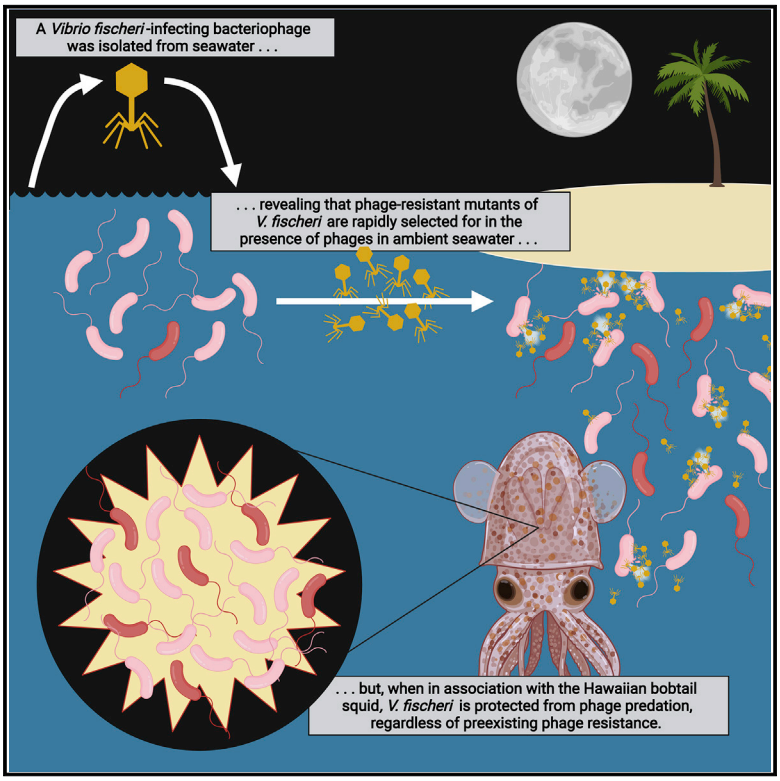
Report

Cell Reports

Independent host- and bacterium-based determinants protect a model symbiosis from phage predation

Graphical abstract



Highlights

- A *Vibrio fischeri*-infecting bacteriophage, HNL01, is isolated and characterized
- HNL01 does not disrupt the mutualism between *V. fischeri* and *Euprymna scolopes*
- HNL01-resistant *V. fischeri* mutants rapidly emerge *in vitro*
- When HNL01 is present, squid colonization by HNL01-resistant *V. fischeri* is favored

Authors

Jonathan B. Lynch, Brittany D. Bennett, Bryan D. Merrill, Edward G. Ruby, Andrew J. Hryckowian

Correspondence

hryckowian@medicine.wisc.edu

In brief

Lynch et al. isolate and characterize a bacteriophage that infects the marine bacterium *Vibrio fischeri.* They show that the mutualism between *V. fischeri* and the Hawaiian bobtail squid *Euprymna scolopes* is not disrupted by phages present in the environment and highlight both bacterium- and squid-based determinants of protecting this mutualism against phage predation.

Lynch et al., 2022, Cell Reports *38*, 110376
February 15, 2022
https://doi.org/10.1016/j.celrep.2022.110376

CellPress

Cell Reports

CellPress
OPEN ACCESS

Report

Independent host- and bacterium-based determinants protect a model symbiosis from phage predation

Jonathan B. Lynch,[1,2] Brittany D. Bennett,[1,3] Bryan D. Merrill,[4] Edward G. Ruby,[1] and Andrew J. Hryckowian[5,6,7,*]

[1]Pacific Biosciences Research Center, University of Hawai'i at Manoa, Honolulu, HI 96822, USA
[2]Department of Integrative Biology and Physiology, University of California, Los Angeles, Los Angeles, CA 90095, USA
[3]Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089, USA
[4]Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94304, USA
[5]Department of Medicine, University of Wisconsin School of Medicine and Public Health, Madison, WI 53792, USA
[6]Department of Medical Microbiology and Immunology, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706, USA
[7]Lead contact
*Correspondence: hryckowian@medicine.wisc.edu
https://doi.org/10.1016/j.celrep.2022.110376

SUMMARY

Bacteriophages (phages) are diverse and abundant constituents of microbial communities worldwide, capable of modulating bacterial populations in diverse ways. Here, we describe the phage HNL01, which infects the marine bacterium *Vibrio fischeri.* We use culture-based approaches to demonstrate that mutations in the exopolysaccharide locus of *V. fischeri* render this bacterium resistant to infection by HNL01, highlighting the extracellular matrix as a key determinant of HNL01 infection. Additionally, using the natural symbiosis between *V. fischeri* and the squid *Euprymna scolopes*, we show that, during colonization, *V. fischeri* is protected from phages present in the ambient seawater. Taken together, these findings shed light on independent yet synergistic host- and bacterium-based strategies for resisting symbiosis-disrupting phage predation, and we present important implications for understanding these strategies in the context of diverse host-associated microbial ecosystems.

INTRODUCTION

Bacteriophages (phages) are viruses that infect bacteria, influencing broad areas of bacterial physiology and ecology. These effects can manifest through bacterial lysis, development of bacterial resistance to phage infection, and horizontal gene transfer and can have positive, negative, or neutral effects on bacterial fitness (Brum et al., 2015; Matilla et al., 2014; Roossinck, 2011; Suttle, 2007) . Phages are abundant across diverse environments, including the mammalian gastrointestinal tract, rhizosphere, and oceanic plankton, where they dramatically influence bacterial physiology and community function (Al-Shayeb et al., 2020; Breitbart et al., 2018; Dion et al., 2020; Reyes et al., 2010).

Given the known impacts of phages in microbial communities (microbiotas), there is great interest in the roles that phages play in eukaryote-associated microbiotas in particular. In these associations, phages can select for or against certain bacteria, alter bacterial gene expression, or influence spatial organization of the microbiota (Barr et al., 2013), suggesting that "phage therapy" could be used to selectively alter the composition and function of diverse host-associated microbiotas.

Phage biology is commonly studied using a variety of well-described model bacterium-phage pairs, such as *Escherichia coli* (phages T4, T7, and λ (Kutter et al., 2018)), *Vibrio cholerae* (phages ICP1, ICP2, and ICP3 (Yen and Camilli, 2017)), and others (Ofir and Sorek, 2018). Despite these efforts, we lack knowledge of how most phages interact with their bacterial hosts in their natural environments. This knowledge gap is due both to the high diversity of phages and their target bacteria and to the difficulty of inferring these relationships from sequence homology or other bioinformatic techniques (Dion et al., 2020; Reyes et al., 2010). This difficulty is largely driven by the fact that phages can be restrictive or promiscuous, with host ranges spanning from specific strains to multiple bacterial species (Beumer and Robinson, 2005; Flores et al., 2011; de Jonge et al., 2019). In addition, bacterially encoded phage resistance can be either broadly or narrowly protective (e.g., alteration of extracellular structures or CRISPR spacer acquisition, respectively), and phages have evolved many ways to counter these bacterial defenses (Black, 1988; Fan et al., 2018; Morona and Henning, 1984; Parent et al., 2014; Porter et al., 2020; Wang et al., 2019; Xu et al., 2014; Zborowsky and Lindell, 2019). Finally, bacterial evasion strategies can be constitutive or differentially expressed under particular environmental conditions (Reyes-Robles et al., 2018), further complicating an understanding of phage-bacterium interactions and highlighting the need for diverse and tractable model systems to study them.

In animal-bacterial symbioses, the animal host also plays key, but understudied, roles in phage dynamics. For example, phages can adhere to mucus, creating regions of high and low phage abundance and activity (Barr et al., 2013, 2015). Animals can also produce effectors like immunoglobin A that bind

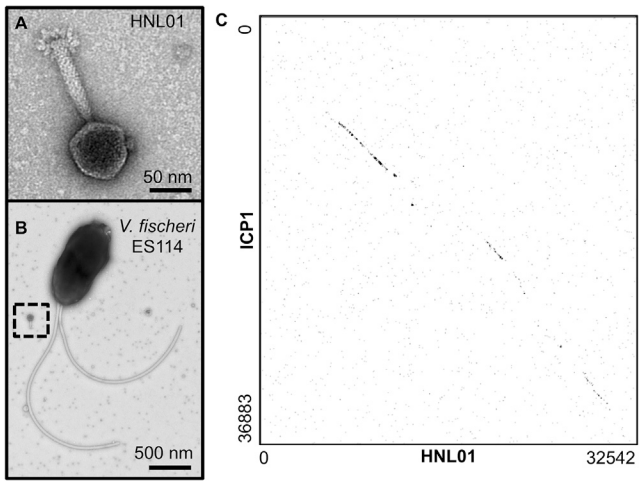


Figure 1. Isolation and genomic characterization of HNL01

(A and B) Transmission electron micrographs of (A) HNL01 and (B) *V. fischeri* ES114 and HNL01 (dotted box surrounds phage).

(C) The amino acid sequences of all annotated protein-coding open reading frames (ORFs) in HNL01 were concatenated and compared with the concatenated protein-coding ORFs in *V. cholerae* phage ICP1 with a dot plot. Numbers denote position within the concatenated amino acid sequence for each phage. See also Figure S1, Tables S1, and S2.

bacteria and protect them from phage infection (Rollenske et al., 2021). Indirectly, the host environment influences expression of surface molecules by bacterial symbionts, which changes their susceptibility to phages (Porter et al., 2020).

While many animal-associated microbiotas are highly diverse communities, rendering them difficult to study, some animals develop mutualisms with narrowly defined sets of bacteria that enable controlled experimental approaches. One well-studied example is the bioluminescence-based mutualism between the Hawaiian bobtail squid, *Euprymna scolopes,* and the marine γ-proteobacterium *Vibrio (Aliivibrio) fischeri.* Within hours after the squid hatch from their eggs, *V. fischeri* cells from the surrounding seawater colonize the crypt spaces of a specialized symbiotic tissue (the light organ [LO]), where the bacteria multiply and reside extracellularly for the approximately 9-month duration of the squid's life (Essock-Burns et al., 2020; McFall-Ngai, 2014). This colonization process is marked by an extreme bottlenecking, in which $\sim 10^3$–10^6 cells/mL of *V. fischeri* in seawater are whittled down to a few cells that initiate the colonization (Wollenberg and Ruby, 2009). These successfully colonizing bacteria are further sorted into the six distinct crypts of the LO, so that each crypt ordinarily contains one or two clonal populations of *V. fischeri* cells (Bongrand and Ruby, 2019; Speare et al., 2018; Sun et al., 2016). To ensure successful colonization of symbiosis-competent *V. fischeri* while simultaneously restricting LO access of non-*V. fischeri* microbes, *E. scolopes* employs several strategies, such as cilia-derived flow patterns, that physically control LO access (Nawroth et al., 2017).

To leverage this tractable partnership for the study of phages in beneficial host-microbe associations, we isolated and characterized a phage with marked specificity for a symbiosis-competent *V. fischeri* strain, as well as spontaneous phage-resistant mutants of this strain. We hypothesized that the near-clonality of *V. fischeri* in the LO would predispose these symbionts to decimation upon the introduction of a virulent phage, potentially by reducing or removing the resident *V. fischeri* population and undoing the previously stable host-symbiont relationship. Surprisingly, our data demonstrate that, although phages prey on populations of *V. fischeri* in the ambient seawater, LO-resident *V. fischeri* are protected from phage predation. We posit that host-mediated protection of symbionts from phages provides a fitness advantage by discouraging phage sweeps of the symbiont population (e.g., no light production in the case of the squid-*Vibrio* symbiosis) or where general microbiota stability, rather than persistent phage-driven oscillations in community structure, promotes host homeostasis. This work identifies how animal hosts and their symbiotic bacteria can respond to and protect highly specific mutualisms from phages, and it establishes a framework to understand the mechanisms governing these relationships in this and other host-associated microbial ecosystems.

RESULTS

Isolation and genomic characterization of HNL01 and comparative analysis with *Vibrio cholerae* phage ICP1

Using a previously reported protocol (Hryckowian et al., 2020), we isolated HNL01 from coastal Hawaiian seawater (Figure 1A). HNL01 was propagated on *V. fischeri* ES114, the well-studied LO isolate of this species (Figure 1B). We assayed the bacterial-host range of HNL01 by performing plaque assays on 22 additional strains of *V. fischeri,* as well as five previously described mutants of ES114 (Table S1). As isolates from the ES114 background were the only strains that formed visible plaques, we conclude that HNL01 has a restricted host range within *V. fischeri* strains and that several common extracellular macromolecules do not impact its ability to infect. For example, mutation of neither the O-antigen of lipopolysaccharide (LPS), a well-studied symbiosis polysaccharide (Syp), nor the dominant outer membrane protein (OmpU) affected the ability of HNL01 to infect ES114; similarly, the central trait of the symbiosis, bioluminescence, was not required for phage infection (Table S1). These results suggest that the specificity of HNL01 for ES114 is driven by other effectors.

Transmission electron microscopy of HNL01 demonstrated that this phage is a myovirus (e.g., tailed phage with a contractile tail; Figures 1A and 1B), and whole-genome sequencing of HNL01 revealed a 111,792-bp genome consisting of 38% guanine-cytosine (GC) content and 166 predicted protein-coding genes, 33 of which have conserved domains (Figure S1 and Table S2). The virion morphology and genome size of HNL01 resemble the well-studied *Vibrio cholerae*-infecting phage ICP1, a myovirus with a 125,956-bp genome (Seed et al., 2011). Sequence similarity at the amino acid level was apparent between these two phages (Figure 1C and Table S2), with multiple conserved proteins involved in nucleotide metabolism and virion structure (Table S2 and Figure S1). Together, this resemblance suggests a distant evolutionary relationship between HNL01 and ICP1, as has been observed for phages that infect Bacteroidetes (Guerin et al., 2018; Hryckowian et al., 2020).

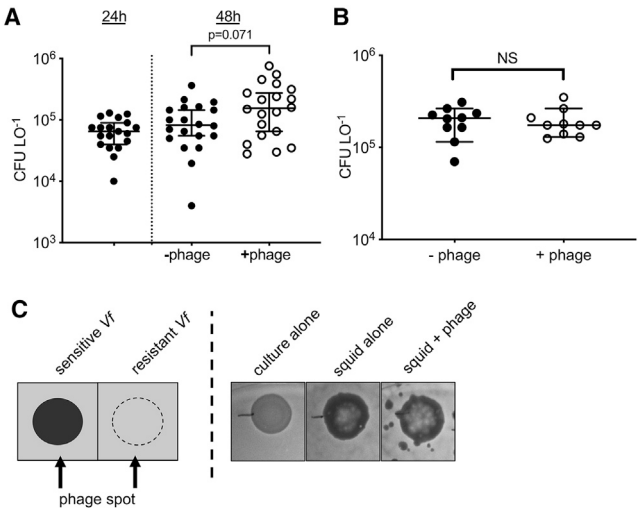


Figure 2. Ambient phage does not reduce squid colonization despite symbiont maintenance of viral sensitivity

(A) Colony-forming units (CFU) of *V. fischeri* ES114 from squid light organ (LO) homogenates. Squid were colonized for 24 h (left) in the absence of phage and then exposed to phage, and CFU were measured at 48 h; n = 19–20 for each experimental condition.

(B) Squid were exposed to HNL01 and *V. fischeri* at the same time, and CFU/LO was measured at 24 h; n = 10 for each condition.

(C) Left of dotted line: schematic showing phage spotting results. Light gray indicates growth of *V. fischeri* lawn in top agar; dark gray indicates lysis of cells by phage. Right of dotted line: HNL01 spotted on top agar overlay of *V. fischeri* from an overnight culture (left), from *V. fischeri* extracted from colonized LOs (middle), or from LOs of squid concurrently exposed to *V. fischeri* and HNL01 (right). Each point represents CFU from the LO of one squid. Bars represent median ± 95% confidence interval (CI). See also Table S1. Statistical significance determined by Mann-Whitney test.

Phage predation does not decrease *V. fischeri* colonization of the *E. scolopes* LO

Because phages are ubiquitous in nature and are increasingly recognized to shape host-associated ecosystems (Koskella and Brockhurst, 2014), we sought to understand how HNL01 impacts the establishment and maintenance of LO colonization. Newly hatched, uncolonized juvenile squid were colonized with *V. fischeri* ES114 for 24 h (Figure 2A, left), and then phages were added to the ambient seawater to a final concentration of 10^7 plaque-forming units per milliliter (PFU/mL). After 24 h of phage exposure (i.e., 48 h after initiation of bacterial colonization), viable *V. fischeri* in the LOs were quantified. The results highlighted that phages in the ambient environment do not impact the maintenance of symbionts (Figure 2A, right; "no phage": 8.25 × 10^4 colony-forming units per LO (CFU/LO); "plus phage": 1.55 × 10^5 CFU/LO, p = 0.07). Importantly, due to the possibility of phage killing during plating, the CFU counts from the plus phage condition are potentially conservative; there may be higher CFU counts than we measured in the phage-exposed squid, emphasizing that ambient phage does not decrease LO colonization. To mimic conditions that might be encountered in coastal seawater, we simultaneously exposed juvenile squid to both excess phage (at ~10^7 PFU/mL) and *V. fischeri* cells (at ~8 × 10^4 CFU/mL) and allowed colonization to proceed for 24 h. We similarly observed no difference in colonization between the two colonization groups (Figure 2B; no phage: 2.1 × 10^5 CFU/LO; plus phage: 1.75 × 10^5CFU/mL, p = 0.78). Subsequently, homogenates from either 10 *V. fischeri*-colonized squid or 10 phage-exposed, *V. fischeri*-colonized squid from the colonizations represented in Figure 2B were tested for phage sensitivity in a soft agar overlay. *V. fischeri* extracted from LOs was similarly sensitive to phage regardless of prior phage exposure during colonization (Figure 2C). These data indicate that LO-colonizing bacteria are protected from ambient phages but that these bacteria do not acquire permanent resistance to phages in the LO.

Phage resistance mutations in ES114 do not impact LO colonization

In addition to the phage protection provided to *V. fischeri* by the LO (Figure 2), we asked whether phage resistance could be acquired *in vitro* to protect *V. fischeri* outside the squid. To address this question, we added HNL01 at a range of multiplicities of infection (MOIs) to cultures of *V. fischeri* and monitored changes in culture density (OD_{600}) over time for 16 h. Independently of the inoculum size or MOI, a decrease in OD_{600} was observed within the first ~1 h of phage exposure in cultures where phage was added, followed by ~5 h, during which bacteria were undetectable, and a subsequent outgrowth of presumably resistant bacteria (Figure 3A). We collected bacteria from these cultures, re-challenged them with HNL01, and compared their phage sensitivity with that of bacteria from the phage-free cultures. As expected, bacteria that had not been previously exposed to HNL01 were susceptible, whereas bacteria that had survived exposure to HNL01 were not cleared by phage, suggesting selection for heritable mutations that result in phage resistance (Figure 3B).

We then retrieved four individual *V. fischeri* ES114 colonies that grew inside HNL01 plaques. After re-streaking them to isolation, we confirmed that they were resistant to HNL01 lysis via plaque assay (similar to Figure 3B). We performed PCR on these isolates with HNL01-specific primers to test for lysogeny, and did not observe any of the tested phage genes in the genomes of these *V. fischeri* cells (data not shown). To identify mutation(s) involved in resistance to HNL01, we performed whole-genome sequencing on these HNL01-resistant clones. A mutation shared among these resistant clones relative to wild type was a missense mutation encoding an E183V substitution in the glycosyltransferase gene VF_0175, which is part of the locus (VF_0157-80) responsible for production of exopolysaccharide (EPS) by strain ES114 (Bennett et al., 2020). Homologs of VF_0175 are predicted in many, but not all, *V. fischeri* genomes (Bongrand et al., 2020), suggesting that this gene may be under selection in different environmental contexts. Targeted deletion of VF_0175 or VF_0157-80, together with the provision of VF_0175 or VF_0175(E183V) *in trans*, confirmed that a functional copy of this gene in the context of a full-length EPS locus is necessary for HNL01 infection (Figure 3C). We will subsequently refer to the VF_0175(E183V) "phage resistance" allele as VF_0175^{PR}.

V. fischeri EPS is associated with HNL01 susceptibility

To evaluate the extent to which the protein encoded by VF_0175 and the wild-type E183 residue contribute to EPS production, we

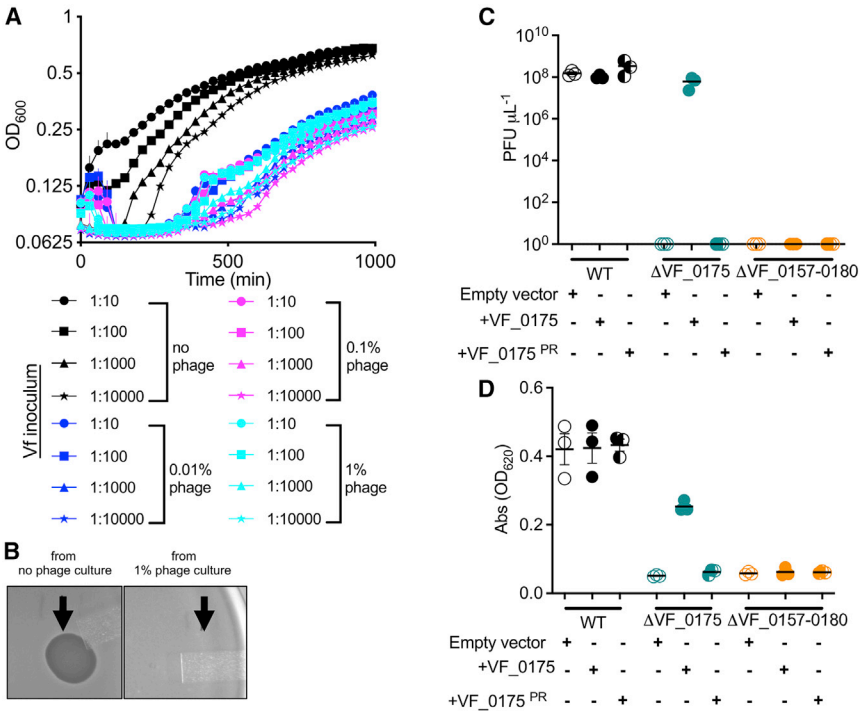


Figure 3. *V. fischeri* rapidly displays phage resistance during exposure to phage during *in vitro* growth

(A) Growth curve of *V. fischeri* ES114 with varying dilutions of phage (0–1% by volume of ~10^{11} PFU/mL stock) and initial *V. fischeri* inoculum (1:10–1:10,000 of an overnight culture). Points with error bars represent mean ± SEM.

(B) Phage suspensions were spotted onto top agar lawns subcultured from *V. fischeri* cultures grown without (left) or with (right) phage. Arrows indicate location where phages were placed onto *V. fischeri*-containing top agar. Note: the white rectangle on the right side of each image is included as an image contrast reference, emphasizing lack of *V. fischeri* clearance in the righthand image.

(C) Plaque assay measurements of HNL01 infection of wild-type *V. fischeri* ES114 (black), ΔVF_0175 (blue), and ΔVF_0157-80 (orange) carrying empty vector (open symbols), vector with wild-type VF_0175 (closed symbols), or vector with VF_0175^{PR} (half-filled symbols). Points along the x-axis represent results falling below the limit of detection (1 PFU/mL).

(D) Alcian blue was used to stain EPS in supernatants of 24-h liquid cultures grown in minimal salts medium supplemented with 6.5 mM *N*-acetylneuraminic acid 0.05% (wt/vol) casamino acids for wild-type *V. fischeri* ES114, ΔVF_0175, and ΔVF_0157-80 carrying empty vector, vector with wild-type VF_0175, or vector with VF_0175^{PR} (same labeling scheme as 3C). Points represent biological replicates, bars represent means ± SEM. See also Tables S1 and S3.

used Alcian blue to stain EPS in liquid culture supernatants of *V. fischeri* ES114-derived strains. Targeted deletion of VF_0175 or VF_0157-80, along with providing VF_0175 or VF_0175^{PR} *in trans*, confirmed that, as was found for phage lysis, a functional copy of VF_0175 in the context of the full-length EPS locus is necessary for wild-type levels of EPS production (Figure 3D).

Autonomous and squid-derived phage resistance contributes to symbiont colonization and maintenance

We next asked whether the VF_0175^{PR} allele impacted competition with phage-sensitive *V. fischeri.* We co-cultured wild-type *V. fischeri* ES114 and its resistant VF_0175^{PR} derivative in the presence or absence of HNL01. In the presence of phage, wild-type ES114 was outcompeted by the VF_0175^{PR} strain, consistent with our observations of *V. fischeri* ES114 mutants in Figure 3 (Figure 4A). In addition, we observed that the VF_0175^{PR} strain monocolonized *E. scolopes* LOs equally well as wild-type *V. fischeri* in both the presence and the absence of phage in the ambient water (Figure S2), suggesting that, rather than impacting LO colonization, the variation in this locus observed across *V. fischeri* strains is important for evading phages like HNL01.

To extend our previous observations that LO-resident bacteria are protected from phages, we conducted co-colonization experiments using differentially labeled wild-type and resistant VF_0175^{PR} *V. fischeri.* Both strains colonized LOs similarly well in the absence of phages at both 24 and 48 h post-colonization (Figure 4B; median wild type/VF_0175^{PR} competition index, 1.28 at 24 h and 2.68 at 48 h). However, when squid were simultaneously exposed to bacteria and phage, as in Figure 2B, the wild-type strain was outcompeted by the VF_0175^{PR} strain (Figure 4B; median wild type/VF_0175^{PR} competition index, 2.24 × 10^{-4} at 24 h and <1 × 10^{-4} at 48 h, $p < 0.0001$ compared with no phage at either time point, Mann-Whitney test; Figure 4B). Finally, we conducted a sequential-exposure experiment, where squid that were initially co-colonized with wild type and VF_0175^{PR} were then exposed to phage at 24 h post-inoculation. Although phages present during the initiation of colonization led to the dominance of phage-resistant VF_0175^{PR} *V. fischeri* in the symbiosis, equal ratios of resistant and susceptible bacteria were present in LOs initially colonized without phage, regardless of later phage addition (Figure 4B, median wild type: VF_0175^{PR} competitive index without phage, 2.68; median competitive index with phage after co-colonization, 1.37, $p = 0.187$, Mann-Whitney test).

DISCUSSION

We describe two synergistic means by which a mutualistic bacterium can be protected from phage predation: a cell-intrinsic, exopolysaccharide-based resistance factor and a host-derived shielding effect. Using the highly specific mutualism between *E. scolopes* and *V. fischeri,* we have shown that these strategies protect the normal host-microbe association despite high

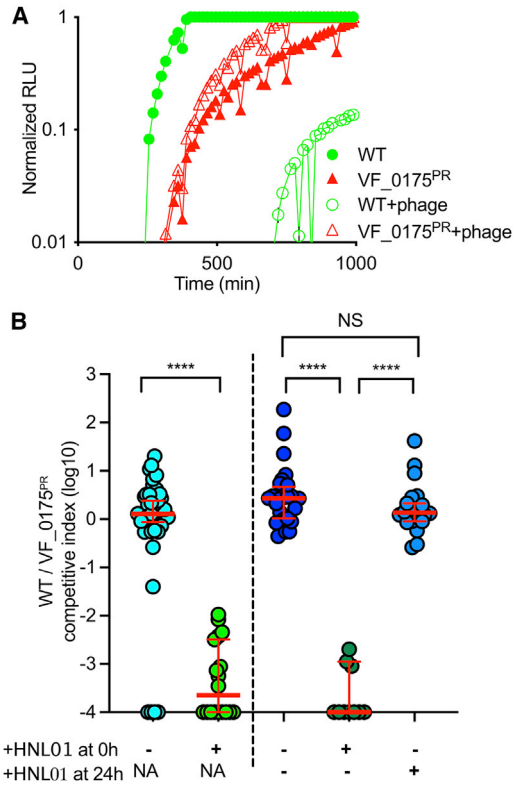


Figure 4. Bacteria-derived phage resistance is beneficial to *V. fischeri* during planktonic lifestyle, but host-derived protection dominates after colonization

(A) Growth curves from co-cultured, differentially labeled wild-type (WT) *V. fischeri* ES114 and the VF_0175^{PR} mutant with or without phage exposure. WT was labeled with GFP, and the VF_0175^{PR} mutant was labeled with RFP to distinguish them during co-culture. Data presented are normalized relative light units (RLU): (RLU – RLU$_{t=0}$)/(maximum RLU range for that strain). Points represent means ± SEM.

(B) Relative ratios of CFU from WT or strain VF_0175^{PR} from LO homogenates of co-colonized squid. Phages were introduced at time = 0, time = 24 h, or neither, and homogenates were measured at t = 24 h (left two columns) or t = 48 h (right three columns); n = 10–38 for each condition. Bars represent median ± 95% CI. ****p < 0.0001, Mann-Whitney test. See also Figure S2, Tables S1, and S3.

symbiont clonality and a continuous connectivity between the symbiont culture and ambient seawater. We have also shown that these types of phage resistance are important at different stages of colonization: bacterium-determined resistance is important during the transition from free living to the initial colonization, while symbiotic host-determined resistance is important during the maintenance of an already established colonization.

We were surprised that HNL01 shared genomic features with ICP1, a phage that is prevalent and abundant in the stool of humans with cholera infections and may play a role in the seasonality of cholera through selection against the *V. cholerae* O-antigen, the primary receptor for ICP1 (Seed et al., 2011, 2012). While we found that some membrane components are not required for HNL01 infection (e.g., mutations in *waaL* (the O-antigen ligase) and *eptA,* both of which are involved in LPS decoration, do not affect ES114 susceptibility to HNL01; Table S1), we did identify other surface-associated infection resistance genes (e.g., mutations in the EPS gene cluster), suggesting that surface-exposed polysaccharides are important for phage infection in both *V. cholerae* and *V. fischeri.* Because *V. fischeri* ES114 is the only HNL01 host we identified, broader studies of *V. fischeri* phages around different areas of Hawai'i and other geographic regions where *V. fischeri* is endemic will reveal whether related phages exist for other *V. fischeri* strains and the ways in which *V. fischeri* evades phages among its various symbiotic relationships (Bongrand et al., 2020).

Envelope and extracellular matrix modifications are major determinants of host-phage relationships at the individual and population levels (Díaz-Pascual et al., 2019; Dunsing et al., 2019; Hryckowian et al., 2020; Kim et al., 2019; Porter et al., 2020; Simmons et al., 2020), emphasizing the importance of the cell surface in phage susceptibility and community dynamics. Resistance factors are important with respect to the survival of individual bacterial cells, but they likely become even more critical in areas with high densities of related and susceptible cells, where even inefficient infections could quickly sweep through a population. Scenarios like this not only include highly specific symbioses like the squid-*Vibrio* system but may also include spatially organized, complex communities like biofilms and microcolonies.

Given the ubiquity of phages in the biosphere and their enrichment at mucosal surfaces (Barr et al., 2013, 2015), we asked whether associating with *E. scolopes* promoted infection of *V. fischeri* by HNL01, perhaps as a regulator of symbiont density or function. Instead, our work demonstrated that *E. scolopes* is a safe haven from viral infection and that LO colonization protects *V. fischeri* from phages. This host-mediated protection may most directly benefit *V. fischeri,* but it also is likely to benefit the squid by protecting its mutualist asset. This type of shielding may be especially important when a specific symbiont is not easily replaceable, because of a colonization refractory period, niche competition, or ecological dynamics with the rest of the microbiota (Koch et al., 2014; Rao et al., 2021; Speare et al., 2018), as has been reported with the squid-*Vibrio* symbiosis. Similarly, it was previously hypothesized that the distribution of gastrointestinal bacteria between phage-accessible and phage-inaccessible sites is a driver of long-term co-existence of phage and bacteria (Lourenço et al., 2020). Conversely, whereas protection of some mutualists from phages could benefit the host, it could similarly backfire if pathogens were protected, especially if they were desired to be targeted via phage therapy.

Although the exact means by which the squid protects *V. fischeri* remains undefined, we speculate that several processes play a role. First, the LO epithelium is proficient at uptake of small particles, which might allow non-specific consumption or transport of phages before they can infect *V. fischeri* in the crypts, as has been shown with other eukaryotic cells (Bichet et al., 2021; Cohen et al., 2020; Nguyen et al., 2017). Second, mucus has been shown in other models to adsorb phages. *V. fischeri* both stimulates mucus production in the squid and aggregates on this mucus at the onset of colonization; this

mucus might be a means of sequestering phages (Barr et al., 2013; Nyholm and McFall-Ngai, 2003; Nyholm et al., 2002). Third, *E. scolopes* generates flow fields through the beating of cilia on the exterior of the LO. This promotes aggregation of *V. fischeri*-sized particles around LO entry points. It is possible that these flow patterns may actively exclude phage-sized particles from the LO surface, mechanically protecting *V. fischeri* as it initiates LO colonization (Nawroth et al., 2017). These processes of symbiont protection hold broad implications for phage infections in other symbioses, such as the mammalian gastrointestinal tract, where hosts might non-specifically protect symbionts by using similar strategies. Future work with HNL01, such as visually tracking phage particles engineered to produce fluorescent proteins or phage particles labeled via fluorescence *in situ* hybridization as they interact with *V. fischeri* and the squid during colonization, will help to define specific protection mechanisms deployed during this and other symbioses.

This work also shows that, in *V. fischeri,* mutation-derived resistance to phages is readily selected for under phage predation (Figures 3A and 3B). In four independent HNL01-resistant clones, we identified a point mutation in VF_0175, which encodes a putative glycosyltransferase. Although we did not identify any fitness costs imposed by the VF_0175^{PR} mutation during *in vitro* growth or experimental LO colonization, it is possible that such naturally arising strains would be outcompeted by wild-type *V. fischeri* in the absence of phages in nature, but random or specifically induced mutation to VF_0175 is strongly selected for during phage attacks.

Wild-type VF_0175 is necessary for plaque formation by HNL01 on *V. fischeri* ES114, but introducing VF_0175 in the absence of the surrounding VF_0157–0180 locus does not confer phage sensitivity, suggesting that carriage of a VF_0175 homolog is not sufficient for infection (Figure 3C). Interestingly, these mutant strains produce a lower level of EPS than wild-type ES114 (Figure 3D), suggesting that EPS itself may be necessary for infection by HNL01-like phages. However, similar changes in EPS production have differing effects on bacteria-prey interactions in other systems (Deveau et al., 2002; Roach et al., 2013), and another polysaccharide type produced by *V. fischeri*—the Syp polysaccharide—is required during LO aggregation and colonization (Yip et al., 2006), demonstrating that *V. fischeri* needs to balance multiple selective pressures in symbiosis. Importantly, EPS production appears to be upregulated by genes expressed by *V. fischeri* in the LO (Bennett et al., 2020), where the bacteria are at high density and susceptible to a population-crashing phage sweep. These regulatory genes are downregulated soon after *V. fischeri* is vented into the water column (Thompson et al., 2017), highlighting the importance of host-mediated protection of bacteria in the LO.

Using a binary symbiotic system, we show that host- and bacteria-derived features protect a host-microbe mutualism in the face of phage exposure. This work illuminates key approaches that members of a host-associated microbiota can use to escape or avoid phage predation, providing a resilience mechanism for these communities. This work answers long-standing questions about the maintenance of symbiont populations, and it serves as a foundation for investigating specific parameters that lead to protection in the LO. More broadly, this helps us to understand rules that modulate phage susceptibility in a specific host-associated niche, which is necessary for understanding the roles that phages play in microbiotas and for harnessing phages therapeutically.

Limitations of the study

Our work demonstrates that the mutualism between *V. fischeri* and *E. scolopes* is protected from phages in the ambient seawater but is by no means a comprehensive description of the ways the squid-*Vibrio* mutualism—or systems like it—are maintained in the presence of phages. As such, we did not define the mechanism by which the squid protects its symbionts from phages. In part, this is due to technical challenges of using *Euprymna* hosts. For example, there is a lack of genetic tools that would allow for the inhibition of specific host effectors to probe relevant interactions. In addition, autofluorescence in the squid LO conflicts with imaging techniques like SYBR Gold labeling, limiting our understanding of whether phages are excluded from the LO.

Although we identified a mutation (VF_0175^{PR}) in *V. fischeri* ES114 that successfully confers resistance to HNL01 and identified that this mutation reduced EPS production, it remains to be determined whether EPS is a receptor for HNL01 or whether the EPS machinery (encoded by VF_0157-80) modifies another extracellular epitope that is the phage receptor. We partially addressed this question by using mutants that lack some surface-exposed epitopes, including the LPS O-antigen and dominant outer-membrane protein OmpU, which were still phage sensitive. This work narrows the list of possible HNL01 receptors/co-receptors but does not conclusively identify them.

STAR★METHODS

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

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Animal husbandry
  - Bacterial strains and culture conditions
- METHOD DETAILS
  - Isolation of *Vibrio fischeri*-infecting bacteriophage HNL01
  - Plaque assays
  - HNL01 genome sequencing
  - HNL01 genome annotation and comparative analysis with *Vibrio cholerae* phage ICP1
  - Plasmid and mutant construction
  - Alcian blue detection of extracellular polysaccharides
  - Identification of phage-resistance genes in *Vibrio fischeri*
  - *Vibrio fischeri* colonization and phage exposure
  - *In vitro* phage exposures
  - PCR detection of phage genes

- Electron microscopy
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.110376.

ACKNOWLEDGMENTS

We thank Randall Scarborough for assistance in collecting seawater samples, Daniel Russell and Rebecca Garlena for sequencing phage and bacterial genomes, and Tina Carvalho for assistance with transmission electron microscopy. This work was funded by NIH grant F32 GM119238 and a Ford Foundation Postdoctoral Fellowship to J.B.L., a National Science Foundation Graduate Research Fellowship DGE-114747 to B.D.M., NIH R01 GM135254–02 and R01 AI050661 to E.G.R., and startup funding from the University of Wisconsin-Madison to A.J.H. Microscopy was performed at the University of Hawai'i's MICRO facility, supported under COBRE grant P20 GM125508. The graphical abstract was created with biorender.com.

AUTHOR CONTRIBUTIONS

J.B.L., B.D.B., B.D.M., and A.J.H. performed experiments/computational analyses and analyzed the data. J.B.L., B.D.B., B.D.M., and A.J.H. prepared the display items. E.G.R. provided key insights, tools, and reagents. J.B.L., B.D.B., and A.J.H. wrote the paper. All authors edited the manuscript prior to submission.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. One or more of the authors of this paper received support from a program designed to increase minority representation in science.

Received: July 14, 2021
Revised: November 8, 2021
Accepted: January 20, 2022
Published: February 15, 2022

REFERENCES

Al-Shayeb, B., Sachdeva, R., Chen, L.X., Ward, F., Munk, P., Devoto, A., Castelle, C.J., Olm, M.R., Bouma-Gregson, K., Amano, Y., et al. (2020). Clades of huge phages from across Earth's ecosystems. Nature *578*, 425–431.

Ast, J.C., Urbanczyk, H., and Dunlap, P.V. (2009). Multi-gene analysis reveals previously unrecognized phylogenetic diversity in aliivibrio. Syst. Appl. Microbiol. *32*, 379–386.

Barr, J.J., Auro, R., Furlan, M., Whiteson, K.L., Erb, M.L., Pogliano, J., Stotland, A., Wolkowicz, R., Cutting, A.S., Doran, K.S., et al. (2013). Bacteriophage adhering to mucus provide a non-host-derived immunity. Proc. Natl. Acad. Sci. U. S. A. *110*, 10771–10776.

Barr, J.J., Auro, R., Sam-Soon, N., Kassegne, S., Peters, G., Bonilla, N., Hatay, M., Mourtada, S., Bailey, B., Youle, M., et al. (2015). Subdiffusive motion of bacteriophage in mucosal surfaces increases the frequency of bacterial encounters. Proc. Natl. Acad. Sci. U. S. A. *112*, 13675–13680.

Bennett, B.D., Essock-Burns, T., and Ruby, E.G. (2020). HBTR, a heterofunctional homolog of the virulence regulator TCPP, facilitates the transition between symbiotic and planktonic lifestyles in *Vibrio fischeri*. mBio *11*, e01624–e01720.

Bertani, G. (1951). Studies on lysogenesis. I: the mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. *62*, 293–300.

Besemer, J., and Borodovsky, M. (2005). GeneMark: web software for gene finding in prokaryotes, eukaryotes and viruses. Nucleic Acids Res. *33*, W451–W454.

Beumer, A., and Robinson, J.B. (2005). A broad-host-range, generalized transducing phage (SN-T) acquires 16S rRNA genes from different genera of bacteria. Appl. Environ. Microbiol. *71*, 8301–8304.

Bichet, M.C., Chin, W.H., Richards, W., Lin, Y.W., Avellaneda-Franco, L., Hernandez, C.A., Oddo, A., Chernyavskiy, O., Hilsenstein, V., Neild, A., et al. (2021). Bacteriophage uptake by mammalian cell layers represents a potential sink that may impact phage therapy. iScience *24*, 102287.

Black, P.N. (1988). The fadL gene product of *Escherichia coli* is an outer membrane protein required for uptake of long-chain fatty acids and involved in sensitivity to bacteriophage T2. J. Bacteriol. *170*, 2850–2854.

Boettcher, R. (1994). Occurrence of plasmid DNA in the sepiolid squid symbiont *Vibrio fischeri*. Curr. Microbiol. *29*, 279–286.

Boettcher, K.J., and Ruby, E.G. (1990). Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. J. Bacteriol. *172*, 3701–3706.

Bongrand, C., and Ruby, E.G. (2019). Achieving a multi-strain symbiosis: strain behavior and infection dynamics. ISME J. *13*, 698–706.

Bongrand, C., Moriano-Gutierrez, S., Arevalo, P., McFall-Ngai, M., Ruby, E.G., Visick, K.L., and Polz, M. (2020). Using colonization assays and comparative genomics to discover symbiosis behaviors and factors in *Vibrio fischeri*. mBio *11*, e03407–e03419.

Bose, J.L., Rosenberg, C.S., and Stabb, E.V. (2008). Effects of luxCDABEG induction in *Vibrio fischeri*: enhancement of symbiotic colonization and conditional attenuation of growth in culture. Arch. Microbiol. *190*, 169–183.

Bose, J.L., Wollenberg, M.S., Colton, D.M., Mandel, M.J., Septer, A.N., Dunn, A.K., and Stabb, E.V. (2011). Contribution of rapid evolution of the luxR - luxI intergenic region to the diverse bioluminescence outputs of *Vibrio fischeri* strains isolated from different environments. Appl. Environ. Microbiol. *77*, 2445–2457.

Breitbart, M., Bonnain, C., Malki, K., and Sawaya, N.A. (2018). Phage puppet masters of the marine microbial realm. Nat. Microbiol. *3*, 754–766.

Brum, J.R., Cesar Ignacio-Espinoza, J., Roux, S., Doulcier, G., Acinas, S.G., Alberti, A., Chaffron, S., Cruaud, C., De Vargas, C., Gasol, J.M., et al. (2015). Ocean plankton. patterns and ecological drivers of ocean viral communities. Science *348*, 1261498.

Cohen, S.K., Aschtgen, M.S., Lynch, J.B., Koehler, S., Chen, F., Escrig, S., Daraspe, J., Ruby, E.G., Meibom, A., and McFall-Ngai, M. (2020). Tracking the cargo of extracellular symbionts into host tissues with correlated electron microscopy and nanoscale secondary ion mass spectrometry imaging. Cell. Microbiol. *22*, e13177.

Coursolle, D., and Gralnick, J.A. (2010). Modularity of the Mtr respiratory pathway of shewanella oneidensis strain MR-1. Mol. Microbiol. *77*, 995–1008.

Cresawn, S.G., Bogel, M., Day, N., Jacobs-Sera, D., Hendrix, R.W., and Hatfull, G.F. (2011). Phamerator: a bioinformatic tool for comparative bacteriophage genomics. BMC Bioinformatics *12*, 395.

Delcher, A.L., Harmon, D., Kasif, S., White, O., and Salzberg, S.L. (1999). Improved microbial gene identification with GLIMMER. Nucleic Acids Res. *27*, 4636–4641.

Deveau, H., Van Calsteren, M.R., and Moineau, S. (2002). Effect of exopolysaccharides on phage-host interactions in Lactococcus lactis. Appl. Environ. Microbiol. *68*, 4364–4369.

Díaz-Pascual, F., Hartmann, R., Lempp, M., Vidakovic, L., Song, B., Jeckel, H., Thormann, K.M., Yildiz, F.H., Dunkel, J., Link, H., et al. (2019). Breakdown of *Vibrio cholerae* biofilm architecture induced by antibiotics disrupts community barrier function. Nat. Microbiol. *4*, 2136–2145.

Dion, M.B., Oechslin, F., and Moineau, S. (2020). Phage diversity, genomics and phylogeny. Nat. Rev. Microbiol. *18*, 125–138.

Dunn, A.K., Millikan, D.S., Adin, D.M., Bose, J.L., and Stabb, E.V. (2006). New rfp- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and lux expression in situ. Appl. Environ. Microbiol. *72*, 802–810.

Dunsing, V., Irmscher, T., Barbirz, S., and Chiantia, S. (2019). Purely polysaccharide-based biofilm matrix provides size-selective diffusion barriers for nanoparticles and bacteriophages. Biomacromolecules *20*, 3842–3854.

Essock-Burns, T., Bongrand, C., Goldman, W.E., Ruby, E.G., McFall-Ngai, M.J., and Graf, J. (2020). Interactions of symbiotic partners drive the development of a complex biogeography in the squid-vibrio symbiosis. mBio *11*, e00853–e00920.

Fan, F., Li, X., Pang, B., Zhang, C., Li, Z., Zhang, L., Li, J., Zhang, J., Yan, M., Liang, W., et al. (2018). The outer-membrane protein TolC of *Vibrio cholerae* serves as a second cell-surface receptor for the VP3 phage. J. Biol. Chem. *293*, 4000–4013.

Fidopiastis, P.M., Von Boletzky, S., and Ruby, E.G. (1998). A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus Sepiola. J. Bacteriol. *180*, 59–64.

Flores, C.O., Meyer, J.R., Valverde, S., Farr, L., and Weitz, J.S. (2011). Statistical structure of host-phage interactions. Proc. Natl. Acad. Sci. U. S. A. *108*, E288–E297.

Guerin, E., Shkoporov, A., Stockdale, S.R., Clooney, A.G., Ryan, F.J., Sutton, T.D.S., Draper, L.A., Gonzalez-Tortuero, E., Ross, R.P., and Hill, C. (2018). Biology and Taxonomy of crAss-like bacteriophages, the most abundant virus in the human gut. Cell Host Microbe *24*, 653–664.e6.

Hryckowian, A.J., Merrill, B.D., Porter, N.T., Van Treuren, W., Nelson, E.J., Garlena, R.A., Russell, D.A., Martens, E.C., and Sonnenburg, J.L. (2020). Bacteroides thetaiotaomicron-infecting bacteriophage isolates inform sequence-based host range predictions. Cell Host Microbe *28*, 371–379.e5.

Hussa, E.A., O'Shea, T.M., Darnell, C.L., Ruby, E.G., and Visick, K.L. (2007). Two-component response regulators of *Vibrio fischeri*: identification, mutagenesis, and characterization. J. Bacteriol. *189*, 5825–5838.

de Jonge, P.A., Nobrega, F.L., Brouns, S.J.J., and Dutilh, B.E. (2019). Molecular and evolutionary determinants of bacteriophage host range. Trends Microbiol. *27*, 51–63.

Kim, H., Kim, M., Bai, J., Lim, J.A., Heu, S., and Ryu, S. (2019). Colanic acid is a novel phage receptor of Pectobacterium carotovorum subsp. carotovorum phage POP72. Front. Microbiol. *10*, 143.

Koch, E.J., Miyashiro, T., McFall-Ngai, M.J., and Ruby, E.G. (2014). Features governing symbiont persistence in the squid-vibrio association. Mol. Ecol. *23*, 1624–1634.

Koskella, B., and Brockhurst, M.A. (2014). Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities. FEMS Microbiol. Rev. *38*, 916–931.

Kutter, E., Bryan, D., Ray, G., Brewster, E., Blasdel, B., and Guttman, B. (2018). From host to phage metabolism: hot tales of phage T4's takeover of *E. coli*. Viruses *10*, 387.

Lee, K.H., and Ruby, E.G. (1992). Detection of the light organ symbiont, *Vibrio fischeri*, in Hawaiian seawater by using lux gene probes. Appl. Environ. Microbiol. *58*, 942–947.

Lee, K.H., and Ruby, E.G. (1994). Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl. Environ. Microbiol. *60*, 1565–1571.

Lourenço, M., Chaffringeon, L., Lamy-Besnier, Q., Pédron, T., Campagne, P., Eberl, C., Bérard, M., Stecher, B., Debarbieux, L., and De Sordi, L. (2020). The spatial heterogeneity of the gut limits predation and fosters coexistence of bacteria and bacteriophages. Cell Host Microbe *28*, 390–401.

Lowe, T.M., and Eddy, S.R. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. *25*, 955–964.

Lynch, J.B., Schwartzman, J.A., Bennett, B.D., McAnulty, S.J., Knop, M., Nyholm, S.V., and Ruby, E.G. (2019). Ambient pH alters the protein content of outer membrane vesicles, driving host development in a beneficial symbiosis. J. Bacteriol. *201*, e00319-19.

Mandel, M.J., Stabb, E.V., and Ruby, E.G. (2008). Comparative genomics-based investigation of resequencing targets in *Vibrio fischeri*: focus on point miscalls and artefactual expansions. BMC Genomics *9*, 138.

Matilla, M.A., Fang, X., and Salmond, G.P.C. (2014). Viunalikeviruses are environmentally common agents of horizontal gene transfer in pathogens and biocontrol bacteria. ISME J. *8*, 2143–2147.

McFall-Ngai, M.J. (2014). The importance of microbes in animal development: lessons from the squid-Vibrio symbiosis. Annu. Rev. Microbiol. *68*, 177–194.

Montgomery, M.K., and McFall-Ngai, M. (1993). Embryonic development of the light organ of the sepiolid squid *Euprymna scolopes* berry. Biol. Bull. *184*, 296–308.

Morona, R., and Henning, U. (1984). Host range mutants of bacteriophage Ox2 can use two different outer membrane proteins of *Escherichia coli* K-12 as receptors. J. Bacteriol. *159*, 579–582.

Nawroth, J.C., Guo, H., Koch, E., Heath-Heckman, E.A.C., Hermanson, J.C., Ruby, E.G., Dabiri, J.O., Kanso, E., and McFall-Ngai, M. (2017). Motile cilia create fluid-mechanical microhabitats for the active recruitment of the host microbiome. Proc. Natl. Acad. Sci. *114*, 9510–9516.

Nguyen, S., Baker, K., Padman, B.S., Patwa, R., Dunstan, R.A., Weston, T.A., Schlosser, K., Bailey, B., Lithgow, T., Lazarou, M., et al. (2017). Bacteriophage transcytosis provides a mechanism to cross epithelial cell layers. mBio *8*, e01874–e01917.

Nishiguchi, M.K., and Nair, V.S. (2003). Evolution of symbiosis in the Vibrionaceae: a combined approach using molecules and physiology. Int. J. Syst. Evol. Microbiol. *53* (*Pt 6*), 2019–2026.

Nishiguchi, M.K., Ruby, E.G., and McFall-Ngai, M.J. (1998). Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-vibrio symbioses. Appl. Environ. Microbiol. *64*, 3209–3213.

Nyholm, S.V., and McFall-Ngai, M.J. (2003). Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. Appl. Environ. Microbiol. *69*, 3932–3937.

Nyholm, S.V., Deplancke, B., Gaskins, H.R., Apicella, M.A., and McFall-Ngai, M.J. (2002). Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. Appl. Environ. Microbiol. *68*, 5113–5122.

Ofir, G., and Sorek, R. (2018). Contemporary phage biology: from classic models to new insights. Cell *172*, 1260–1270.

Parent, K.N., Erb, M.L., Cardone, G., Nguyen, K., Gilcrease, E.B., Porcek, N.B., Pogliano, J., Baker, T.S., and Casjens, S.R. (2014). OmpA and OmpC are critical host factors for bacteriophage Sf6 entry in Shigella. Mol. Microbiol. *92*, 47–60.

Porter, N.T., Hryckowian, A.J., Merrill, B.D., Fuentes, J.J., Gardner, J.O., Glowacki, R.W.P., Singh, S., Crawford, R.D., Snitkin, E.S., Sonnenburg, J.L., et al. (2020). Phase-variable capsular polysaccharides and lipoproteins modify bacteriophage susceptibility in bacteroides thetaiotaomicron. Nat. Microbiol. *5*, 1170–1181.

Post, D.M.B., Yu, L., Krasity, B.C., Choudhury, B., Mandel, M.J., Brennan, C.A., Ruby, E.G., McFall-Ngai, M.J., Gibson, B.W., and Apicella, M.A. (2012). O-antigen and core carbohydrate of *Vibrio fischeri* lipopolysaccharide. J. Biol. Chem. *287*, 8515–8530.

Preheim, S.P., Boucher, Y., Wildschutte, H., David, L.A., Veneziano, D., Alm, E.J., and Polz, M.F. (2011). Metapopulation structure of Vibrionaceae among coastal marine invertebrates. Environ. Microbiol. *13*, 265–275.

Rao, C., Coyte, K.Z., Bainter, W., Geha, R.S., Martin, C.R., and Rakoff-Nahoum, S. (2021). Multi-kingdom ecological drivers of microbiota assembly in preterm infants. Nature *591*, 633–638.

Reyes-Robles, T., Dillard, R.S., Cairns, L.S., Silva-Valenzuela, C.A., Housman, M., Ali, A., Wright, E.R., and Camilli, A. (2018). *Vibrio cholerae* outer membrane vesicles inhibit bacteriophage infection. J. Bacteriol. *200*, 1–9.

Reyes, A., Haynes, M., Hanson, N., Angly, F.E., Heath, A.C., Rohwer, F., and Gordon, J.I. (2010). Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature *466*, 334–338.

Roach, D.R., Sjaarda, D.R., Castle, A.J., and Svircev, A.M. (2013). Host exopolysaccharide quantity and composition impact erwinia: amylovora bacteriophage pathogenesis. Appl. Environ. Microbiol. *79*, 3249–3256.

Rollenske, T., Burkhalter, S., Muerner, L., von Gunten, S., Lukasiewicz, J., Wardemann, H., and Macpherson, A.J. (2021). Parallelism of intestinal secretory IgA shapes functional microbial fitness. Nature *598*, 657–661.

Roossinck, M.J. (2011). The good viruses: viral mutualistic symbioses. Nat. Rev. Microbiol. *9*, 99–108.

Ruby, E.G., and Lee, K.H. (1998). The *Vibrio fischeri-Euprymna scolopes* light organ association: current ecological paradigms. Appl. Environ. Microbiol. *64*, 805–812.

Saltikov, C.W., and Newman, D.K. (2003). Genetic identification of a respiratory arsenate reductase. Proc. Natl. Acad. Sci. *100*, 10983–10988.

Schwartzman, J.A., Lynch, J.B., Flores Ramos, S., Zhou, L., Apicella, M.A., Yew, J.Y., and Ruby, E.G. (2019). Acidic pH promotes lipopolysaccharide modification and alters colonization in a bacteria–animal mutualism. Mol. Microbiol. *112*, 1326–1338.

Seed, K.D., Bodi, K.L., Kropinski, A.M., Ackermann, H.W., Calderwood, S.B., Qadri, F., and Camilli, A. (2011). Evidence of a dominant lineage of *Vibrio cholerae*-specific lytic bacteriophages shed by cholera patients over a 10-year period in Dhaka, Bangladesh. mBio *2*, e00334-10.

Seed, K.D., Faruque, S.M., Mekalanos, J.J., Calderwood, S.B., Qadri, F., and Camilli, A. (2012). Phase variable O antigen biosynthetic genes control expression of the major protective antigen and bacteriophage receptor in *Vibrio cholerae* O1. Plos Pathog. *8*, e1002917.

Simmons, E.L., Bond, M.C., Koskella, B., Drescher, K., Bucci, V., and Nadell, C.D. (2020). Biofilm structure promotes coexistence of phage-resistant and phage-susceptible bacteria. mSystems *5*, e00877-19.

Speare, L., Cecere, A.G., Guckes, K.R., Smith, S., Wollenberg, M.S., Mandel, M.J., Miyashiro, T., and Septer, A.N. (2018). Bacterial symbionts use a type VI secretion system to eliminate competitors in their natural host. Proc. Natl. Acad. Sci. U. S. A. *115*, E8528–E8537.

Stabb, E.V., Reich, K.A., and Ruby, E.G. (2001). *Vibrio fischeri* genes hvnA and hvnB encode secreted NAD+-glycohydrolases. J. Bacteriol. *183*, 309–317.

Sun, Y., LaSota, E.D., Cecere, A.G., LaPenna, K.B., Larios-Valencia, J., Wollenberg, M.S., and Miyashiro, T. (2016). Intraspecific competition impacts *Vibrio fischeri* strain diversity during initial colonization of the squid light organ. Appl. Environ. Microbiol. *82*, 3082–3091.

Suttle, C.A. (2007). Marine viruses - major players in the global ecosystem. Nat. Rev. Microbiol. *5*, 801–812.

Thompson, L.R., Nikolakakis, K., Pan, S., Reed, J., Knight, R., and Ruby, E.G. (2017). Transcriptional characterization of *Vibrio fischeri* during colonization of juvenile *Euprymna scolopes*. Environ. Microbiol. *19*, 1845–1856.

Wang, C., Tu, J., Liu, J., and Molineux, I.J. (2019). Structural dynamics of bacteriophage P22 infection initiation revealed by cryo-electron tomography. Nat. Microbiol. *4*, 1049–1056.

Wollenberg, M.S., and Ruby, E.G. (2009). Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* squid from two oahu (Hawaii) populations. Appl. Environ. Microbiol. *75*, 193–202.

Xu, D., Zhang, J., Liu, J., Xu, J., Zhou, H., Zhang, L., Zhu, J., and Kan, B. (2014). Outer membrane protein OmpW is the receptor for typing phage VP5 in the *Vibrio cholerae* O1 el tor biotype. J. Virol. *88*, 7109–7111.

Yen, M., and Camilli, A. (2017). Mechanisms of the evolutionary arms race between *Vibrio cholerae* and Vibriophage clinical isolates. Int. Microbiol. *20*, 116–120.

Yip, E.S., Geszvain, K., DeLoney-Marino, C.R., and Visick, K.L. (2006). The symbiosis regulator RscS controls the syp gene locus, biofilm formation and symbiotic aggregation by *Vibrio fischeri*. Mol. Microbiol. *62*, 1586–1600.

Zborowsky, S., and Lindell, D. (2019). Resistance in marine cyanobacteria differs against specialist and generalist cyanophages. Proc. Natl. Acad. Sci. U. S. A. *116*, 16899–16908.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Vibrio fischeri ES114	Boettcher and Ruby, 1990	BioProject: PRJNA12986
V. fischeri ES213	(Boettcher, 1994)	BioProject: PRJNA311605
V. fischeri MJ11	Mandel et al., 2008	BioProject: PRJNA19393
V. fischeri SR5	Fidopiastis et al., 1998	BioProject: PRJNA73351
V. fischeri KB2B1	Wollenberg and Ruby, 2009	BioProject: PRJNA675878
V. fischeri KB4B5	Wollenberg and Ruby, 2009	BioProject: PRJNA311605
V. fischeri MB13B1	Wollenberg and Ruby, 2009	BioProject: PRJNA311605
V. fischeri MB13B2	Wollenberg and Ruby, 2009	BioProject: PRJNA311605
V. fischeri MB13B3	Wollenberg and Ruby, 2009	BioProject: PRJNA311605
V. fischeri MB14A3	Wollenberg and Ruby, 2009	BioProject: PRJNA311605
V. fischeri MB15A4	Wollenberg and Ruby, 2009	BioProject: PRJNA311605
V. fischeri MB14A3	Wollenberg and Ruby, 2009	BioProject: PRJNA311605
V. fischeri MB11B1	Wollenberg and Ruby, 2009	BioProject: PRJNA311605
V. fischeri EM17	Lee and Ruby, 1994, Ruby and Lee, 1998	BioProject: PRJNA212806
V. fischeri EM24	Lee and Ruby, 1994, Ruby and Lee, 1998	GenBank: JF509755
V. fischeri EM30	Lee and Ruby, 1994	GenBank: JF509756
V. fischeri ET101	Nishiguchi et al., 1998	GenBank: AY292923
V. fischeri ET401	Nishiguchi and Nair, 2003	GenBank: AY292943
V. fischeri 9CS99	Wollenberg 2012, Preheim et al., 2011	GenBank: JF717859
V. fischeri H905	Lee and Ruby, 1992	BioProject: PRJNA316342
V. fischeri VLS2	Lee and Ruby, 1994, Bose et al., 2011	BioProject: PRJNA311605
V. fischeri ZF73	Gift from Martin Polz, MIT	BioProject: PRJNA318805
V. fischeri 5F7	Gift from Martin Polz, MIT	BioProject: PRJNA318805
V. fischeri emors.6.1	Ast et al., 2009	BioProject: PRJNA590810
V. fischeri emors.6.2	Ast et al., 2009	BioProject: PRJNA590810
V. fischeri JAS340	Schwartzman et al., 2019	N/A
V. fischeri KV1787	Hussa et al., 2007	N/A
V. fischeri JG01	Lynch et al., 2019	N/A
V. fischeri MB06859	Post et al., 2012	N/A
V. fischeri EVS102	Bose et al., 2008	N/A
V. fischeri BDB127	Bennett et al., 2020	N/A
V. fischeri BDB233	This paper	N/A
V. fischeri BDB234	This paper	N/A
V. fischeri BDB129	Bennett et al., 2020	N/A
V. fischeri BDB186	Bennett et al., 2020	N/A
V. fischeri BDB235	This paper	N/A
V. fischeri BDB236	This paper	N/A
V. fischeri BDB222	This paper	N/A
V. fischeri BDB227	This paper	N/A
V. fischeri BDB235	This paper	N/A
V. fischeri BDB236	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Escherichia coli WM3064	Saltikov and Newman, 2003	N/A
Vibrio fischeri phage HNL01	This paper	BioProject: PRJNA741526
Deposited data		
Vibrio fischeri phage HNL01 genome sequence	This paper	BioProject: PRJNA741526
Complete genome sequences of HNL01-resistant *V. fischeri* clones	This paper; BioProject: PRJNA751213	BioSample: SAMN20511716
		BioSample: SAMN20511717
		BioSample: SAMN20511718
		BioSample: SAMN20511719
Experimental models: Organisms/strains		
Euprymna scolopes	Raised in house	N/A
Oligonucleotides		
See Table S3 for oligonucleotide information	N/A	N/A
Recombinant DNA		
pVSV105 (Cloning vector; Cam^r)	Dunn et al., 2006	N/A
pVSV105::VF_1075 (VF_0175, 17 bp upstream, 49 bp downstream; Cam^r)	This paper	N/A
pVSV105::VF_0175(E183V) (VF_0175 with codon 183 GAG→GTT, 17 bp upstream, 49 bp downstream; Cam^r)	This paper	N/A
pSMV3 (Deletion vector, Kan^r, *sacB*)	Coursolle and Gralnick, 2010	N/A
pVSV102 (GFP expression vector; Kan^r)	Dunn et al., 2006	N/A
pVSV208 (RFP expression vector; Cam^r)	Dunn et al., 2006	N/A
pVSV105-H (Empty experssion vector; Kan^r)	Dunn et al., 2006	N/A
Software and algorithms		
Geneious Prime (2021.1.1)	N/A	https://www.geneious.com/prime/
DNA Master	N/A	http://cobamide2.pitt.edu
Genemark	Besemer and Borodovsky, 2005	http://topaz.gatech.edu/GeneMark/license_download.cgi
Glimmer	Delcher et al., 1999	https://sourceforge.net/projects/glimmer/
tRNAscan-SE	Lowe and Eddy, 1997	http://lowelab.ucsc.edu/tRNAscan-SE/
Phamerator	Cresawn et al., 2011	https://phamerator.org/
Graphpad Prism 9.1.0	https://www.graphpad.com/	https://www.graphpad.com/scientificsoftware/prism/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Andrew Hryckowian (hryckowian@medicine.wisc.edu).

Materials availability

HNL01 and strains generated as part of this study are available upon request from the Lead Contact (hryckowian@medicine.wisc.edu).

Data and code availability

- The HNL01 genome sequence and the genome sequences of the 4 HNL01-resistant ES114 clones are deposited in GenBank and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal husbandry

Adult *Euprymna scolopes* were collected from the waters of Maunalua Bay, O'ahu, Hawai'i, and maintained at Kewalo Marine Laboratory, where they would lay eggs as previously described (Montgomery and McFall-Ngai, 1993). Juvenile squid were collected <3 hours after hatching and placed in filter-sterilized ocean water (FSOW) and maintained on a 12:12-h light-dark photic cycle for the duration of experiments.

Bacterial strains and culture conditions

The LO symbiont *V. fischeri* strain ES114 (Boettcher and Ruby, 1990) was used for all experiments unless otherwise noted. Bacteria were grown in Lysogeny broth-salt medium (LBS) (10 g tryptone L^{-1}, 5 g yeast extract L^{-1}, 342 mM NaCl, and 20 mM Tris, pH 7.5) (Stabb et al., 2001) or seawater-tryptone medium (SWT) (70% ocean water, 32.5 mM glycerol, 5 g tryptone L^{-1}, 3 g yeast extract L^{-1}) (Boettcher and Ruby, 1990) at 28°C, either on agar plates or in liquid culture shaking at 225 rpm. *E. coli* WM3064 was grown in lysogeny broth (Bertani, 1951) supplemented with 300 μM diaminopimelic acid at 37°C.Where appropriate, chloramphenicol (2.5 μg mL^{-1}) or kanamycin (50 μg mL^{-1}) was added to the media.

METHOD DETAILS

Isolation of *Vibrio fischeri*-infecting bacteriophage HNL01

Approximately 700 mL of coastal ocean water was collected each from Kewalo Basin and Kāne'ohe Bay on O'ahu, Hawai'i, USA, and was centrifuged at 5,500 × g for 10 minutes at room temperature to precipitate solids. The supernatants were then sequentially filtered through 0.45 μm and 0.22 μm pore polyvinylidene fluoride (PVDF) filters. The filtered water was concentrated 750-fold via 100 kDa PVDF size exclusion columns. The initial screening of plaques was performed using a soft agar overlay method in which 35 μL of filtered and concentrated seawater was combined with 200 μL of overnight *V. fischeri* ES114 LBS culture and with 4.5 mL molten SWT top agar (cooled to ~50°C, 3.5 g L^{-1} agar) and poured onto a warmed SWT agar plate (15 g L^{-1} agar). Soft agar overlays were incubated aerobically at room temperature overnight.

Single isolated plaques were picked into 100 μL autoclaved Instant Ocean (IO; Spectrum Brands, Madison, WI, USA), and serial dilutions were prepared and spotted onto a solidified top agar overlay. Isolated plaques were picked after overnight growth. This procedure was repeated for a total of 3 times to purify HNL01.

A high-titer stock of HNL01 was generated by flooding a soft-agar overlay plate that yielded a 'lacy' pattern of bacterial growth (near confluent lysis). After overnight incubation of the plate, 5 mL of sterile IO were added to the plate to resuspend the phage. After at least 2 hours of incubation at room temperature, the lysate was extracted and filter sterilized through a 0.22 μm PVDF filter.

Plaque assays

Isolated colonies from freshly streaked −80°C *V. fischeri* stocks (Table S1) were used to inoculate liquid LBS cultures. Strains used were either generated in this work (see plasmid and mutant construction, below) or in previous studies (Ast et al., 2009; Bose et al., 2008, 2011; Dunn et al., 2006; Fidopiastis et al., 1998; Hussa et al., 2007; Lee and Ruby, 1992, 1994; Mandel et al., 2008; Nishiguchi et al., 1998; Nishiguchi and Nair, 2003; Post et al., 2012; Preheim et al., 2011; Ruby and Lee, 1998; Schwartzman et al., 2019).

After ~16 h, 200 μL of culture were mixed with 4 mL molten SWT top agar and poured onto warmed SWT agar plates. 1 μL of serial 1:10 dilutions of phage lysate was spotted onto solidified top agar, and plates were incubated at 28°C for 3–5 h before counting plaques. Alternatively, 500 μL of bacteria (either from a stationary-phase culture of *V. fischeri* in SWT as in Figure 3B or pooled squid homogenates as in Figure 2C) were added to 4.5 mL of molten SWT top agar, then spread onto a warmed SWT plate and allowed to solidify. 5 μL of diluted phage (10^9 PFU/mL unless otherwise noted) were spotted onto plates, and after drying, plates were incubated at 28°C for 24 hours to allow for active growth of *V. fischeri* before checking for lysis. PFU counts and phage susceptibility assays were performed on SWT plates, as plaques were noticeably smaller or non-observable on LBS plates.

HNL01 genome sequencing

DNA was extracted from a high-titer HNL01 lysate and sequencing libraries were prepared using the Ultra II FS Kit (New England Biolabs, Ipswich, MA, USA). Libraries were quantified using a BioAnalyzer (Agilent, Santa Clara, CA, USA) and subsequently sequenced using 150-base single-end reads (Illumina MiSeq). Reads were imported into Geneious Prime (2021.1.1) and quality-trimmed at an error rate of 0.001%. The Geneious assembler was used to assemble 30% of the surviving reads ≥150bp, with the options ''Medium sensitivity/Fast'' and "circularize contigs" selected. Coverage for the genome assembly reported by Geneious was 170 +- 21.

HNL01 genome annotation and comparative analysis with *Vibrio cholerae* phage ICP1

A 383-bp short direct terminal repeat was identified in HNL01 by visualizing read pileups in Geneious Prime (2021.1.1), and the genome was arranged to place this repeat at the 5' end of the genome. Protein-coding genes and tRNAs were predicted and annotated using DNA Master default parameters (http://cobamide2.pitt.edu), which incorporates Genemark (Besemer and Borodovsky,

2005), Glimmer (Delcher et al., 1999), and tRNAscan-SE (Lowe and Eddy, 1997). Phage genomes were annotated and compared on the basis of shared gene phamily (pham) membership within Phamerator using default parameters (Cresawn et al., 2011). Phams are groups of related protein-encoding genes where pham membership is built and expanded when a candidate protein shares $\geq$32.5% identity or a BLASTp e-value $\leq 1e^{-50}$ with one or more existing members of the pham. Genome maps of HNL01 and ICP1 were visualized in Phamerator using default parameters. Amino acid sequences for HNL01 and ICP1 were concatenated and visualized as a dot plot using word size 5 in Geneious Prime (2021.1.1)

Plasmid and mutant construction

Primers used to create the *V. fischeri* gene deletion and expression plasmids used in this study are listed in Tables S1 and S3, respectively. In-frame deletion of VF_0175 from the *V. fischeri* genome was performed as previously described (Bennett et al., 2020; Saltikov and Newman, 2003). Briefly, ~1kb fragments surrounding VF_0175 were fused via an internal *Eco*RI site and inserted via *Bam*HI and *Sac*I sites into pSMV3 (Coursolle and Gralnick, 2010), which has kanamycin resistance and *sacB* cassettes. Plasmids were introduced to *V. fischeri* ES114 through conjugation with *E. coli* WM3064, and counter-selection to remove pSMV3 and VF_0175 was performed on low-salt, high-sucrose LB plates (Bennett et al., 2020) at room temperature. The wild-type VF_0175 complementation plasmid was constructed by cloning VF_0175 from the *V. fischeri* ES114 genome, and inserting it into the expression plasmid pVSV105 (Dunn et al., 2006) via *Sph*I and *Kpn*I sites. The VF_0175^{PR} allele was ordered as a gblock from Integrated DNA Technologies (Coralville, IA, USA), and inserted into pVSV105 via *Sph*I and *Kpn*I sites.

Alcian blue detection of extracellular polysaccharides

EPS was detected as previously described (Bennett et al., 2020). Briefly, overnight bacterial cultures grown in minimal salts medium supplemented with 6.5 mM *N*-acetylneuraminic acid and 0.05% (wt/vol) casamino acids were centrifuged for 15 min at 12,000 × g and 4°C. 250 μL culture supernatant was incubated with 1 mL Alcian blue reagent on a rocker for one hour at room temperature, then centrifuged 10 min at 10,000 rpm and 4°C. Pellets were washed with 1 mL 100% ethanol and centrifuged 10 min at 10,000 × g and 4°C. Pellets were solubilized in 200 μL SDS-acetate and the absorbance read at OD_{620}.

Identification of phage-resistance genes in *Vibrio fischeri*

Four colonies from were picked from the center of HNL01 phage spots containing at least 10^5 PFU. Colonies were streaked for isolation over two more passages on LBS plates. Isolates were grown overnight in LBS medium and were used to make glycerol stocks and confirm phage insensitivity. An overnight LBS culture was used for DNA extraction using the Qiagen Blood and Tissue Kit (Qiagen, Germantown, MD, USA). Sequencing libraries were prepared from genomic DNA from each HNL01-resistant isolate, as well as the parental *V. fischeri* ES114 strain, using the Ultra II FS Kit (New England Biolabs). Libraries were quantified using a BioAnalyzer (Agilent) and subsequently sequenced using 151-base single-end reads (Illumina MiSeq). Differences between wild-type and resistant *V. fischeri* ES114 was identified through genomic comparison using CLC Genomics Workbench (Qiagen).

Vibrio fischeri colonization and phage exposure

Juvenile squid were incubated with ~10^4–10^5 CFU/mL of wild-type *V. fischeri* ES114 and/or the VF_0175^{PR} mutant (as noted) for 24 h. For co-colonizations, ~1:1 inocula of each strain were used, and competitive index was measured relative to strain ratio in the inoculum (competitive index = (wildtype:mutant ratio in LO)/(wild-type: mutant ratio in inoculum). When appropriate, animals were transferred to fresh FSOW after 24 h. Animals were frozen at −80°C in FSOW immediately before dark (*i.e.*, at maximal colonization), then thawed and homogenized, and serial dilutions were plated on LBS-agar plates to determine *V. fischeri* CFU counts. GFP- and RFP-positive CFU were counted using a fluorescence dissecting microscope. When appropriate, phage was added to the water at ~10^7 PFU/mL.

In vitro phage exposures

HNL01-sensitive and resistant *V. fischeri* ES114 strains received pVSV102 (GFP, Kan^r), pVSV208 (RFP, Cam^r), or pVSV104-H (Kan^r) (Dunn et al., 2006) via conjugation with donor strain *E. coli* WM3064 (Lynch et al., 2019). Cultures of wild-type and/or HNL01-resistant *V. fischeri* ES114 strains were streaked onto LBS plates with the appropriate antibiotic and then cultured in LBS overnight. Cells were then diluted in SWT from 1:10–1:10,000. 100μL of diluted cells were placed in wells of a 96-well flat-bottom, transparent plate with or without HNL01 at the noted dilutions of a ~10^{11} PFU/mL stock, and were incubated at 28°C with periodic shaking for 24–48 h. A Tecan GenIOS plate reader (Tecan, Baldwin Park, CA, USA) was used to measure GFP and RFP fluorescence and/or overall cell density (OD_{600}).

PCR detection of phage genes

Taq DNA polymerase was used perform to PCR against HNL01-specific genes (*gp47, gp61, gp96, gp147*; see Table S3) on glycerol stocks of HNL01-resistant *V. fischeri* isolates to assess their potential lysogen status (see Table S3 for primer sequences). Cycling conditions were: 95°C for 5 min, 35 cycles of 95°C for 30 sec → 55°C for 30 sec → 68°C for 1 min, then 68 °C for 5 min followed by a 4°C hold. PCR was also run directly on phage samples and on wild-type ES114 as controls. Products were run on a 1% agarose gel to determine PCR product presence/absence, and lack of integrated prophages was confirmed by whole genome sequencing.

Electron microscopy

Serial dilutions of phages were spotted onto *V. fischeri* ES114 + SWT top-agar lawns as described above and allowed to form plaques overnight. Dilutions with the most easily distinguishable plaques (*i.e.*, the lowest dilution that still produced plaques) were briefly covered with 10 μL of FSOW to resuspend phage, then removed to a clean PCR tube. A 4 μL portion of the suspension was spotted onto a charged formvar-coated copper grid, negatively stained with uranyl acetate, and imaged with a Hitachi HT7700 transmission electron microscope at 100kV at the University of Hawai'i MICRO Facility.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using Graphpad Prism 9.1.0. Details of specific analyses, including statistical tests used, are found in applicable figure legends. All experiments were performed in at least triplicate. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$, **** = $p < 0.0001$.