE, Savina_GE) displayed strict requirements for full O4[5] antigen biosynthesis despite being unrelated (Figure 2BCD). This was expected for both P22Lys and SP6 since the O4[5] antigen is the established receptor for their respective tail fibers [17,19,43]. SP6 and P22Lys had the most stringent LPS and O-antigen requirements, requiring 19 and 18 of the 25 LPS and O-antigen biosynthesis genes respectively across liquid and solid fitness experiments. Relative to P22Lys, SP6 additionally required *yaeD* activity, suggesting that it is less infective on heptose-less LPS [46]. Although *rfaZ* and *rfaQ* gave high mean fitness scores for both phages, these scores were only high when the expected insertion was oriented against transcription, indicating that these fitness values were likely due to polar, transcription-disrupting effects on downstream genes. These results were largely consistent with a related genome-wide screen against P22Lys infection [28]. Compared to this study, host-factor requirement differences for P22Lys were largely attributed to differences in library coverage. Because the coverage of each library is different, each library can interrogate unique genes that the other can not. Due to better coverage in our library, our screens identified *rfaC*, *rfaD*, and *rfaE*. Due to lower coverage in our library, we were unable to analyze effects of mutants for several genes in O-antigen biosynthesis (highlighted in Figure 2A). Despite being analyzed in both studies, our study additionally identified *rfaP* as a novel host-factor for P22Lys (as well as SP6).

Unlike P22Lys and SP6, Reaper_GE and Savina_GE are novel phages investigated in this study and had no prior known host-requirements. The only genes Reaper_GE and Savina_GE required in this study were 16 and 15 of the 25 LPS and O-antigen biosynthesis genes respectively across liquid (both) and solid (Reaper_GE only) fitness experiments. Some additional host-requirement differences were noted between Reaper_GE and Savina_GE versus P22Lys and SP6. For instance, strains with *yaeD*, *pgm*, and *rfbB* (responsible for inner-core heptose biosynthesis and O-antigen precursor biosynthesis (Figure 2A)) disruptions were sensitive to Reaper_GE or Savina_GE phages. Additionally, the only *rfaP* disruptions that were fit against Reaper_GE and Savina_GE were oriented against transcription and are likely polar effects. This absolute

requirement for intact O-antigen including a complete LPS core was validated for Reaper_GE in our LPS chemotype panel (Supplementary Figure 3). Based on these results, we propose O-antigen as the receptor for Reaper_GE phage.

Unlike Reaper_GE, Savina_GE did not require *rfaI* (responsible for the second glucose addition to the outer core (Figure 2A)) and *rfaH* (responsible for activation of *rfa* and *rfb* operons). We found that fitness scores for genes changed in magnitude by position in the proteins' role in LPS biosynthesis. For instance, we found that O-antigen mutants displayed the strongest fitness in the presence of Savina_GE, followed by inner core mutants, and then outer core mutants (Figure 2AB, Supplementary Dataset 4), suggesting differential host interaction with LPS and O-antigen moieties. Unlike all other phages investigated here, solid assays yielded little selection pressure against Savina_GE, suggesting generally poor infection of Savina_GE to wild-type MS1868. We further investigated this fitness pattern on our LPS chemotype panel and found results consistent with our BarSeq data; phage Savina_GE was most infective against strains with an incomplete outer core, but less so against strains without O-antigen or strains missing outer core entirely (Supplementary Figure 5). Based on this data, it appears that phage Savina_GE employs an infection strategy similar to phage PVP-SE1 [48]. Potentially, Savina_GE preferentially employs LPS as a receptor, but branched LPS residues such as those added by *rfaK* and O-antigen biosynthesis hinder adsorption.

FelixO1 gave results consistent with literature identifying outer core GlcNAc, the biosynthetic product of RfaK, as FelixO1's primary receptor [32]. In addition, mutants in 12 of the LPS biosynthesis genes conferred resistance against FelixO1 (*rfaH*, *rfaC*, *yaeD*, *rfaD*, *rfaP*, *rfaE*, *rfaF*, *rfaG*, *rfaB*, *galE*, *rfaI*, and *rfaK*) in both liquid and solid fitness experiments. High fitness score of *yaeD* (responsible for inner-core heptose biosynthesis), suggested the importance of inner core integrity for FelixO1 infection [46]. We also found an additional 61 non-LPS genes important in FelixO1 infection, which will be discussed below (see Discovery of Novel Cross-Resistant Genotypes Between Diverse Phages).

S16, Chi, and Aji_GE all likely employ protein-based receptors (see Protein Receptors and Their Regulation), but also each had some degree of reliance on LPS. *rfaI* and *rfaG* mutants provided small, but significant, fitness benefits against phage S16 (Figure 2). Plaque assays against defined LPS chemotypes suggested decreased infectivity due to *rfaF* and *rfaG* mutants (Supplementary Figure 6), but overall, these results are consistent with literature suggesting that inner core *Salmonella* LPS assist in, but are not strictly required for, S16 adsorption [18]. Mutants in *rfaH*, *yaeD*, *rfaP*, *rfaQ*, *rfaY*, *rfaG*, and *pgm* all provided fitness benefits against phage Chi, results not before observed for Chi. Nonetheless, it is likely that these mutants primarily impact motility, thus reducing Chi infectivity (see Protein Receptors and Regulation) [40]; additionally, homologs of *rfaH*, *rfaP*, *rfaQ*, *rfaY*, and *rfaG* are among the least motile mutants in experiments for *E. coli* BW25113 [27]. Since LPS disruptions induce stress responses, this pleiotropy may be stress-induced downregulation of outer membrane proteins and flagellar activity [26].

Phage Aji_GE, a T5-like phage, showed minimal requirements for LPS or O-antigen except for disruptions in *oafA* (Figure 2BC). OafA performs an acylation reaction on the abequose residue (Figure 2A) of O5-antigen in LT2-derived strains [49], but loss-of-function of this gene doesn't yield other impacts on O-antigen biosynthesis [47]. We propose that Aji_GE employs *oafA* O-antigen acetylation to enhance access to its primary receptor. A similar receptor archetype has been observed in T5 and T5-like phage SPC35, employing different O-antigen modifications to enhance access to their primary receptor [50,51]. To confirm Aji_GE's dependence on *oafA*, but not necessarily on O-antigen and LPS, we screened Aji_GE infectivity on an *oafA* mutant and our LPS chemotype panel. Of this panel, only the *oafA* mutant gave resistance to this phage (Supplementary Figures 7, 11). Thus, OafA activity is important for Aji_GE infection, but the acetylation provided by OafA activity is not critical in the absence of complete LPS and O-antigen. This makes Aji_GE the first phage shown to be reliant on OafA activity and suggests naturally occurring *oafA* mutants may emerge from phage predation as previously speculated [47].

Uncovering Host-Factors of Rough Phages – Implications for Exploiting Collateral Sensitivity Between LPS-Binding Phages

Br60, Ffm, and the newly isolated Shishito_GE phage are T7-like rough phages. Against O-antigen positive strains, such as our library base strain, MS1868, none of these phages successfully infect as they are occluded from their native receptor by the O-antigen structure. However, they can infect strains without O-antigen [14] (Figure 3AB, Supplementary Figures 8-10). Thus, the resistance landscape between O-antigen requiring phages (for instance P22Lys, Reaper_GE, or SP6) and rough-LPS-requiring phages (Br60, Ffm, and Shishito_GE) presents an evolutionary trade-off through collateral sensitivity. In other words, many mutants resistant to O-antigen requiring phages display increased sensitivity to the rough-LPS requiring phages. However, the extent of collateral-sensitivity depends on infection requirements of the rough phages, which remain unknown.

As expected with our MS1868 library primarily consisting of O-antigen positive mutants, the vast majority of gene disruptions in MS1868 showed no significant fitness benefit against these phages. However, we noticed strong fitness defects in many of the LPS and O-antigen mutants in our library (Supplementary Dataset 4), consistent with optimal adsorption and infection in O-antigen-defective *Salmonellae*.

Many lab model strains of *E. coli* lost their ability to produce O-antigen, but have a similar inner and outer core LPS structure to *S. typhimurium* [52]. Thus we postulated and confirmed that these phages may additionally infect strains such as *E. coli* K-12 BW25113 (BW25113) (Figure 3C). Thus, we repeated experiments for these 3 phages using an earlier reported *E. coli* RB-TnSeq library as a model "rough LPS" library analog to the MS1868 library [26,53]. Consistent with related phages T3 and T7 from earlier library analyses, few host-factors were required for infection. All 3 phages showed slightly different LPS requirements for infection. Against Ffm and Shishito_GE, mutants in *gmhA*, *hldE*, *hldD*, and *waaC* were phage-resistant (*ghmA*, *rfaE*, *rfaD*, *rfaC* MS1868 homologs respectively). Ffm additionally required *waaG* (*rfaG* MS1868 homolog) for infectivity.

However, no LPS or protein mutants were fit against Br60. *trxA* mutants were fit against all three rough phages (Figure 3D) (Supplementary Dataset 4).

To confirm that these results were extensible to *S. typhimurium*, we assayed infectivity against our LPS mutant collection (SGSC, Supplementary Figures 8-10). Indeed, the results observed against the *S. typhimurium* LPS panel were perfectly analogous to the fitness experiments observed against the BW25113 library, with the exception of *rfaB*, which has a known heterogenous, smooth phenotype in *S. typhimurium* and likely occludes adsorption [45]. *S. typhimurium* homologs of host-factors inferred from the BW25113 library experiments were important for infection of these phages. Ffm required function of *rfaC*, *rfaD*, *rfaG*, *rfaB*, and loss of O-antigen (Supplementary Figure 8). Shishito_GE required function of *rfaC*, *rfaD*, *rfaB*, and loss of O-antigen (Supplementary Figure 9). Br60 only required loss of O-antigen (Supplementary Figure 10). From our *E. coli* and *S. typhimurium* LOF libraries and *S. typhimurium* LPS mutant collection results, we conclude that these related T7-like bacteriophages require distinct LPS moieties, providing additional resolution to the putative receptors of these phages. Br60 emerged from these experiments as a particularly interesting phage due to the inability to detect important host-factors other than *trxA* from the saturated BW25113 transposition library; potentially this phage binds to LPS KDO sugars. These LPS residues are synthesized by enzymes encoded by essential genes, making host-side loss-of-function a futile way for a pathogen to escape this phage. Because the loss of function cross-resistance with O-antigen requiring phages (Figure 2) is minimized with Br60, Br60 appears to be a better candidate for exploiting collateral sensitivity than Ffm or Shishito_GE if applied alongside an O-antigen requiring phage.

Identification of Salmonella Phage Protein Receptors and Receptor Regulation

Phages S16, Aji_GE, and Chi showed dependence on outer protein structures in addition to LPS. Prior genetic and biochemical characterization of T4-like phage S16 identified OmpC as the primary receptor [18]. Concordantly, in our screens, *ompC* loss of function mutants were highly fit against S16. In addition, positive regulators of *ompC* expression, *ompR* and *envZ* [54], were enriched against S16 (Figure 4AB). Our screens are consistent with earlier findings of OmpC as the primary receptor with influence from LPS for S16, similar to similar screens against related phage T4 (Figure 2A, Figure 4ABC) [18,26].

For T5-like phage Aji_GE, we find *fepA* (that encodes a TonB-dependent enterobactin receptor) as a high-scoring host-factor in addition to *oafA*. As FepA is known to form a complex with TonB and function as a receptor for some T5-like phages, such as phage H8 [55], we believe the FepA-TonB complex is the primary receptor for phage Aji_GE (Figure 4AC). As our RB-TnSeq library lacked *tonB* mutants, we created *fepA* and *tonB* deletions to validate the role of FepA and to assess the requirement of TonB. Indeed, individual *fepA* or *tonB* deletion mutants gave resistance to Aji_GE, confirming their essentiality for Aji_GE infection (Figure 4D, Supplementary Figure 11). These results indicate that, potentially similar to phages T5 and SPC35, phage Aji_GE employs LPS modifications (here, added by OafA) to enhance infection [50,51] and gain access to the FepA-TonB complex for efficient infection.

Bacteriophage Chi is a model flagellar-binding bacteriophage that employs flagellar activity to approach the *S. typhimurium* outer membrane [56]. Because flagellar assembly and activity is dependent on the concerted activity of around 50 genes (for a review on enteric flagella, see Macnab et al. (2003) [57]), we postulated that a systems-level view may provide efficient insight into the host-requirements for Chi-like phage versus individual genetic mutant studies. As expected, 36 genes across *flg*, *flh*, *fli*, and *mot* operons responsible for flagellar biosynthesis and regulation gave strong fitness scores, implicating the importance of flagellar biosynthesis for phage Chi infection (Figure 4A).

In addition, we found a number of flagellar activity-modulating factors important against phage Chi infection. For example, we observed *cheZ* mutants fit against Chi phage (Supplementary Figure 12). CheZ functions by dephosphorylating CheY-phosphate, biasing flagella counterclockwise towards smooth swimming [57]. Putatively, loss of CheZ activity biases flagellar activity clockwise, leading to tumbling and subsequent Chi resistance, consistent with CheY activity studies and Chi phage [31]. In addition, several mutants in guanosine penta/tetraphosphate ((p)ppGpp) biosynthesis and metabolism were enriched in Chi infection experiments, potentially due to impacts on motility [58]. Other top-scoring mutants against Chi infection include *nusA*, *tolA*, and *cyaA,* that were earlier found to greatly decrease *E. coli* motility [27], suggesting that indirect motility defects provide numerous routes to resist flagella-dependent phages. Because phase I flagellin, *fliC*, show high fitness scores, but phase II flagellin, *fljB*, and *fliC*'s repressor, *fljA*, did not, Chi infection could be specific to phase I flagellar phenotypes [59]. However, further studies are needed to dive deeper and mechanistically confirm this result.

As high-throughput genetic screens to infer interactions between bacteriophage and their hosts grow more commonplace, we expect to see further enrichments in our understanding of bacteriophage adsorption requirements. For instance, we doubt that dual LPS + protein receptor combinations are unique to S16, Aji_GE, and Chi - many such combinations likely remain to be discovered as more phage are characterized. Additionally, many Chi-like bacteriophage variants exist, some of which depend on different flagellar rotations or other flagellin filaments [58,60]. Due to the pooled nature of RB-TnSeq experiments, we are able to characterize in a single experiment what would have otherwise taken over 75 individual mutant experiments for some of these phages.

Discovery of Novel Cross-Resistant Genotypes Between Diverse Phages

Cross-resistance can occur between phages when resistant mutants to one phage display resistance to a different phage [23,26]. Generally discussion of phage cross-resistance is focused on phage receptors - combinations of phages that employ distinct receptors yield minimal cross-resistance, and therefore yield decreased rates of resistance emerging [9,10,12]. While non-receptor mutants can display phage-cross resistance, they have not been identified in *Salmonella* phage [23,29]. Nonetheless, an increased understanding of phage cross-resistance could impact therapeutic design by identifying hard-to-predict incompatibilities between unrelated phages.

To illustrate that our phage characterization platform can uncover cross-resistance profiles, we looked at phage resistance genotypes enriched in our assay conditions. Two

phages, Aji_GE and FelixO1, showed a large degree of cross-resistance between their fitness profiles independent of their primary receptors. Out of 52 non-receptor LOF genotypes conferring resistance to FelixO1 and 32 non-receptor LOF genotypes conferring resistance to Aji_GE, 29 genotypes were resistant to both Aji_GE and FelixO1 (Figure 5). Functions included disruptions across central metabolism (*aceEF*, *pta*, *ackA*, *fabF*), amino acid biosynthesis and regulation (*rpoN*, *glnDLG*, *ptsIN*, *aroM*), global regulators (*himAD*, *crp*, *rpoN*, *lon*, *arcB*), ion transport (*trkAH*), secondary messenger signaling (*gppA*, *cyaA*), translation (*trpS*) among many other genes with less clear functions (*sapABCF*, *nfuA*, *yfgL*, *ytfP*).

To investigate if these mutants were indeed cross-resistant to both Aji_GE and FelixO1, we selected a few top scoring genes to investigate further: *trkH*, *sapB*, *aceE*, *rpoN*, *himA*, and *himD*. Several of these genes were recently implicated as host-factors in phage resistance in related organisms [23,25], although the extent of phage cross-resistance and mechanisms were not determined. *himA* and *himD* (i.e. integration host factor subunits alpha and beta) were known to be involved in temperate phage infection pathways, though never shown for obligately lytic phages such as Aji_GE and FelixO1 [61].

To validate their role in Aji_GE and FelixO1 infectivity in *Salmonella*, we created individual mutants of *trkH*, *sapB*, *aceE*, *rpoN*, *himA*, and *himD* through genetic replacement and assayed Aji_GE and FelixO1 infectivity. Indeed, all *trkH*, *sapB*, *aceE*, *rpoN*, *himA* and *himD* mutants showed resistance to FelixO1 and Aji_GE (Figure 5C, Supplementary Figures 13, 14). Consistent with prior reports of high fitness costs being associated with non-receptor phage cross-resistant mutants [23], mutants in *aceE*, *rpoN*, and *himA* displayed significant growth defects during planktonic growth. Many of these genes are known to play an important ecological role in *Salmonella* virulence and fitness in infection contexts [62,63], indicating these phage resistance loci may exhibit evolutionary trade-offs with virulence or ecological fitness in application contexts.

RpoS Activity Mediates Phage Cross-Resistance

Towards discovering the specifically regulated factors responsible for the phage cross-resistance phenotype imparted by *trkH*, *sapB*, *rpoN*, and *himA* mutants, we carried out RNA-Seq experiments and investigated whole-genome expression-level differences compared to wild-type MS1868 (N=3 for all except for *himA*, which was N=2). We observed many shared regulatory changes compared to wild-type MS1868 (Figure 6A) (Supplementary Dataset 6). In all cases, neither of the known innate immunity defense mechanisms in *S. typhimurium* (type I CRISPR or type I BREX), were found to be differentially expressed [64,65]. However, in *trkH*, *sapB*, and *rpoN* mutant backgrounds, we observed that a number of known RpoS-regulated genes [66] were significantly upregulated versus wild-type (log2FC > 2, p_adj < 0.001) (Figure 6B, Supplementary Dataset 6), implicating RpoS involvement in cross-module phage resistance for both Aji_GE and FelixO1 (Figure 6B).

The sigma-factor RpoS activity in *Salmonella* is critical for many aspects of its lifestyle, including general virulence, host cell takeover, and general stress response [66,67]. However, recent studies in *Salmonella* found decreased RpoS activity in model strain LT2

versus related virulent strains due to a suboptimal start codon [68]. Being a LT2 derivative, MS1868 has this suboptimal codon, so it is intriguing to find signatures of elevated RpoS activity in phage resistant candidates. We suspect that, in the 28 cross-resistant mutants, a facet of RpoS activity itself is decreasing susceptibility to phages Aji_GE and FelixO1. Potentially, a similar mechanism of elevated RpoS in the *trk* and *sap* mutants provided resistance to T4-like phages seen in transposon library experiments of *E. coli* O157 [25].

To validate the impact of RpoS on phage infection, we created a *rpoS* deletion mutant and additional double gene replacement mutants of *rpoS* with one of *trkH*, *sapB*, *rpoN*, or *himA*. The single *rpoS* deletion mutant displayed increased sensitivity to both FelixO1 and Aji_GE phage (Figure 6CD). In addition, *rpoS* deletion was also sufficient to restore infectivity in *trkH*, *sapB*, and *himA* mutants to levels observed in *rpoS* mutants (Figure 6D, Supplementary Figures 13, 14). While *himA* mutants did not show elevated levels of RpoS activity in our RNA-Seq data, we suspect that phage-resistant RpoS activity may be playing a role in intermediate phage-resistance phenotypes that are typically difficult to quantify in our pooled fitness assays [26].

In the *rpoN* (encoding sigma factor-54) mutant background, an additional *rpoS* mutation was sufficient to restore infectivity of phage Aji_GE, but insufficient to restore infectivity of FelixO1 (Figure 6C). Like RpoS, the alternate sigma factor RpoN is known to regulate a diverse set of pathways involved in adaptation and survival in unfavorable environmental conditions including nitrogen starvation. Because *rpoN* mutants decrease glutamine uptake and biosynthesis and have significant growth defects, the phage resistance phenotype observed in *rpoN* mutants potentially indicate the importance of glutamine levels on successful phage infection (Supplementary Dataset 4) (see also: [24,69]). To assess the dependence of glutamine on phage resistance mechanism, we repeated phage infection supplemented with glutamine or casamino acids. Both FelixO1 and Aji_GE were able to successfully plaque on *rpoN* mutants supplemented with glutamine, but not in the presence of casamino acids. In the *rpoN*, *rpoS* double mutant background, additional glutamine supplementation was able to nearly restore FelixO1 infectivity to the *rpoS* mutant's baseline (Figure 6C). Thus, we propose *rpoN* loss-of-function manifests two avenues of phage resistance. First, nutrient limitation to the cell can "starve" phage replication, such as FelixO1 but not Aji_GE, during infection. Second, elevated RpoS activity (likely induced by nutrient limitation) confers further resistance to phage infection, extending to diverse phages such as FelixO1 and Aji_GE.

Discussion

Here, we employed a high-throughput approach to uncover the genetic determinants important in phage infection and resistance in a model enteric *Salmonella* species across 11 distinct dsDNA phages. We identified known receptors in model *Salmonella* phages, rapidly characterized host requirements in uncharacterized phages, and discovered novel host factors essential for successful phage infection. We unveiled a highly resolved view of differential LPS layer requirements for diverse *Salmonella* phages, suggesting a multitude of adsorption strategies to this complex structure. We additionally uncovered an extensive network of cross-resistant genotypes against unrelated phages. This network implicated an expanded role of phage-specific metabolic requirement and global

transcription factor interplay in phage cross-resistance. Additional resistance factors could be identified through high-throughput gain-of-function screens [26,70]. By minimizing cross-resistance factors between phages, we anticipate these insights and methods will be directly useful towards rational formulation of phage cocktails.

As high-throughput genetic screens to understand phage-host interactions grow more commonplace across diverse bacteria [25–29,53,58,70–72], leveraging fitness data across phages and bacterial genetic diversity constitutes a major challenge and opportunity. Although not systematically investigated here, we highlighted several examples of how the scale of barcoded screens allowed systems-level hypotheses to be efficiently formulated. Employing additional libraries [26,53] identified important infection factors for rough phages Ffm, Shishito_GE, and Br60. Analyzing previous motility assays [27], provided rationale for many indirect motility defects genotypes conferring resistance to Chi phage. Additional screens against antibiotics, such as those presented in Price et al., [27], could rapidly discover collateral sensitivity patterns wherein phage resistant genotypes display sensitization to antibiotics or ecologically-relevant conditions (for instance sera or bile salts). Such information has the potential to form the basis of successful combinations or treatments [6]. We posit that phage-host interaction studies across diverse hosts, phages, and conditions enable much needed rational therapeutic and biocontrol solutions.

Author contributions

B.A.A., A.M.D., V.K.M., and A.P.A. conceived the project.
B.A.A. led the experimental work, analysis, and manuscript preparation.
B.A.A., V.K.M., C.Z., and H.L. built and characterized the MS1868 RB-TnSeq library.
B.A.A. performed experiments, processed, and analyzed data.
E.B.K. provided critical reagents and advice.
B.A.A., E.B.K., A.M.D., V.K.M., and A.P.A. wrote the paper.

Competing Interests

V.K.M., A.M.D., and A.P.A. consult for and hold equity in Felix Biotechnology Inc..

Acknowledgments

The authors gratefully thank Kenneth Sanderson (Salmonella Genetic Stock Center), Michael McClelland, Sylvain Moineau (Félix d'Hérelle Reference Center for Bacterial Viruses), Richard Calendar, Ian J. Molineux, and Jason J. Gill for sharing bacterial strains and phages and supplying valuable advice. Additionally, we would like to thank Morgan Price for helpful conversations during analysis and preparation of the manuscript.

This project was funded by the Microbiology Program of the Innovative Genomics Institute, Berkeley. The initial concepts for this project were funded by ENIGMA, a Scientific Focus Area Program at Lawrence Berkeley National Laboratory, supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under contract DE-AC02- 05CH11231.

RNA sample processing and library creation was performed at Functional Genomics Lab, Vincent J. Coates Genomics Sequencing Lab, & Computational Genomics Resources Lab (University of California at Berkeley). Sequencing was performed at: Vincent J. Coates Genomics Sequencing Laboratory (University of California at Berkeley), supported by NIH S10 Instrumentation Grants S10RR029668, S10RR027303, and OD018174.

Methods

Bacterial strains and growth conditions

Strains, primers, and plasmids are listed in Supplementary Tables 3, 4, and 5, respectively. In general, all *Salmonella* strains were grown in Luria-Bertani (LB-Lennox) broth (Sigma) at 37 ˚C, 180 rpm unless stated otherwise. When appropriate, 50 µg/mL kanamycin sulfate and/or 34 µg/mL Chloramphenicol were supplemented to media. For strains containing an ampR selection marker, Carbenicillin was employed at 100 µg/mL, but exclusively used during isolation of clonal mutants to avoid mucoidy phenotypes. All bacterial strains were stored at -80ºC for long term storage in 25% sterile glycerol (Sigma).

Bacteriophages and propagation

Bacteriophages employed in this study and sources are listed in Table 1. All phages were either successively serially diluted or streaked onto 0.7% LB-agar overlays for isolation. For bacteriophage Chi, 0.35% LB-agar overlays were employed. Bacteriophage Aji_GE_EIP16, Reaper_GE_8C2, Savina_GE_6H2, and Shishito_GE_6F2 were isolated from an Intesti Bacteriophage formulation from Georgia (M2-601). All phages isolated from this source are denoted with "_GE" (to recognize being sourced from Georgia). All other bacteriophages were re-isolated from lysates provided from stock centers or gifts from other labs (Table 1). Bacteriophage Aji_GE_EIP16, Chi, FelixO1, P22lys, Reaper_GE_8C2, S16, and SP6 were isolated and scaled on *S. typhimurium* MS1868 (*S. typhimurium* LT2 *(leuA414(Am) Fels2- hsdSB(r-m+)*) (MS1868). Bacteriophage Br60, Ffm, Savina_GE_6H2, and Shishito_GE_6F2 were isolated and rough-LPS mutant *S. typhimurium.* SL733 (BA1256). We followed standard protocols for propagating phages [73]. Br60, Chi, Ffm, P22lys, Reaper_GE_8C2, S16, Savina_GE_6H2, Shishito_GE_6F2, and SP6 were propagated in LB-Lennox liquid culture on their respective strains. Reaper_GE_8C2, Savina_GE_6H2, and Shishito_GE_6F2 were additionally buffer-exchanged into SM-Buffer (Teknova) via ultrafiltration (Amicron 15) and resuspension. Bacteriophage Aji_GE_EIP16 and FelixO1 were propagated in LB-Lennox liquid culture on their respective strains and further propagated through a standard overlay method. Whenever applicable, we used SM buffer without added salts (Tekova) as a phage resuspension or dilution buffer and routinely stored phages as filter-sterilized (0.22um) lysates at 4ºC.

Construction of MS1868 RB-TnSeq library

We created the *Salmonella enterica* subsp. Typhimurium serovar MS1868 (MS1868_ML3) transposon mutant library by conjugating with *E. coli* WM3064 harboring pHLL250 mariner transposon vector library (strain AMD290). To construct pHLL250, we used the magic pools approach we outlined previously[74]. Briefly, pHLL250 was

assembled via Golden Gate assembly using BbsI from part vectors pHLL213, pHLL216, pHLL238, pHLL215, and pJW14[74]. We then incorporated millions of DNA barcodes into pHLL250 with a second round of Golden Gate assembly using BsmBI. Briefly, we grew *S. typhimurium* LT2 MS1868 at 30ºC to mid-log-phase and combined equal cell numbers of *S. typhimurium* LT2 MS1868 and donor strain AMD290, conjugated them for 5 hrs at 30°C on 0.45-µm nitrocellulose filters (Millipore) overlaid on LB agar plates containing diaminopimelic acid (DAP) (Sigma). The conjugation mixture was then resuspended in LB and plated on LB agar plates with 50 ug/ml kanamycin to select for mutants. After 1 day of growth at 30°C, we scraped the kanamycin-resistant colonies into 25 mL LB and processed them as detailed earlier to make multiple 1-mL 80°C freezer stocks. To link random DNA barcodes to transposon insertion sites, we isolated the genomic DNA from cell pellets of the mutant libraries with the DNeasy kit (Qiagen) and followed published protocol to generate Illumina compatible sequencing libraries[53]. We then performed single-end sequencing (150 bp) with the HiSeq 2500 system (Illumina). Mapping the transposon insertion locations and the identification of their associated DNA barcodes was performed as described previously [27].

Liquid culture "competitive" fitness experiments

Competitive, phage-stress fitness experiments were performed in liquid culture, as phage progeny from an infection of one genotype could subsequently infect other host genotypes. All bacteriophages were tested against the MS1868 library. Bacteriophage Br60, Ffm, and Shishito_GE_6F2 were additionally tested against the previously described *E. coli* BW25113 library [53]. To avoid jackpot effects, at least two replicate experiments were performed per phage-host library experiment as presented earlier [26]. Briefly, a 1 mL aliquot of RB-TnSeq library was gently thawed and used to inoculate a 25 mL of LB supplemented with kanamycin. The library culture was allowed to grow to an OD600 of ~1.0 at 37˚C. From this culture we collected three, 1 mL pellets, comprising the 'Time-0' or reference samples in BarSeq analysis. The remaining cells were diluted to a starting OD600 of 0.04 in 2X LB with kanamycin. 350 µL of cells were mixed with 350 µL phage diluted in SM buffer to a predetermined MOI and transferred to a 48-well microplate (700 µL per well) (Greiner Bio-One #677102) covered with breathable film (Breathe-Easy). Phage infection progressed in Tecan Infinite F200 readers with orbital shaking and OD600 readings every 15 min for 3 hours at 37˚C. At the end of the experiment, each well was collected as a pellet individually. All pellets were stored at -80˚C until prepared for BarSeq.

Solid agar "noncompetitive" fitness experiments

Noncompetitive, phage-stress fitness experiments were performed on solid-agar plate culture as presented earlier [26]. Solid plate fitness experiments were performed by assaying all 11 bacteriophages against the MS1868 library. Bacteriophage Br60, Ffm, and Shishito_GE_6F2 were additionally assayed on the *E. coli* BW25113 library [53]. For the solid plate experiments a 1 mL aliquot of the RB-TnSeq library was gently thawed and used to inoculate a 25 mL LB supplemented with kanamycin. The library culture was allowed to grow to an OD600 of ~1.0 at 37˚C. From this culture we collected three, 1 mL pellets, comprising the 'Time-0' for data processing in BarSeq analysis. The remaining

cells were diluted to a starting OD600 of 0.01 in LB with kanamycin. 75 µL of cells were mixed with 75 µL of phage diluted in SM buffer to a predetermined MOI and allowed to adsorb for 10 minutes. The entire culture was spread evenly over a LB agar plate with kanamycin and grown overnight at 37˚C. The next day, all resistant colonies were collected and suspended in 1.5 mL LB media before pelleting. All pellets were then stored at -80˚C until prepared for BarSeq.

BarSeq of RB-TnSeq pooled fitness assay samples

Genomic DNA was isolated from stored pellets of enriched and 'Time 0' RB-TnSeq samples using the DNeasy Blood and Tissue kit (Qiagen). We performed 98°C BarSeq PCR protocol as described previously [26,53]. BarSeq PCR in a 50 uL total volume consisted of 20 umol of each primer and 150 to 200 ng of template genomic DNA. For the HiSeq4000 runs, we used an equimolar mixture of four common P1 oligos for BarSeq, with variable lengths of random bases at the start of the sequencing reactions (2–5 nucleotides). Equal volumes (5 uL) of the individual BarSeq PCRs were pooled, and 50 uL of the pooled PCR product was purified with the DNA Clean and Concentrator kit (Zymo Research). The final BarSeq library was eluted in 40 uL water. The BarSeq samples were sequenced on Illumina HiSeq4000 instruments with 50 SE runs. Typically, 96 BarSeq samples were sequenced per lane of HiSeq.

Data processing and analysis of BarSeq reads

Fitness data for the RB-TnSeq library was analyzed as previously described [53]. Briefly, the fitness value of each strain (an individual transposon mutant) is the normalized log2(strain barcode abundance at end of experiment/strain barcode abundance at start of experiment). The fitness value of each gene is the weighted average of the fitness of its strains. Further analysis of BarSeq data was carried out in Python3 and visualized employing matplotlib and seaborn packages. For heatmap visualizations, genes with under 25 BarSeq reads in the phage samples had their fitness values manually set to 0 to avoid artificially high fitness scores (due to the strong selection pressure imposed by phage predation).

Due to the strong selection pressure and subsequent fitness distribution skew resulting from phage infection, a couple additional heuristics were employed during analysis. Initially, per phage experiment, fitness scores were filtered for log2-fold-change thresholds, aggregated read counts, and t-like-statistics. Experiments using phages Ffm, Shishito_GE, and Br60 (which cannot infect wild-type MS1868, but can infect specific MS1868 mutants) against the MS1868 library employed negative thresholds to identify sensitized genotypes. A summary of log2-fold-change fitness and t-like statistic thresholds are provided in Supplementary Dataset 2. Each reported hit per phage was further processed via manual curation to minimize reporting of false-positive results due to the strong phage selection pressure. Here, all individual barcodes per genotype were investigated simultaneously for each experiment through both barcode-level fitness scores and raw read counts. First, genotypes were analyzed for likely polar effects. If the location and orientation of each fit barcode were exclusively against the orientation of

transcription and/or exhibited strong fitness at the C-terminus of a gene, while being transcriptionally upstream of another fit gene, the genotype was likely a polar effect and eliminated. Second, genotypes were analyzed for jackpot fitness effects that could indicate a secondary site mutation. These cases were identified by investigating consistency between individual strains within a genotype. If the vast majority of reads per genotype belonged to a singular mutant (of multiple), we attributed the aggregate fitness score to secondary-site mutation effects and eliminated those genotypes from reported results. Genotypes where there were too few strains to make a judgment call on within genotype strain consistency (ie 1-3 barcodes) were generally excluded from analysis unless they were genotypes consistent with other high-scoring genotypes. Next, we investigated for consistency between read counts and fitness scores at both the strain level. In general, we found that strains with read counts under 25 often had inflated fitness scores under strong phage selection pressure and the subsequent fitness distribution skew resulting from phage infection. Cases where high fitness scores were attributed to a couple of strains with reads under 25 were eliminated as false positives as well. Finally, all genotypes were loosely curated for consistency across liquid experiments. Cases that barely passed confidence thresholds as described above that were inconsistent across replicate experiments were eliminated from reporting. A summary of fit genotypes that passed automated filtering and manual curation are reported in Supplementary Dataset 4. No fit genotypes were added during manual analyses.

Individual Mutant Creation

All individual deletion mutants in *S. typhimurium* were created through lambda-red mediated genetic replacement [75]. Per deletion, primers were designed to PCR amplify either kanamycin or ampicillin selection markers with ~30-40 bp of homology upstream and downstream of the targeted gene locus, leaving the native start and stop codons intact preserving directionality of gene expression at the native locus (Supplementary Table 3). PCRs were generated and gel-purified through standard molecular biology techniques and stored at -20˚C until use. All strains (including mutants) employed in this study are listed in Supplementary Table 5.

Deletions were performed by incorporating the above dsDNA template into the *Salmonella* genome through standard pSIM5-mediated recombineering methods [75]. First temperature-sensitive recombineering vector, pSIM5, was introduced into the relevant *Salmonella* strain through standard electroporation protocols and grown with chloramphenicol at 30˚C. Recombination was performed through electroporation with an adapted pSIM5 recombineering protocol. Post-recombination, clonal isolates were streaked onto plates without chloramphenicol at 37˚C to cure the strain of pSIM5 vector, outgrown at 37˚C and stored at -80˚C until use. For double deletions, this process was repeated two times in series with kanamycin followed by ampicillin selection markers. Gene replacements were verified by colony PCR followed by Sanger sequencing at the targeted locus (both loci if a double deletion mutant) and 16S rDNA regions (primers provided in Supplementary Table 3).

Assessing Phage Sensitivity

Phage-resistance and -sensitivity was assessed through efficiency of plating experiments. Bacterial hosts were grown overnight at 37˚C. 100 µL of these overnight cultures were added to 5 mL of top-agar with appropriate antibiotics and allowed to solidify at room temperature. For assays including supplements such as glutamine, the supplement was added directly to the top agar layer. Phages were ten-fold serially diluted in SM Buffer, two microliters spotted out on the solidified lawn, and incubated the plates overnight at 37˚C. Efficiency of plating was calculated as the ratio of the average effective titer on the tested host to the titer on the propagation host. For some assay strains, plaques showed diffused morphology and were difficult to count, or displayed plaque phenotypes distinct from its propagation host. In all cases, representative images are presented (Supplementary Figures 1-14). All plaquing experiments were performed with at least three biological replicates, each replicate occurring on a different day from a different overnight host culture.

RNA-Seq experiments

Samples for RNA-Seq analysis were collected and analyzed for wild-type MS1868 (BA948) (N=3), knockout mutants for trkH (BA1124) (N=3) *sapB* (BA1136) (N=3), *rpoN* (BA1139) (N=3), and *himA* (BA1142) (N=2). All cultures for RNA-Seq were grown on the same day from unique overnights and subsequent outgrowths. Strains were diluted to OD600 ~0.02 in 10 mL LB with appropriate selection marker, and then grown at 30˚C at 180 RPM until they reached an OD600 0.4-0.6. Samples were collected as follows: 400 µL of culture was added to 800 µL RNAProtect (Qiagen), incubated for 5 minutes at room temperature, and centrifuged for 10 minutes at 5000xg. RNA was purified using RNeasy RNA isolation kit (Qiagen) and quantified and quality-assessed by Bioanalyzer. Library preparation was performed by the Functional Genomics Laboratory (FGL), a QB3-Berkeley Core Research Facility at UC Berkeley. Illumina Ribo-Zero rRNA Removal Kits were used to deplete ribosomal RNA. Subsequent library preparation steps of fragmentation, adapter ligation and cDNA synthesis were performed on the depleted RNA using the KAPA RNA HyperPrep kit (KK8540). Truncated universal stub adapters were used for ligation, and indexed primers were used during PCR amplification to complete the adapters and to enrich the libraries for adapter-ligated fragments. Samples were checked for quality on an Agilent Fragment Analyzer, but ribosome integrity numbers were ignored. This is routine for *Salmonella* sp., since they natively have spliced 23S rRNA [76]. Sequencing was performed at the Vincent Coates Sequencing Center, a QB3-Berkeley Core Research Facility at UC Berkeley on a HiSeq4000 using 100PE runs.

RNA-Seq Data Analysis

For all RNA-Seq experiments, analyses were performed through a combination of KBase-[77] and custom jupyter notebook-based methods. The data processing narrative in KBase will be made publicly available during and after peer review. StringTie and DESeq2 KBase outputs are currently available in Supplementary Datasets 5 and 6 (10.6084/m9.figshare.12185031). Briefly, Illumina reads were trimmed using Trimmomatic v0.36 [78] and assessed for quality using FASTQC. Trimmed reads were subsequently mapped to the *S. typhimurium* LT2 along with PSLT genome (NCBI Accession: AE006468.2 and AE006471.2 respectively) with HISAT2 v2.1.0 [79]. Alignments

were quality-assessed with BAMQC. From these alignments, transcripts were assembled and abundance-estimated with StringTie v1.3.3b [80]. Tests for differential expression were performed on normalized gene counts by DESeq2 (negative binomial generalized linear model) [81]. Additional analyses for all experiments were performed in Python3 and visualized employing matplotlib and seaborn packages. Conservative thresholds were employed for assessing differentially expressed genes. Conclusions were considered differentially expressed if they possessed a Bonferoni-corrected p-value below a threshold of 0.001 and an absolute log2 fold change greater than 2. Assembled transcripts from StringTie and differential expression from RNA-Seq analyses can be found in Supplementary Dataset 5 and 6 respectively.

Data Availability

At time of bioRxiv submission, Supplementary Information can be found here: 10.6084/m9.figshare.12185001. Complete Supplementary Datasets can be found here: 10.6084/m9.figshare.12185031. Upon peer-reviewed publication, all NGS reads will be deposited and made publicly accessible via the Sequence Read Archive (SRA). The RNA-Seq data processing narrative in KBase will be made publicly available during and after peer review.

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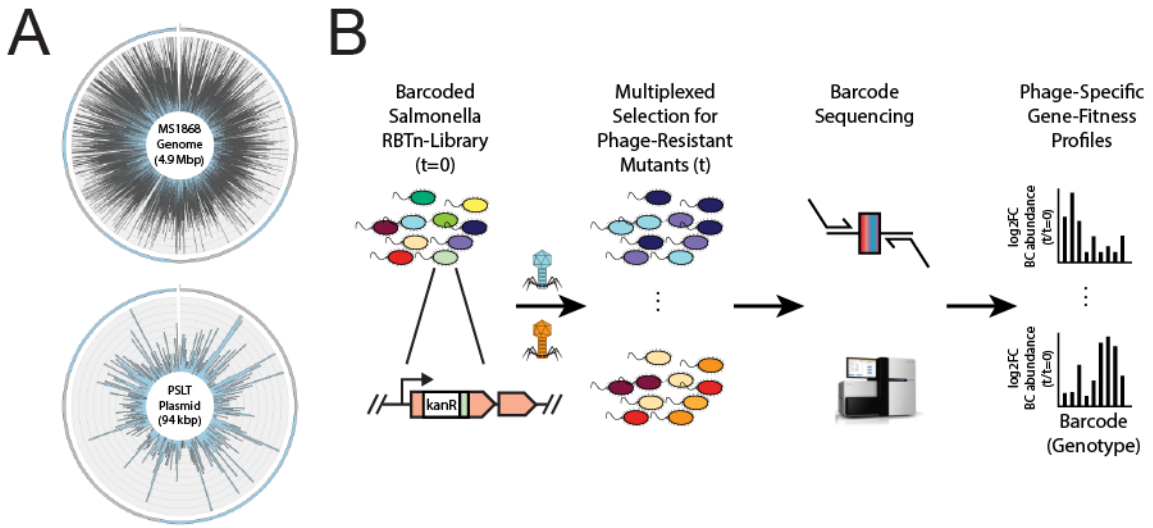

Figure 1. Overview of *Salmonella* RB-TnSeq Library and Experimental Workflow. (A) Insertion density maps of new RB-TnSeq library in *S. typhimurium* MS1868 mapped against *S. typhimurium* LT2 reference genome (top) and PSLT plasmid (bottom). The gap in insertion density in quadrant III against the *S. typhimurium* LT2 reference genome is attributed to the absence of prophage Fels2 in MS1868 relative to the LT2 reference genome. (B) Overview of multiplexed screens employed to find host factors for phage infection. For additional details, see methods. Briefly, for each experiment, an exponentially growing *S. typhimurium* RB-TnSeq library was exposed to a high MOI of one of eleven dsDNA *Salmonella* phages. RB-TnSeq disruption of specific genes provided fitness against phage predation. Strains were tracked by quantifying the abundance of DNA barcodes associated with each strain by Illumina sequencing. Phage-specific gene fitness profiles were calculated by taking the log2-fold-change of barcode abundances post- (t) to pre- (t=0) phage predation. High fitness scores indicate that loss of genetic function in *Salmonella* confers fitness against specific phage predation.

Table 1. Bacteriophages employed in this study.

Phage	Established Receptor?	Source	Reference
Aji_GE_EIP16 (Aji_GE)	No	Intesti Bacteriophage formulation M2-601	This study
Br60	“Rough Salmonella”	Salmonella Genetic Stock Center (SGSC)	[14]
Chi	Flagella	Gift from Jason Gill	[56]
FelixO1	Outer core LPS	Félix d'Hérelle Reference Center for Bacterial Viruses	[32]
Ffm	“Rough Salmonella”	Salmonella Genetic Stock Center (SGSC)	[14]
P22Lys	O-Antigen LPS	Gift from Richard Calendar	[17,43]
Reaper_GE_8C2 (Reaper_GE)	No	Intesti Bacteriophage formulation M2-601	This study
S16	OmpC	Félix d'Hérelle Reference Center for Bacterial Viruses	[18]
Savina_GE_6H2 (Savina_GE)	No	Intesti Bacteriophage formulation M2-601	This study
Shishito_GE_6F2 (Shishito_GE)	No	Intesti Bacteriophage formulation M2-601	This study
SP6	O-Antigen LPS	Gift from Ian J Molineaux	[19]

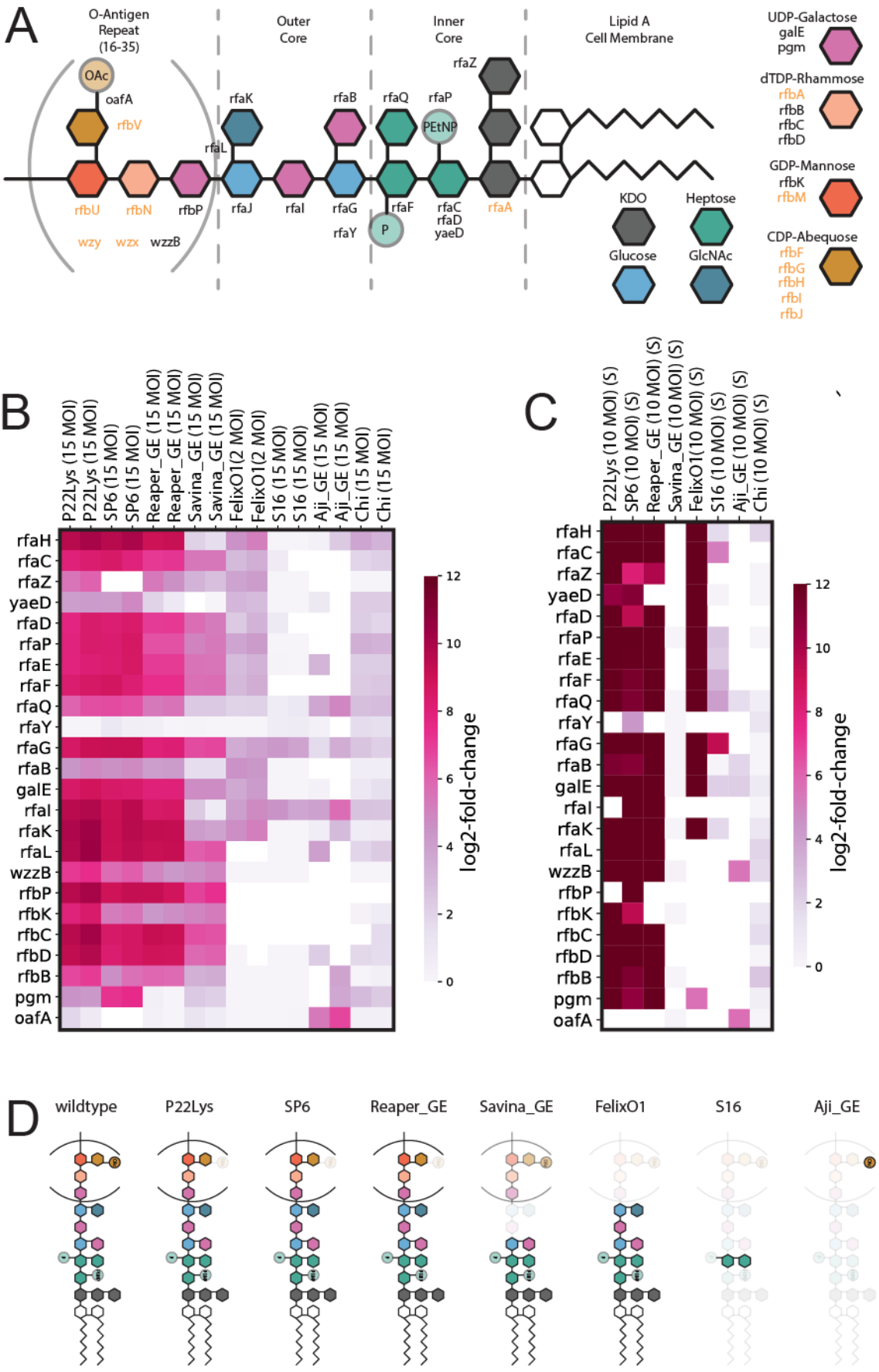
A
O-Antigen Repeat (16-35)
Outer Core
Inner Core
Lipid A Cell Membrane
OAc
oafA
rfbV
rfbU
rfbN
rfbP
wzy
wzx
wzzB
rfaK
rfaL
rfaJ
rfaI
rfaB
rfaG
rfaY
rfaQ
rfaF
P
rfaP
PEtNP
rfaC
rfaD
yaeD
rfaZ
rfaA
KDO
Heptose
Glucose
GlcNAc
UDP-Galactose
galE
pgm
dTDP-Rhammose
rfbA
rfbB
rfbC
rfbD
GDP-Mannose
rfbK
rfbM
CDP-Abequose
rfbF
rfbG
rfbH
rfbI
rfbJ
B
P22Lys (15 MOI)
P22Lys (15 MOI)
SP6 (15 MOI)
SP6 (15 MOI)
Reaper_GE (15 MOI)
Reaper_GE (15 MOI)
Savina_GE (15 MOI)
Savina_GE (15 MOI)
FelixO1(2 MOI)
FelixO1(2 MOI)
S16 (15 MOI)
S16 (15 MOI)
Aji_GE (15 MOI)
Aji_GE (15 MOI)
Chi (15 MOI)
Chi (15 MOI)
rfaH
rfaC
rfaZ
yaeD
rfaD
rfaP
rfaE
rfaF
rfaQ
rfaY
rfaG
rfaB
galE
rfaI
rfaK
rfaL
wzzB
rfbP
rfbK
rfbC
rfbD
rfbB
pgm
oafA
log2-fold-change
0
2
4
6
8
10
12
C
P22Lys (10 MOI) (S)
SP6 (10 MOI) (S)
Reaper_GE (10 MOI) (S)
Savina_GE (10 MOI) (S)
FelixO1(10 MOI) (S)
S16 (10 MOI) (S)
Aji_GE (10 MOI) (S)
Chi (10 MOI) (S)
rfaH
rfaC
rfaZ
yaeD
rfaD
rfaP
rfaE
rfaF
rfaQ
rfaY
rfaG
rfaB
galE
rfaI
rfaK
rfaL
wzzB
rfbP
rfbK
rfbC
rfbD
rfbB
pgm
oafA
log2-fold-change
0
2
4
6
8
10
12
D
wildtype
P22Lys
SP6
Reaper_GE
Savina_GE
FelixO1
S16
Aji_GE

Figure 2. Diverse LPS-Specificity Requirements for Bacteriophages Characterized in this Study. (A) Overview of O5 *S. typhimurium* LPS and O-antigen biosynthesis as characterized previously. The four sugars in brackets comprise the O-antigen, which repeats 16-35 times per LPS molecule under standard growth conditions. Specialized O-antigen precursor biosynthesis genes are described to the right. Genes covered in our library and used for analysis are written in black. Genes not covered in our library, and thus not analyzed in this study are written in orange. (B) Heatmap overview of gene fitness data for LPS-biosynthesis genes covered in the MS1868 RB-TnSeq library under liquid growth conditions. Genes with under 25 BarSeq reads in the phage samples had their fitness values set to 0 for visualization purposes. **(**C) Heatmap overview of gene fitness data for LPS-biosynthesis genes covered in the MS1868 RB-TnSeq library under solid media fitness conditions. Genes with under 25 BarSeq reads in the phage samples had their fitness values set to 0 for visualization purposes. **(**D) Rendition of LPS-receptor for different Salmonella bacteriophages based on RB-TnSeq fitness values and validations from a defined chemotype panel. For the modification catalyzed by OafA, a new gene replacement mutant was employed. Opaque sugar residues are strictly required for phage infection, lighter sugar and PTM residues are not required.

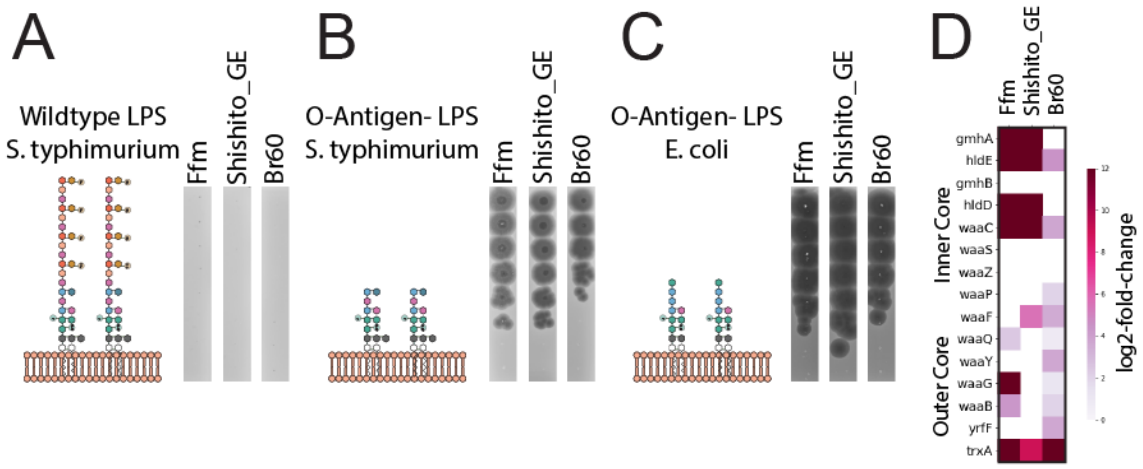

Figure 3. Characterization of Ffm, Shishito_GE, and Br60 Adsorption Through Comparative RB-TnSeq Library Analysis. (A) Overview of LPS structure in smooth, O5 *S. typhimurium* (key in Figure 2A) and representative plaque assays of Ffm, Shishito_GE, and Br60. Presumably rough phage cannot access their true receptor due to presence of O-Antigen. (B) Overview of LPS structure in rough, O5 *S. typhimurium* (key in Figure 2A) (*rfaL* mutant). Rough *S. typhimurium* strains (lacking O-antigen) show efficient phage infection. (C) Overview of rough LPS structure in, *E. coli* BW25113 (same key in Figure 2A), which shares most of the residues in inner and outer core as rough *Salmonellae*. Despite host phylogenetic distance, we observe that Ffm, Shishito_GE, and Br60 phages efficiently infect *E. coli* BW25113. (D) Heatmap overview of functional data for LPS-biosynthesis genes covered in the *E. coli* BW25113 RB-TnSeq library under short-time-adsorption conditions on solid media for rough phage Ffm, Shishito_GE, and Br60. Genes with under 25 BarSeq reads in the phage samples had their fitness values set to 0 for visualization purposes.

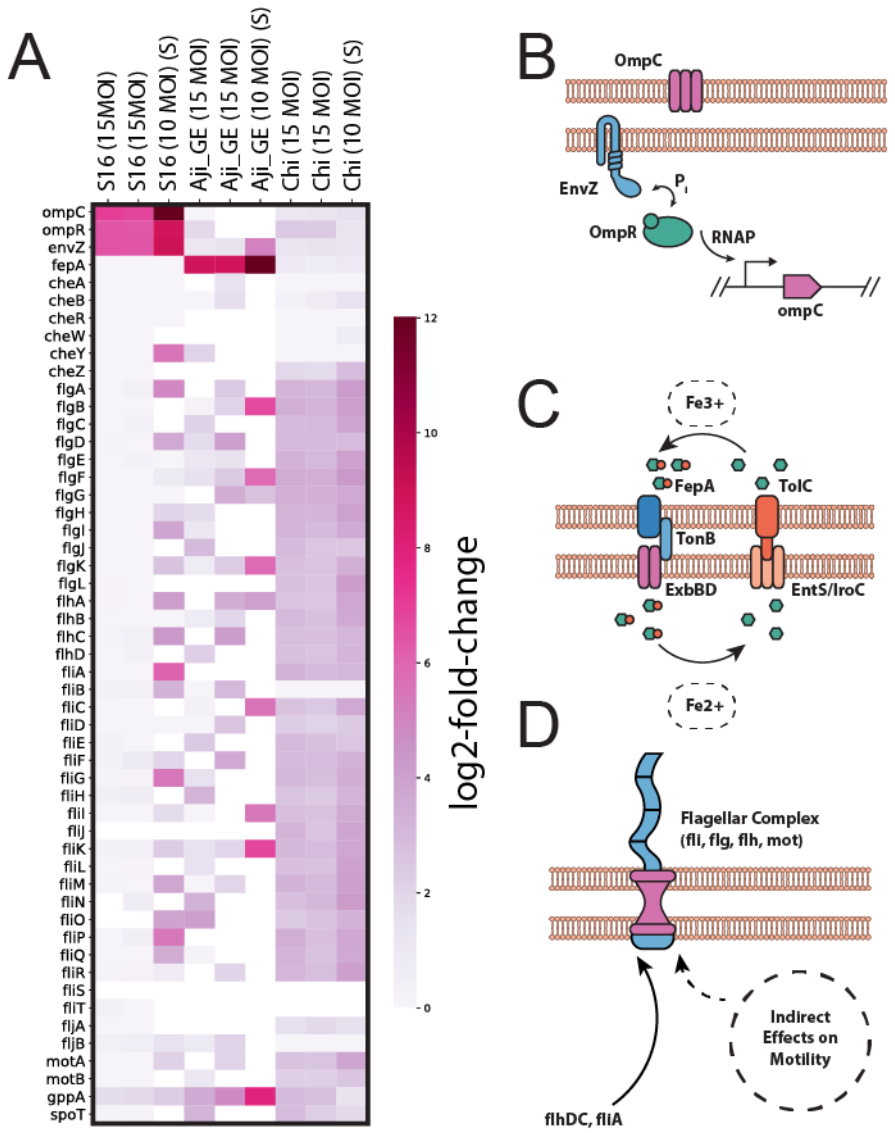

Figure. 4. Diverse protein receptors and their regulation observed for bacteriophages characterized in this study. (A) Heatmap overview of gene fitness data for putative protein receptors and their regulators in experiments against the MS1868 RB-TnSeq library under liquid and solid growth conditions. Noncompetitive, solid agar growth experiments are marked with a (S). Genes with under 25 BarSeq reads in the phage samples had their fitness values set to 0 for visualization purposes. (B) Schematic overview for OmpC regulation observed against phage S16. Two component system EnvZ-OmpR positively regulates expression of OmpC. If EnvZ or OmpR are disrupted, lower levels of OmpC are expressed. (C) Schematic overview for FepA, a critical host-requirement for phage Aji_GE_EIP16. FepA mediates iron scavenging through import of Fe-enterobactin and is indirectly regulated by internal iron levels. (D) Schematic overview for flagellar regulation and activity observed against phage Chi. The type I flagellar complex is assembled from proteins expressed from the multiple *flg*, *fli*, *flh*, and mot operons. Disruption of positive flagellar biosynthesis regulators *flhD*, *flhC*, and *fliA* hinders Chi infection, while disruption of negative flagellar biosynthesis regulators, *fliT* and *flgM*

do not. Phase II flagellar genes *fljAB* do not appear to important for Chi infection. Other motility promoting genes such as *cheZ* are important for Chi infection as well.

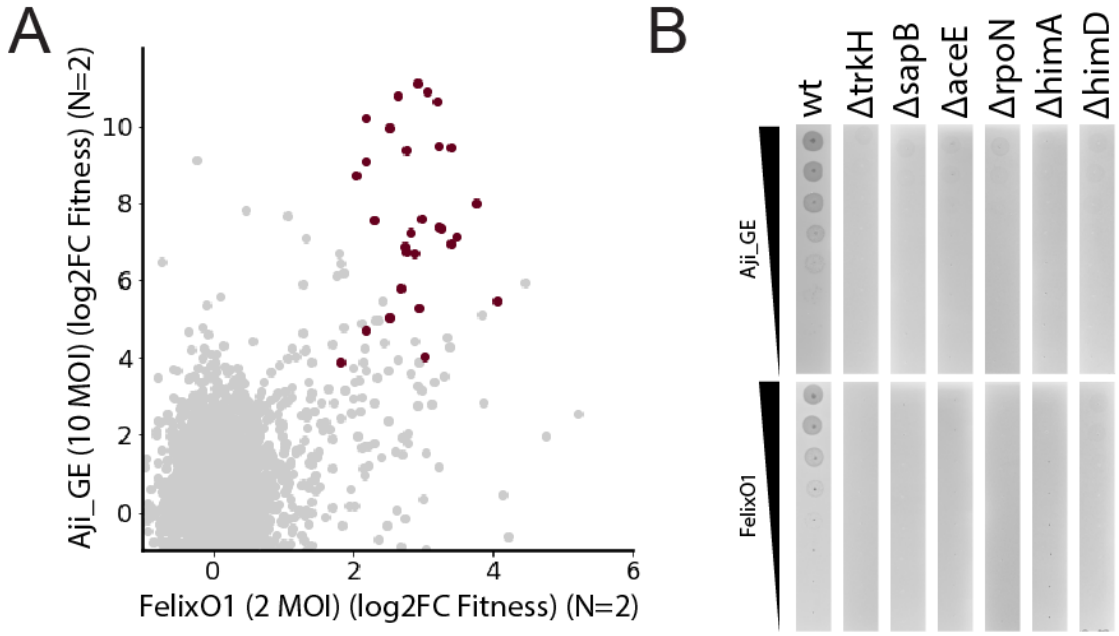

Figure 5. Loss of function mutants in Salmonella MS1868 provide cross-resistance to unrelated bacteriophage Aji_GE and FelixO1. (A) Scatter visualization of FelixO1-Aji_GE cross-resistant genotypes identified in BarSeq experiments. Fit, loss of function genotypes passing both significance thresholds and manual curation for both FelixO1 and Aji_GE experiments are shown in maroon (see Supplementary Datasets 5 and 6 for thresholds and genotypes respectively). Fitness data for other genes are shown in grey. Values shown are the average fitness across two liquid culture experiments. (B) Phenotypes inferred from the experiments in Figure 5A are validated through single gene deletions in *trkH*, *sapB*, *aceE*, *rpoN*, *himA*, and *himD* and plaquing efficiency gauged for Aji_GE and FelixO1. Images shown are representative of 3 biological replicate experiments.

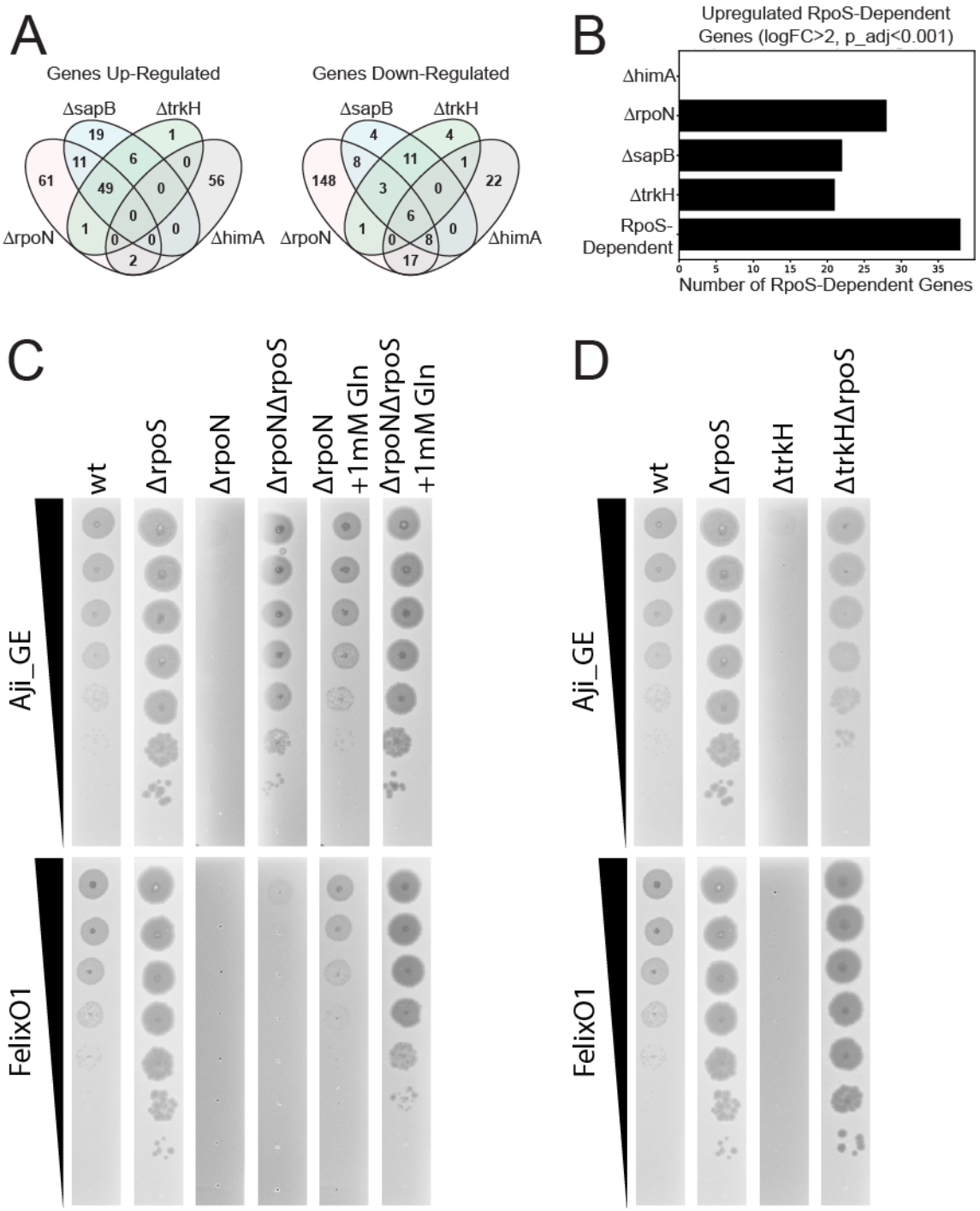

Figure 6. Cross-module mechanisms are mediated by rpoS. (A) Summary of genes with significant up- and down-regulation relative to wild-type for *trkH*, *rpoN*, and *himA* mutants. Reported values are genes with log2-fold changes over 2 and Bonferroni-corrected p values below 0.001. (B) Δ*rpoN*, Δ*sapB*, Δ*trkH*, but not Δ*himA* are enriched for upregulated genes dependent on RpoS activity. RpoS-dependent regulation genes are based off of a curated list from Lucchini et al., 2009. (C) A secondary deletion in *rpoS* is sufficient to restore Aji_GE infectivity in a Δ*rpoN* strain. However, FelixO1 is only restored

with additional supplementation of glutamine. (D) A secondary deletion in *rpoS* is sufficient to restore both Aji_GE and FelixO1 infectivity in a Δ*trkH* strain.