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Prophage regulation of *Shewanella fidelis* 3313 motility and biofilm formation: implications for gut colonization dynamics in *Ciona robusta*

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eLife Assessment

This **valuable** study presents findings linking prophage carriage to lifestyle regulation in the marine bacterium Shewanella fidelis, with potential implications for niche occupation within a host (Ciona robusta) and mediation of host immune responses. The study leverages a unique animal model system that offers distinct advantages in identifying select phenotypes to present overall **solid** evidence that supports findings relating to the impact of a prophage on host-microbe interaction. Understanding the role of integrated lysogenic phages in bacterial fitness, both within a host and in the environment, is a significant concept in bacterial eco-physiology, potentially contributing to the success of certain strains.

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Abstract

Lysogens, bacteria with one or more viruses (prophages) integrated into their genomes, are abundant in the gut of animals. Prophages often influence bacterial traits; however, the influence of prophages on the gut microbiota-host immune axis in animals remains poorly understood. Here, we investigate the influence of the prophage SfPat on *Shewanella fidelis* 3313, a persistent member of the gut microbiome of the model marine tunicate, *Ciona robusta*. Establishment of a SfPat deletion mutant (ΔSfPat) reveals the influence of this prophage on bacterial physiology *in vitro* and during colonization of the *Ciona* gut. *In vitro*, deletion of SfPat reduces *S. fidelis* 3313 motility and swimming while increasing biofilm formation. To understand the *in vivo* impact of these prophage-induced changes in bacterial traits, we exposed metamorphic stage 4 *Ciona* juveniles to wildtype (WT) and ΔSfPat strains.

During colonization, ΔSfPat localizes to overlapping and distinct areas of the gut compared to the WT strain. We examined the differential expression of various regulators of cyclic-di-GMP, a secondary signaling molecule that mediates biofilm formation and motility. The *pdeB* gene, which encodes a bacterial phosphodiesterase known to influence biofilm formation and motility by degrading cyclic-di-GMP, is upregulated in the WT strain but not in ΔSfPat when examined *in vivo*. Expression of the *Ciona* gut immune effector, VCBP-C, is enhanced during colonization by ΔSfPat compared to the WT strain; however, VCBP-C binding to the WT strain does not promote the excision of SfPat in an SOS-dependent pathway. Instead, VCBP-C binding significantly reduces the expression of a phage major capsid protein. Our findings suggest that SfPat influences host perception of this important colonizing commensal and highlights the significance of investigating tripartite dynamics between prophages, bacteria, and their animal hosts to better understand the gut microbiota-host immune axis.

Introduction

An epithelial layer with a mucus-rich surface lines the gastrointestinal tract (or gut) of animals. This dynamic environment is a primary interface between host immunity, symbiotic microbes, and dietary antigens Li et al. (2015); Johansson et al. (2011). During colonization of the gut, bacteria encounter physical, chemical, and biological forces. They must compete for nutrients and niche space while managing the stress of digestive enzymes and host immune factors Duncan et al. (2021); Harrington et al. (2021); Hornung et al. (2018); Moran et al. (2019). Gut-colonizing bacteria also encounter bacteriophages (phages), or viruses that infect bacteria Mirzaei and Maurice (2017); e.g., over 10^{12} viruses have been estimated in the human gut Shkoporov and Hill (2019).

Phages display both lytic and temperate lifestyles. While the former impacts bacterial community dynamics through lysis, the effect of temperate phages on bacterial communities, especially those associated with animals, remains poorly understood. Conventionally, temperate phages integrate into bacterial genomes as prophages and remain 'dormant' until an external trigger activates them to enter the lytic cycle Lwoff (1953); Boling et al. (2020); Howard-Varona et al. (2017); these bacteria are considered 'lysogenized' and referred to as lysogens Lwoff (1953); Howard-Varona et al. (2017). Prophages often encode accessory genes that can influence bacterial traits and behaviors Mills et al. (2013). These genes can encode virulence factors, antibiotic resistance determinants, and those that provide superinfection exclusion, thereby protecting their bacterial hosts from infections by related phages Bondy-Denomy and Davidson (2014). Based on the site of integration, prophages can also impact the expression of bacterial genes Aziz et al. (2005). Bacterial lysogens exist in every environment Jiang and Paul (1998); Silveira et al. (2021); Leigh et al. (2018) and are particularly prevalent in the microbiomes of diverse animals Kim and Bae (2018); Shkoporov and Hill (2019).

Prophage induction results in bacterial lysis and can be mediated by various stressors, including antibiotics and inflammatory processes Allen et al. (2011); Banks et al. (2003); Diard et al. (2017); Fang et al. (2017); Garcia-Russell et al. (2009); Maiques et al. (2006); Nanda et al. (2015); Wang et al. (2010); Zhang et al. (2000). Because prophages can influence the phenotypes of their bacterial hosts, such as biofilm formation Nanda et al. (2015), understanding the impact of lysogens in animal microbiomes is becoming a research priority Fortier and Sekulovic (2013); Hu et al. (2021); Lin et al. (1999). In the gut, biofilms that associate with host mucus may benefit the host by enhancing epithelial barriers against pathogenic invasion Swidsinski et al. (2007). Integration of prophages into bacterial genomes may impart functional changes that could be important in surface colonization. For example, in *E. coli* K-12, integrating the Rac prophage into a tRNA thioltransferase region disrupts biofilm functions Liu et al. (2015); deleting this prophage decreases resistance to antibiotics, acids and

oxidative stress. Some of these traits may be affected by prophage-specific genes Wang et al. (2010). Prophages have also been shown to influence biofilm life cycles in Pseudomonas aeruginosa Rice et al. (2009).

Lysogenized *Shewanella* species colonize the gut of *Ciona robusta*, an ascidian collected in Southern California waters Dishaw et al. (2014b); Leigh et al. (2017) and referred to here as *Ciona*. Tunicates like *Ciona* are a subphylum (Tunicata) of chordates that are well-established invertebrate model systems for studies of animal development Chiba et al. (2004); Davidson (2007); Liu et al. (2006) and are now increasingly leveraged for gut immune and microbiome studies Liberti et al. (2021). Armed with only innate immunity, *Ciona* maintains stable gut bacterial and viral communities Dishaw et al. (2014b); Leigh et al. (2018) despite continuously filtering microbe-rich seawater. Previous efforts to define gut immunity in *Ciona* revealed the presence of a secreted immune effector, the variable immunoglobulin (V-Ig) domain-containing chitin-binding protein, or VCBP, that likely plays important roles in shaping the ecology of the gut microbiome by binding bacteria and fungi (as well as chitin-rich mucus) on opposing functional domains Dishaw et al. (2011); Liberti et al. (2019). VCBP-C is one of the best-studied VCBPs expressed in the stomach and intestines of *Ciona*, shown to bind bacteria *in vitro* and in the gut lumen Dishaw et al. (2016, 2011). Based on various *in vitro* and *in vivo* observations, it was proposed previously that VCBPs likely modulate bacterial settlement and/or biofilm formation Dishaw et al. (2016); Liberti et al. (2019, 2021). The potential influence of soluble immune effectors on host-bacterial-viral interactions is particularly interesting. However, the possibility that prophages may influence interactions between bacteria and secreted immune effectors like VCBPs remains to be explored.

Shewanella fidelis strain 3313 was isolated previously from the gut of *Ciona* and found to posses two inducible prophages, SfPat and SfMu Leigh et al. (2017). Furthermore, *in vitro* experiments demonstrated enhanced biofilm formation in *S. fidelis* 3313 in the presence of extracellular DNA (eDNA) that may originate from lytic phage activity Leigh et al. (2017). Other *Shewanella* species have previously demonstrated a link between phagemediated lysis and biofilm formation Gödeke et al. (2011). For example, in *S. oneidensis* strain MR-1, Mu, and Lambda prophages enhance biofilm formation via eDNA released during prophage-induced lysis, with genomic DNA likely serving as a scaffold for biofilms Gödeke et al. (2011). Similarly, the P2 prophage of *S. putrefaciens* strain W3-18-1 influences biofilm formation via spontaneous induction at low frequencies, resulting in cell lysis and contributing eDNA that can mediate biofilm formation Liu et al. (2019).

Here, we set out to isolate and characterize the influence of the prophage, SfPat, on its host, *S. fidelis* 3313. Since the last description Leigh et al. (2017), the genome of *S. fidelis* 3313 was improved by combining long-read and short-read sequencing, which resulted in improved resolution of the genomic landscape within and around the prophages. A homologous recombination-based deletion strategy was designed to generate a deletion mutant (i.e., knockout) of SfPat. We report that deletion of SfPat results in reduced bacterial motility and increased biofilm formation *in vitro*. These changes in bacterial traits and behaviors are associated with the expression of genes regulating important signaling molecules and a corresponding impact on host immune gene expression during gut colonization in *Ciona* juveniles. Gut colonization experiments in laboratory-reared *Ciona* juveniles comparing wild-type (WT) and SfPat prophage knockout (ΔSfPat) mutant strains demonstrate that SfPat influences gut colonization outcomes, e.g., niche preference and retention. These effects are influenced by host gene expression. The results reported herein reflect complex tripartite interactions among prophages, bacterial hosts, and animal immune systems.

Results

Sequence verification of prophage deletion mutant strains

Colony PCR and single primer extension sequencing were both used to validate the prophage deletion, using primers EDK81/82 for SfPat (**Table 2**)(**Figure 1** a). All recovered amplicon sizes were consistent with the predictions for SfPat deletion (**Figure 1** b). The SfPat deletion (ΔSfPat) strain was named JG3862 (**Table 1**). Genome sequencing of the deletion mutant strain did not reveal the significant introduction of additional mutations or DNA modifications (**Figure 1** b). The WT and ΔSfPat strains were then used for *in vitro* and *in vivo* experiments to understand the potential role of prophages in shaping *S. fidelis* 3313 colonization dynamics in the gut of *Ciona*.

Prophage deletion modulates biofilm formation and motility in *S. fidelis* 3313 *in vitro*

Deletion of SfPat from *S. fidelis* 3313 contributed to an overall increase in biofilm formation as quantified by crystal violet staining by over 14% compared to WT strains (**Figure 2** a). Conventionally, to form a biofilm, bacteria will settle and initiate stationary growth dynamics Watnick and Kolter (2000). We studied bacterial swimming on simple semi-solid media to determine if the prophage influenced swimming motility in *S. fidelis* 3313. Bacterial motility was measured by the spread diameter from a primary inoculation point after overnight incubation (**Figure 2** b). The WT strain demonstrated a mean diameter of 8.21 mm, while ΔSfPat resulted in a decrease in the mean diameter of 4.04 mm, demonstrating reduced motility.

The influence of a prophage colonization of the *Ciona* gut by *S. fidelis* 3313

Swimming and biofilm formation behaviors also depend on the modulation of cyclic-di-GMP in the bacteria. Thus, changes in the expression levels of four genes regulating cyclic-di-GMP (*pleD*, *pilZ*, chitinase, and *pdeB*) were measured by qPCR between WT and ΔSfPat. We aimed to identify regulators of the transition from sessile to motile behavior impacted by the deletion of SfPat. No significant change in the expression of the genes in bacteria recovered from 24-hour biofilms (*in vitro*) (**Figure 2** c and Sup fig s1a) was identified. However, when these strain variants were introduced to metamorphic stage 4 (MS4) *Ciona* for 24 hours, the RT-qPCR revealed significant changes in the expression of pdeB (**Figure 2** c and Sup fig s1a). The bacterial gene *pdeB* encodes a phosphodiesterase enzyme that degrades cyclic-di-GMP. By reducing cyclic-di-GMP, pdeB serves as a positive regulator of motility and a negative regulator of biofilm formation Chao et al. (2013). The *pdeB* expression was higher in the colonizing WT *S. fidelis* 3313 than the ΔSfPat mutant strain (**Figure 2c**).

Swimming and biofilm formation often facilitate bacterial colonization of a host. We investigated whether prophages could impact the ability of *S. fidelis* 3313 to colonize the *Ciona* gut. Colonization assays were performed on MS4 *Ciona* juveniles by exposing animals to WT or ΔSfPat strains, repeating the experiments six times to account for diverse genetic backgrounds (**Figure 3** a), that is, using gametes from distinct outbred adults.

After exposure to the bacterial strains, retention was estimated by recovering bacteria from animals and quantifying colony-forming units (cfu) at different time points. The ΔSfPat strain revealed a statistically insignificant 1.3-to-1.5-fold change in retention compared to the WT strain

Figure 1.

General prophage deletion scheme.

(a) Location of upstream, downstream, and flanking primers used in the deletion of SfPat, primer orientation shown with respect to the prophage. (b) Deletion of SfPat from *S. fidelis* 3313 identified after assembling Illumina (short-read) sequenced genomes and mapping onto the improved (short and long-read, PacBio, sequencing) WT genome. The figure illustrates SfPat deletion as revealed by subsequent Illumina sequencing. The solid gray areas on the SfPat genome indicate regions that share identity with the WT.

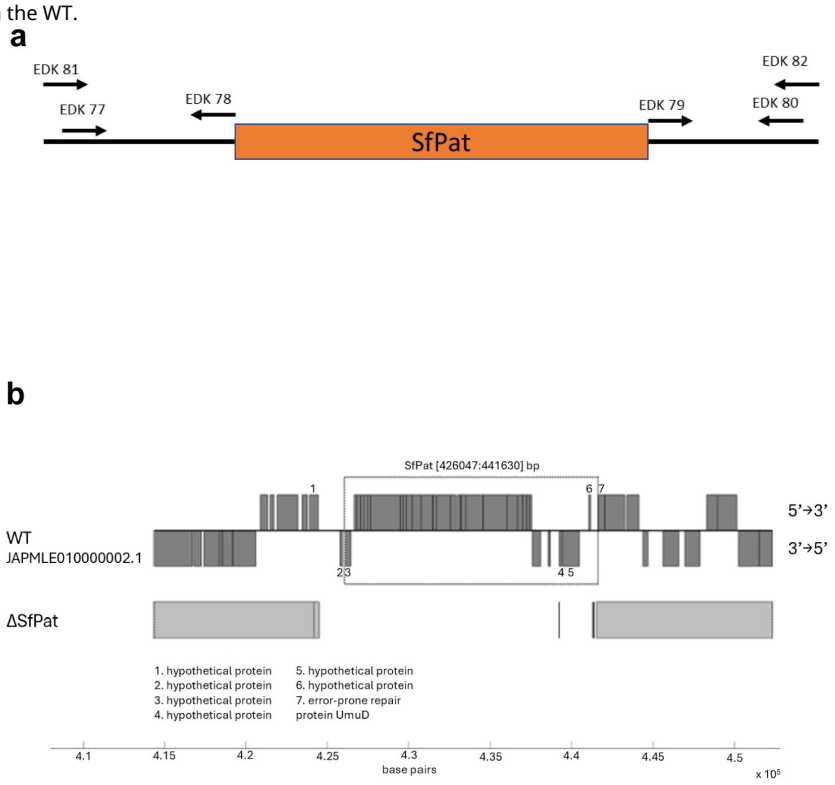

Table 1.

All *S. fidelis* 3313 strains are submitted under the BioProject PRJNA 90327 on NCBI, Accession: SAMN31793880 ID:31793880.

Organisms	Phenotype	NCBI RefSeq Assembly
JG4066	WT	GCF_033441085.1
JG3862	ΔSfPat	GCF_033441065.1

Primer ID	Sequence (5’-3’)
EDK77	AAATGGATCCCGATCAGCCTGCTAGTTTATT
EDK78	ACGGAATAGGTTGAATGCGACTCAGGC
EDK79	TCGCATTCAACCTATTCCGTCATGTTTAGCC
EDK80	ACATGAGCTCGATGCAGATAAAGAGCCGTAAA
EDK81	GTTTATTTTGTGGCAATCGCA
EDK82	GGTAGCAGTGCTTAAACGAT

Table 2.

Primer sequences on *S. flidelis* 3313 used for generating Δ SfPat deletion suicide vector and for deletion verification.

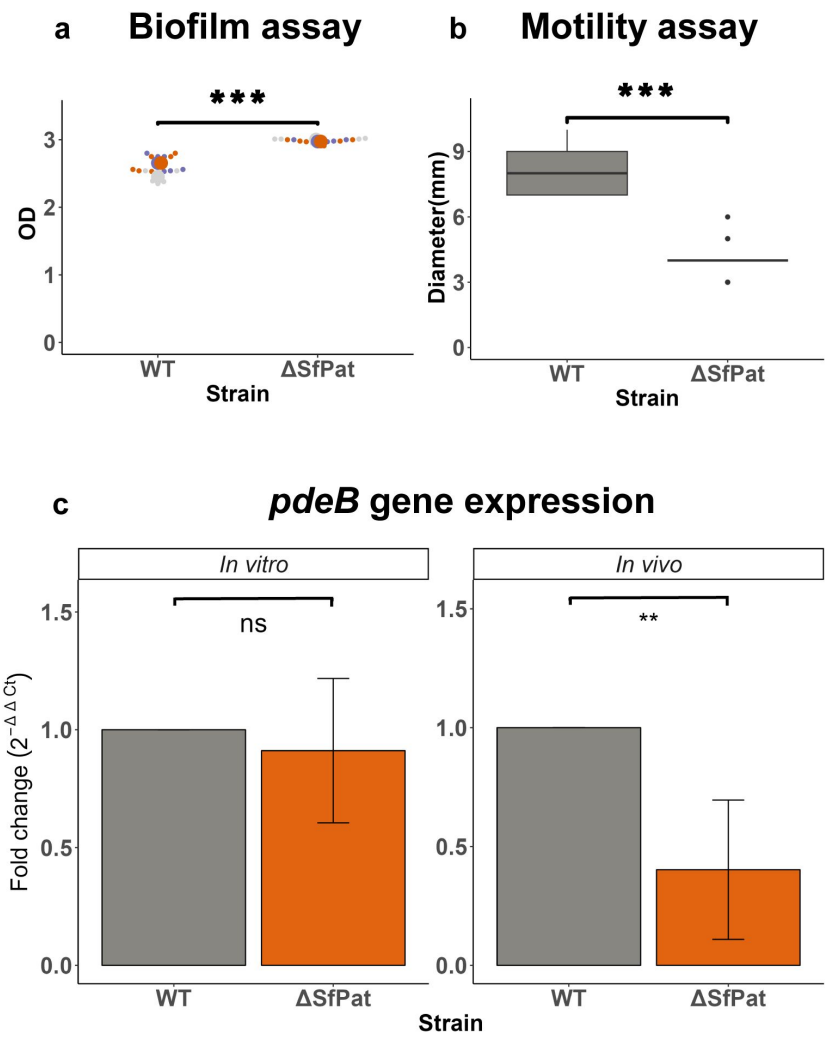

Figure 2.

Effects of prophages on biofilm and swimming in *S. fidelis* 3313.

(a) Influence of prophages on *in vitro* biofilm formation over 24 hours quantified with crystal violet assay (n=3), (b) role of prophages in swimming quantified as the diameter of spread on soft agar after 24-hours (n=6), and (c) fold-change of *pdeB* (with *Rho* as internal control) from 24-hour biofilm (*in vitro*) (n=4) and 24-hour *in vivo* (n=4). (*= p-value<0.05, **= p-value<0.01; ns= not significant).

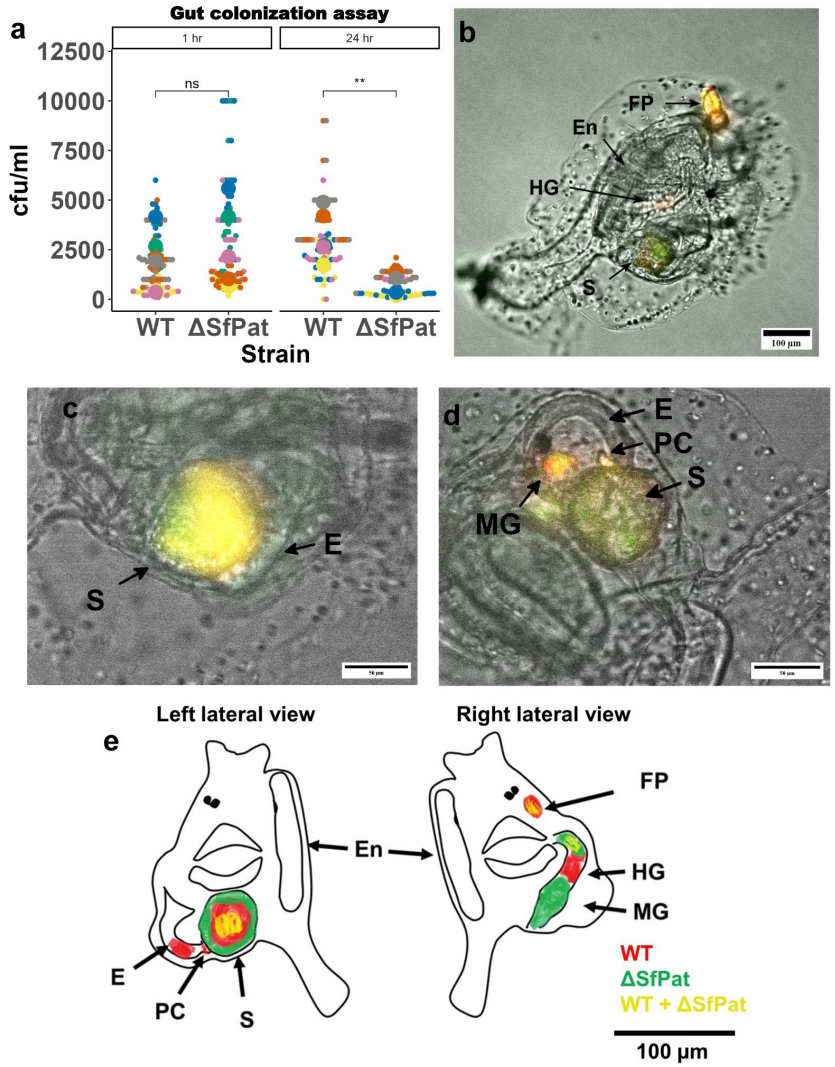

Figure 3.

The influence of SfPat prophage on gut colonization in *Ciona*.

(a) Results of six biological replicates (n=6, each replicate being a pool of ten juvenile tunicates) of the experimental exposure of *Ciona* MS4 juveniles to either WT or ΔSfPat strains for 1 hr and 24 hrs; retention quantification displayed as a Beeswarm plot of colony-forming units (CFUs). There is significant retention observed in WT after 24 hours. The MS4 juveniles reveal differential colonization of WT and ΔSfPat after one hour of exposure (b-e), where WT strain is stained with BacLight Red and ΔSfpat is stained with BacLight Green reveal (b) WT is seen localized in the lower esophagus to anterior stomach, while the ΔSfPat deletion strain localized to the hindgut, while (C) the WT is seen localized mostly as a fecal pellet in the center of the stomach while ΔSfPat prefers to localize to the stomach wall. (d) The WT strain is retained in the pyloric cecum. (e) Summary schematic of asymmetric bilaterial views of MS4 animals; top of image is anterior and stomach is posterior. The ventral side is the 'En' side, and the dorsal side is the opposite side. The findings can be summarized as such: WT is retained in E and S, in PC, and also in the HG, while the ΔSfPat is retained in the stomach folds, MG, and portions of the HG. Some overlap in signal is noted with yellow coloring. (En = Endostyle, E= Esophagus, S= Stomach, MG= Mid Gut, HG = Hind Gut, PC = Pyloric Cecum)

after 1 hour of bacterial exposure, a time point that mimics initial colonization (**Figure 3** a). However, 24 hours after exposure, WT was over two-fold retained in the gut than the ΔSfPat strain($p<0.05$) (**Figure 3** a).

To visualize the localization of WT and ΔSfPat mutant strains in the gut, juveniles of MS4 Ciona were exposed for one hour to BacLight Green-stained WT and BacLight Redstained ΔSfPat strain variants and vice versa (**Figure 3 c-e**). The one-hour time point reflects changes in the initial colonization of juveniles. These experiments revealed a differential localization to the stomach epithelial folds by the WT and ΔSfPat mutant strains. The WT strain typically prefers to occupy the pyloric cecum and the posterior portion of the esophagus and entrance into the stomach (**Figure 3d and e**, Supp Fig. s3a and s3b). Retention of the ΔSfPat mutant was noted at the walls of the stomach during co-exposure (**Figure 3c**). Retention of the ΔSfPat mutant was observed in the stomach and intestines, and less in the esophagus (Supp fig. s3 c and s3d). These studies suggest spatial and temporal differences in retention by differentially lysogenized strains of *S. fidelis* 3313.

Host immune discrimination and impact on lysogenized bacteria

Host immunity also plays an important role in shaping gut homeostasis. Distinct microbes and their antigens and/or metabolites can elicit host immune responses (Rooks and Garrett (2016)). To determine if the *Ciona* immune system discriminates among *S. fidelis* 3313 strains differing only in the presence or absence of the SfPat prophage, we examined the expression patterns of a secreted immune effector, VCBP-C, among juvenile MS4 during intestinal colonization. Under normal healthy conditions, VCBP-C is expressed and secreted by the gut epithelium and can bind (and opsonize) bacteria within the gut lumen (Dishaw et al. (2011)) and influence biofilms *in vitro* (Dishaw et al. (2016)). After one hour of exposure to the *S. fidelis* 3313 WT and mutant strains, changes were detected in the expression of VCBP-C. Up-regulation of VCBP-C was observed when juveniles were exposed to ΔSfPat mutant strains of *S. fidelis* 3313, compared to the WT strain (**Figure 4** a) by qPCR($p<0.05$). As VCBP-C is a major secreted effector of the gut regulating microbial settlement dynamics, the expression of other innate immune genes was evaluated after 24 hours of exposure to the strains and did not reveal statistically significant responses. However, the lack of statistically significant responses may also be attributed to host genetic diversity. i.e., differing responses to the same strains can obscure signal in transcript pools (**Figure 4** b).

Since the presence of SfPat influences host VCBP-C responses, we investigated whether the binding of VCBP-C to bacterial cell surfaces could influence prophage gene expression. *S. fidelis* 3313 was grown in MB *in vitro* for 24 hours in the presence or absence of 50μg/ml of recombinant VCBP-C(Dishaw et al. (2016)). The supernatant was discarded. RNA was extracted from the biofilms, and gene expression among SfPat open reading frames was monitored, as well as the SOS response regulators, *lexA* and *recA*, in *S. fidelis* 3313. Adherent cultures, or biofilms, were analyzed rather than the supernatant since preliminary experiments revealed that the growth of WT *S.fidelis* in stationary culrures is influenced by VCBP-C. Interaction of VCBP-C with the WT strain was found to suppress the expression of the structural phage protein P5 of SfPat (**Figure 5**). It was noted that VCBP-C did not significantly alter the expression of *lexA* and *recA*, indicators of the SOS pathway (**Figure 5**). This suggests that VCBP-C binding to the surface of the *S. fidelis* 3313 strain may not influence prophage structural genes via conventional SOS responses.

Discussion

In this report, we utilized a phage-deletion strategy to study the influence of a prophage (SfPat) on a gut symbiont (*S. fidelis* 3313) of the model invertebrate, *Ciona robusta*. Using RAST annotations and deduced amino acid BLAST (BLASTx), we propose that the SfPat is a distant relative of the PM2 prophage (Supplementary Table 1, Kivelä et al. (2002)). Deletion of the PM2-like SfPat prophage from *S. fidelis* 3313 revealed phage-mediated influence on colonization dynamics. We find that the

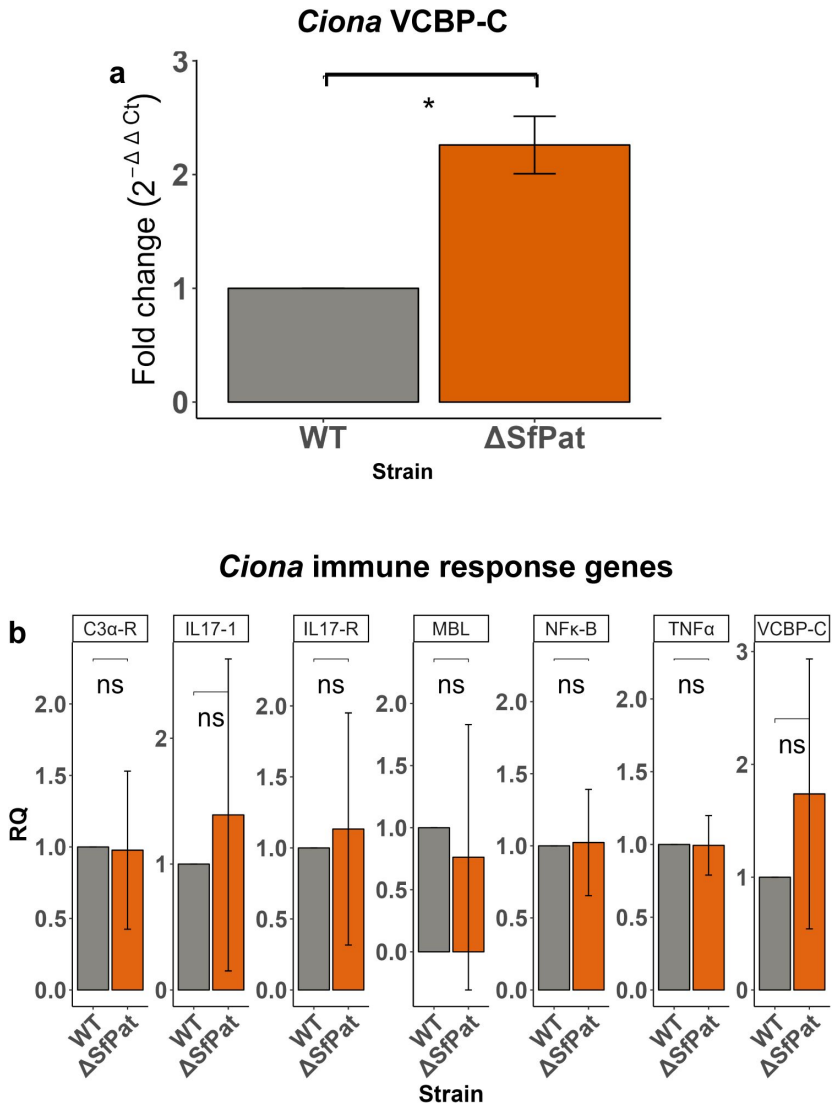

Figure 4.

The influence of prophages on host gene expression.

(a) VCBP-C gene expression in MS4 juveniles after one hour of exposure to *S. fidelis* 3313 strains (n=4), (b) Survey of additional innate immune gene expression in MS4 juveniles after 24-hour exposure to WT or ΔSfPat mutant strains (n=3). Actin is the internal control. (*= p-value<0.05, **= p-value<0.01, ns = not significant).

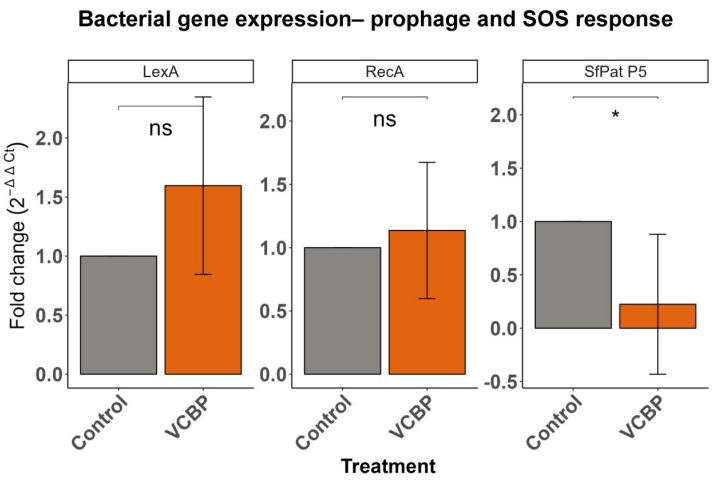

Figure 5.

Lysogen gene expression in response to host immune effector binding.

Gene expression of SfPat structural protein p5, *recA* and *lexA* of WT strain grown as a 24-hour biofilm while exposed to 50 μg/ml VCBP-C. Rho is the internal control (n=4). (*= p-value<0.05, ns = not significant).

deletion of the PM2-like prophage decreases swimming behaviors while increasing biofilm formation. These bacterial phenotypes influence host immune responses in ways that may influence differential retention in the gut if *Ciona*. Our identification of a prophage that interferes with biofilm formation in a *Shewanella* strain contrasts with reports demonstrating an increase in biofilms resulting from the incorporation of exogenous DNA following cell lysis (Gödeke et al. (2011); Liu et al. (2019)). Our study is the first to implicate prophage-induced change in motility for *Shewanella* spp, and it supports the reasoning that prophages can impact their hosts by modulating traits via diverse mechanisms. An influence on motility has also been linked to a prophage in *Pseudomonas aeruginosa* (Tsao et al. (2018)).

SfPat in *S. fidelis* 3313 appears to have a variable influence on bacterial retention in the gut of *Ciona*. As feeding is initiated in MS4 *Ciona* juveniles, food (or bacteria in the environment, natural or artificially introduced) accumulates in the gastrointestinal tract and on average takes about 45 minutes to begin exiting the anus and atrial siphon as fecal pellets. Thus, we monitored and compared transit and retention of introduced bacteria in the gut of MS4 juveniles at one and 24 hrs after introduction. SfPat deletion reveals a prophage-mediated influence on gut retention and localization. For example, within one hour of exposure, fecal pellets begin to form in the stomach that are enriched for the WT *S. fidelis* 3313; however, the WT strain then appears to be retained in the posterior portion of the esophagus (just before the entrance into the stomach) as well as in the pyloric cecum (which is a small outpouching just ventral and posterior to the stomach). Instead, the ΔSfPat mutant strain appears to be retained in the stomach folds and the intestines, and not the esophagus. It should be noted that the base of the stomach folds is where secretion of VCBP-C occurs Dishaw et al. (2016, 2011); Liberti et al. (2015). The overall retention of the two strains did not vary significantly within the first hour of colonization. However, after 24hrs, the retention of the WT strain was significantly greater than that of ΔSfPat despite the continued feeding behavior of the MS4 juveniles. The increase in pdeB activity of the WT *in vivo* (**Figure 2**) might indicate that the secondary messenger, cyclic-di-GMP, may be reduced in the WT, possibly leading to increased biofilm formation and reduced motility(Jenal and Malone (2006)). Thus, the presence of SfPat influences niche preference and retention.

In addition to host genetics obscuring the influence of prophages on colonization of the gut, other biophysical factors that include host immune effectors play crucial and often silent roles in influencing bacterial settlement dynamics. For example, human secretory immunoglobulin A (SIgA) has been shown to enhance and often favor settlement of bacteria both *in vitro* and *in vivo* (Bollinger et al. (2006); Thomas and Parker (2010); Pratt and Kolter (1998); Donaldson et al. (2018)), raising a basic question as to whether this phenomenon is more widespread among other secretory immune effectors present in mucosal environments of animals (Dishaw et al. (2014a)). We speculate that while prophages likely impact the behavior of lysogenized bacteria in ways that can influence colonization dynamics, interaction with VCBP-C on the mucosal surface of the *Ciona* gut likely further influences settlement behaviors (Dishaw et al. (2016, 2014a)). Importantly, we show here that the influence of the SfPat prophage on bacterial physiology (the WT strains) leads to a reduced expression of *Ciona* VCBP-C in the first hours of bacterial retention in the gut (an indicator that the host has detected the exposure). It remains to be shown if prophages stimulate the production of a bacterial metabolite with immunomodulatory properties or if the host immune system responds to differences in bacterial behaviors or traits, as suggested in the ΔSfPat deletion mutant.

Metagenomic sequencing of gut microbes from healthy humans has revealed that temperate lifestyles are prevalent among phages from these ecosystems (Minot et al. (2011, 2013); Reyes et al. (2010)), an observation also made in the *Ciona* gut (Leigh et al. (2018)). Various environmental triggers, such as UV light and mutagenic agents like mitomycin C, have been shown to induce a switch from the temperate to lytic cycle via the SOS response, a cell-wide response to DNA damage that can promote survival (Weinbauer and Suttle (1999)). Since, VCBP-C is an immune molecule in the gut that can interact with bacteria, it could influence prophage induction.

However, we find that VCBP-C binding on the surface of WT *S. fidelis* 3313 leads to a reduction in the expression of an important SfPat structural protein P5, suggesting a limitation in SfPat induction in the presence of VCBP-C. No significant changes in lexA/recA expression were observed upon VCBP-C exposure/binding, suggesting a lack of SOS response when exposed to this immune effector. The various mechanisms by which prophages shape colonization behaviors among gut bacteria of animals remains unclear. While the data reported here are only based on one bacterial strain that colonizes the *Ciona* gut, we find that WT *S. fidelis* colonizes the gut with reduced activation of VCBP-C gene expression compared to ΔSfPat, a trait that may be important in shaping colonization outcomes. We speculate that these observations are more widely applicable since lysogens are so abundant in animal microbiomes. Under normal conditions, VCBP-C protein is present in copious amounts and tethered to chitin-rich mucus lining the gut, as revealed by immunohistochemical staining (Dishaw et al. (2016)). Therefore, overexpression of VCBP-C is not necessarily helpful, and can correspond to the induction of additional inflammatory responses, including an overproduction of mucus. Thus, regulation of the production of additional VCBP-C likely serves important roles in influencing colonization dynamics.

Since colonization of animal mucosal surfaces is an ancient process (Dishaw et al. (2014b)), prophages and their integration into bacterial genomes have likely evolved to provide fitness benefits in often challenging environments like the gut lumen. Determining the role of animal immunity and prophages in these exchanges is of broad interest. Immune effectors like VCBPs, which undoubtedly possess broad specificities, can bind a range of bacterial hosts; however, it remains to be shown if they bind lysogenized bacteria with different affinities than their prophage-free counterparts. Prophages can also be induced to generate lytic particles that can influence gut microbiome structure, serving as an indirect form of protection for the host Wang et al. (2010); Barr et al. (2013). Prophages can also contribute to the transfer of virulence factors (Nanda et al. (2015); Wagner and Waldor (2002)). Retention of lysogens may be preferred if the prophages provide competitive fitness and retention in the gut. Since lysogens are integral in animal development, immunity, and metabolism(Fraune and Bosch (2010)), there is a tripartite interplay required for survival, a snapshot of which is shown here.

Methods and Materials

Culture and growth conditions

S. fidelis 3313 used in this study was originally isolated from the gut of *Ciona robusta* obtained from Mission Bay, CA, USA, as previously described Leigh et al. (2017). The bacterium was cultured using Difco marine agar 2216 (MA) (Fisher Scientific, Hampton, NH) and marine broth (MB) at room temperature (22-24 ˚C). Subsequent genetic manipulations were performed on strains grown in LB/MB, which consists of a mixture of 75% LB (Lysogeny Broth (Luria), Fisher Scientific, Hampton, NH) and 25% MB. Strains are listed in **Table 1**.

Prophage deletion.

SfPat was targeted for deletion from *S. fidelis* 3313 using homologous recombination methods adapted from Saltikov and Newman (2003) to produce knockout mutant strains. First, a pSMV3 suicide vector Saltikov and Newman (2003) was designed with 700 bp regions corresponding to the upstream and downstream sequence of the prophage (**Table 2**). These flanking regions were amplified and ligated using overlap extension PCR, then directionally inserted into the vector with the restriction enzymes BamHI and SacI (**Table 3**) Bryksin (2010). Plasmid conjugation was then performed by inoculating a colony of *S. fidelis* 3313 into a culture of *E. coli* containing the desired suicide vector on an LB/MB agar plate for two hours followed by primary selection after 24 hours at RT on LB/MB + 100 μg/ml Kanamycin plates and a counter selection on LM/MB + 10%

Sucrose at RT. Merodiploids and deletion mutants were verified by PCR. Illumina sequencing (MiGS, University of Pittsburgh) confirmed the deletion of SfPat and the evaluation of any additional genetic changes or mutations (Figure s1a).

S. fidelis crystal violet biofilm assay

WT and ΔSfPat strains were cultured in MB overnight at RT and then diluted to 10^7 cfu/ml in MB. Cultures were brought to a final volume of 2 ml of MB in 12-well dishes and incubated at RT for 24 hours to examine biofilm development. Each variable was tested in technical duplicate. The biofilms were quantified by crystal violet staining as previously described Liberti et al. (2022). Briefly, the supernatants were aspirated after 24 hours of incubation and the biofilms were dried and stained with 0.1% crystal violet for 10 min. The stained biofilms were then gently washed with deionized water and the amount of biofilm produced was quantified as the intensity of the stain ($\boldsymbol{OD}_{570}$) after the biofilmbound crystal violet was extracted from the biofilm with 30% acetic acid. All biofilm assays were performed at least in triplicates.

Motility assay

Soft-agar overlay motility assays were carried out in 12-well dishes to compare swimming behaviors Wolfe and Berg (1989); Kearns (2010). Briefly, a single colony from an overnight streaked LB/MB plate was picked using a sterile tooth pick and then stabbed onto the center of soft agar (containing LB/MB and 0.5% low-melt agarose) and incubated at RT overnight. The results were recorded as the distance traveled (in millimeters) by the bacteria from the inoculation zone. Each variable was tested in duplicate. Two perpendicular distances from the inoculation zone were recorded for each technical replicate and averaged for each well.

Ciona mariculture

The *in vivo* colonization experiments were performed on animals reared under conditions termed “semi-germ-free” (SGF), which include minimal exposure to marine microbes. SGF conditions include animals harvested under conventional approaches Cirino et al. (2002) but permanently maintained in 0.22 μm-filtered, conditioned artificial seawater (cASW), handled with gloves, and lids only carefully removed for water/media changes. cASW is prepared by conditioning ASW made with Instant Ocean^{TM} in an in-house sumpaquarium system containing live rock, growth lights, and sediment from San Diego, California; salinity is maintained at 32-34 parts per thousand (or grams per liter). Compared to germ-free Leigh et al. (2016) or SGF, conventionally-reared (CR) includes a step-up exposure to 0.7μm-filtered cASW that increases exposure to marine bacteria during development. The SGF approach is considered an intermediate method of rearing that includes minimal exposures to microbial signals during development (unpublished observations). The animals were reared at 20 ˚C from larval to juvenile stages. The *Ciona* were collected from Mission Bay, California in order to produce juvenile organisms for each biological replicate. These wild-harvested animals provide a wider genetic diversity compared to traditional model systems, where genetic diversity has been reduced or eliminated through controlled breeding practices.

Gut colonization assays

Both bacterial strains were grown overnight at RT in MB and diluted to 10^7 cfu/ml in cASW after repeated washes. Metamorphic stage 4 (MS4) animals reared in six-well dishes in cASW were exposed to 5 ml of 10^7 cfu/ml bacteria in each well for one hour or 24 hours. Co-exposure studies were also performed, where 2.5 mL (or 1:1) of each culture prepared above was mixed to form a total volume of 5 ml. MS4 animals are considered part of the 1st ascidian stages (post-settlement stages 1-6, whereas stages 7-8 and onwards are 2nd ascidian stages and reflect young adult animals). MS4 juveniles can be identified as having a pair of protostigmata, or gill slits, on each side of the animal (Chiba et al. (2004)). These juveniles first initiate feeding via newly developed

Plasmids/ Strains	Genotype	Source/ Reference
E. coli UQ950	*E. coli* DH5α λ(pir) host for cloning; F$^-$ Δ (argF$^-$ac)169 Φ 80dlacZ58(Δ M15) glnV44(AS) rfbD1 gyrA96(NalR) recA1 endA1 spoT1 thi$^-$1 hsdR17 deoR Λ pir $^+$	***Saltikov and Newman (2003)***
pSMV3−Δ SfPat	pSMV3 with 778 bp upstream and 779 bp downstream of flanking regions of SfPat	This study

Table 3.

Plasmids and strains used in the study.

and opened siphons; before this, the gut remains closed, and the interior lumen is unexposed to the outside world. Following this initial exposure or colonization, for various time intervals, the plates were rinsed multiple times with cASW and replaced with fresh cASW. Ten juveniles were chosen randomly for each treatment, pooled, and homogenized with a plastic pestle; live bacteria were counted by performing serial dilutions and enumerating colony-forming units (cfu) via spot-plating assays (Gaudy Jr et al. (1963); Miles et al. (1938)). Each graphed data represents a biological replicate dataset from genetically distinct/ diverse backgrounds of *Ciona* (represented by separate live animal collection and spawning events). Statistical significance was calculated using the Wilcoxon t-test by pooling data across six genetically diverse biological replicates.

Live bacteria in the gut were visualized using BacLight stains and previously described fluorescently labeled bacteria Moran et al. (2019). For BacLight staining, 1 ml of bacterial cultures were grown overnight at RT, pelleted, washed twice with cASW, and stained with 4 μl of BacLight Red (Invitrogen Cat no B35001) or BacLight Green (Invitrogen Cat no B35000) for 15 mins in the dark. The cultures were stained with alternate dyes in different replicates to get unbiased data from changes in fluorescence. The stained cultures were washed twice with cASW, and then diluted to 10^7 cfu/ml with cASW. MS4 animals were grown in 6-well dishes were then exposed to 5 ml of this culture. Bacteria in the gut of animals were visualized after one hour on a Leica DMI 6000B stereoscope with a CY5 fluorescent filter for BacLight Red and GFP filter for BacLight Green; and imaged and captured with a Hamamatsu ORCAII camera (model C10600-10B-H) and processed with the MetaMorph 7.10.4 imaging suite (Molecular Devices, Downingtown, PA).

Differential transcript level studies

To determine if the *Ciona* innate immune system can recognize and respond to unique mutant strains, which differ only in the presence or absence of prophages, candidate immune response markers were examined using reverse transcription quantitative PCR (RT-qPCR). RNA was extracted using the RNA XS kit (Macherey-Nagel, Cat no 740902) from MS4 *Ciona* juveniles exposed to either the WT or Δ SfPat strain. Complementary DNA (cDNA) synthesis was performed with oligo-dT primers and random hexamers using the First Strand cDNA Synthesis Kit (Promega Cat no A5000) following the manufacturer's instructions. The amplification was set with the qPCR kit (Promega Cat no A6000) and carried out on an ABI7500 with an initial melting temperature of 95˚C for 2 mins and 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The innate immune genes examined and their primers are reported in **Table 4**. Results from four distinct biological replicates are presented. Each replicate includes pooled *Ciona* juveniles from at least two wells of a 6-well dish. *Ciona* actin was referenced as an endogenous control. Data was analyzed using ΔΔCt method Pfaffl (2001) and the ABI7500 software suite. For ΔΔCt method, the Ct values were first normalized to an endogenous control gene and further normalized to the reference samples, here the WT strain.

To understand bacterial genes that are differentially regulated due to the presence or absence of prophages, transcript levels were studied *in vitro* and *in vivo*. The bacterial strains were grown in six-well dishes using the same methodology described for biofilm assays. To understand if the host immune molecule VCBP-C induced prophages, WT was cultured in six-well dishes as described in the biofilm section in the presence or absence of 50 μg/ml VCBP-C in Marine Broth Dishaw et al. (2016). After 24 hours, the supernatant was discarded for both experiments, and RNA from the biofilm was extracted using the Zymo Research Direct-zol kit. cDNA synthesis was performed using random hexamers as primers, and qPCR was conducted as described above. The targeted bacterial genes are described in (**Table 5**). Different housekeeping genes were examined for the different treatments, and the Ct values were used to identify the most stable reference gene to be used as an endogenous control. Rho was identified as the most stable reference gene using RefFinder, which utilizes Bestkeeper, GeNorm, Normfinder, and comparative ΔΔCt methods (**Figure 5—supplement 1**) Andersen et al. (2004); Pfaffl (2001); Vandesompele et al. (2002); Watnick and Kolter (2000).

Table 4.

***Ciona* genes targeted and the necessary reverse transcription-qPCR primers.**

Gene	Function	Genbank accession no.	Primer (5'-3')	Reference
VCBP-C-Fwd	Secreted immune effector in the gut	HQ324151	AGACCAACGCCAACACAGTA	*Liberti et al.* (*2018*)
VCBP-C-Rev			CCCCATACATTGCAGCATTTC	
Actin–Fwd	Cytoskeletal actin	AJ297725	CCCAAATCATGTTCGAAACC	*Liberti et al.* (*2018*)
Actin-Rev	**Reference gene**		ACACCATCACCACTGTCGAA	
IL17-1-Fwd	Interleukin 17	NM_001129875.1	AGGTTAAGAATCCCTATGGTGC	*Liberti et al.* (*2023*)
IL17-1-Rev	Effector cytokine		CAAAGGCACAGACGCAAAGG	
IL17-1R-Fwd IL17-1R-Rev	Interleukin 17 Receptor	NM_001245045	TGTTGGCATGAGTGTTCGGT AGTTGGTTCTGCCCCAAAGT	
NFκ-B-Fwd	Immune regulatory	NM_001078304	TGTCGCTTGTCGTCATGGAA	*Liberti et al.* (*2023*)
NFκ-B-Rev	Transcription factor		AACACCCAAGACCGTCGAAA	
TNFα-Fwd	Tumor Necrosis Factor	NM_001128107	TTCAGAAAGATTGGACGACGA	*Liberti et al.* (*2023*)
TNFα-Rev	Inflammatory effector		TCGTTTAGAAATGCTGCTGTGG	
C3A-R-Fwd C3A-R-Rev	Complement C3 Inflammatory mediator	NM_001078552	TTGTAAGCTGGCACAAGGTGT GACCGTAGTCTGGTAGAGGTC	This study
MBL-Fwd MBL-Rev	Mannose Binding Lectin	NM_001167707.2	TTATTGATGGGAAAGTTTGGT TAACATCTCTGTTCTTGGGTC	This study

Table 5.

***S. fidelis* 3313 genes targeted and the necessary reverse transcription-qPCR primers.**

Gene	Function	Genbank accession no.	Primer (5'-3')	Reference
pleD-Fwd	Regulates production of cyclic di-GMP	NZ_KI912459.1	CTCTTCACCGCCACTTCTT	This study
pleD-Rev			GGTGTGGTCTCTTATGCCTATC	
Chitinase-Fwd Chitinase-Rev	Chitin utilization gene	NF027718.3	CAGTGTAGCTAAGTCGTCATC CGCCAACCAGTGCTTTATTG	This study
pilZ-Fwd	Type IV Pilus control protein	NZ_JADX01000014.1	TGGCAAGGTCGTTTGGATTA	This study
pilZ-Rev			AGGCAAGCTCACTGGAAAG	
pdeB-Fwd pdeB-Rev	Phosphodiesterase	NF012772.3	GCATCAGGGCTCTTACCAATAG GAGGCGGTGATCCTTACAGATA	This study
recA-Fwd	Bacterial recombinase / Bacterial reference gene	NZ_KI912459.1	CGTAGTGGTGCGGTAGATGT	This study
recA-Rev			CGCATTGCTTGGCTCATCAT	
lexA-Fwd	Regulator of recombinase	NZ_JADX01000011.1	TGACCCAGCTATGTTCCGCC	This study
lexA-Rev			GCTCAACCTTGTGTACGGCG	
Rho-Fwd	**Bacterial reference gene**	NZ_JADX01000026.1	CACGTACAAGTTGCCGAAATG	This study
Rho-Rev			CAAGACGGGTGATAGAGTCAAG	
gyrB-Fwd	**Bacterial reference gene**	AM229309.2	TCACGAGCATCATCACCCGT	This study
gyrB-Rev			GGCTTCCGTGGTGCGTTAAC	

Statistical analysis and data visualization

Statistical analysis and data visualization were carried out in R, version 4.2 Team (2021). Data were plotted with ggplot 3.3.5 Kassambara (2020); the Beeswarm plot was constructed using ggbeeswarm 0.6 Eklund (2021). Beeswarm plots and statistics for motility assays were calculated using replicate averages Lord et al. (2020). Statistical significance was calculated using ggsignif package 0.6.3 or ggpubr0.4.0 Kassambara (2020); Constantin (2021). If the data were found to be normally distributed by Shapiro's test, then significance was calculated using an unpaired t-test. The Wilcoxon signed-rank test was used to calculate the significance of non-parametric data.

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Additional information

Author contributions

O.N. designed, executed, and analyzed experiments and wrote and edited the manuscript. S.L.G., N.P., C.G.F.A., F.N, A.L., and E.D.K performed experiments and provided feedback and approved the manuscript. M.N.Y., S.J.L., and B.A.L. performed genome sequence analysis, assembly, and provided feedback and approved the manuscript. M.B. and J.A.G. helped interpret data, provided feedback, and approved the manuscript. L.J.D. helped design experiments, analyze data, and helped write, edit, and approve the manuscript.

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Additional files

SfPat prophage annotations.

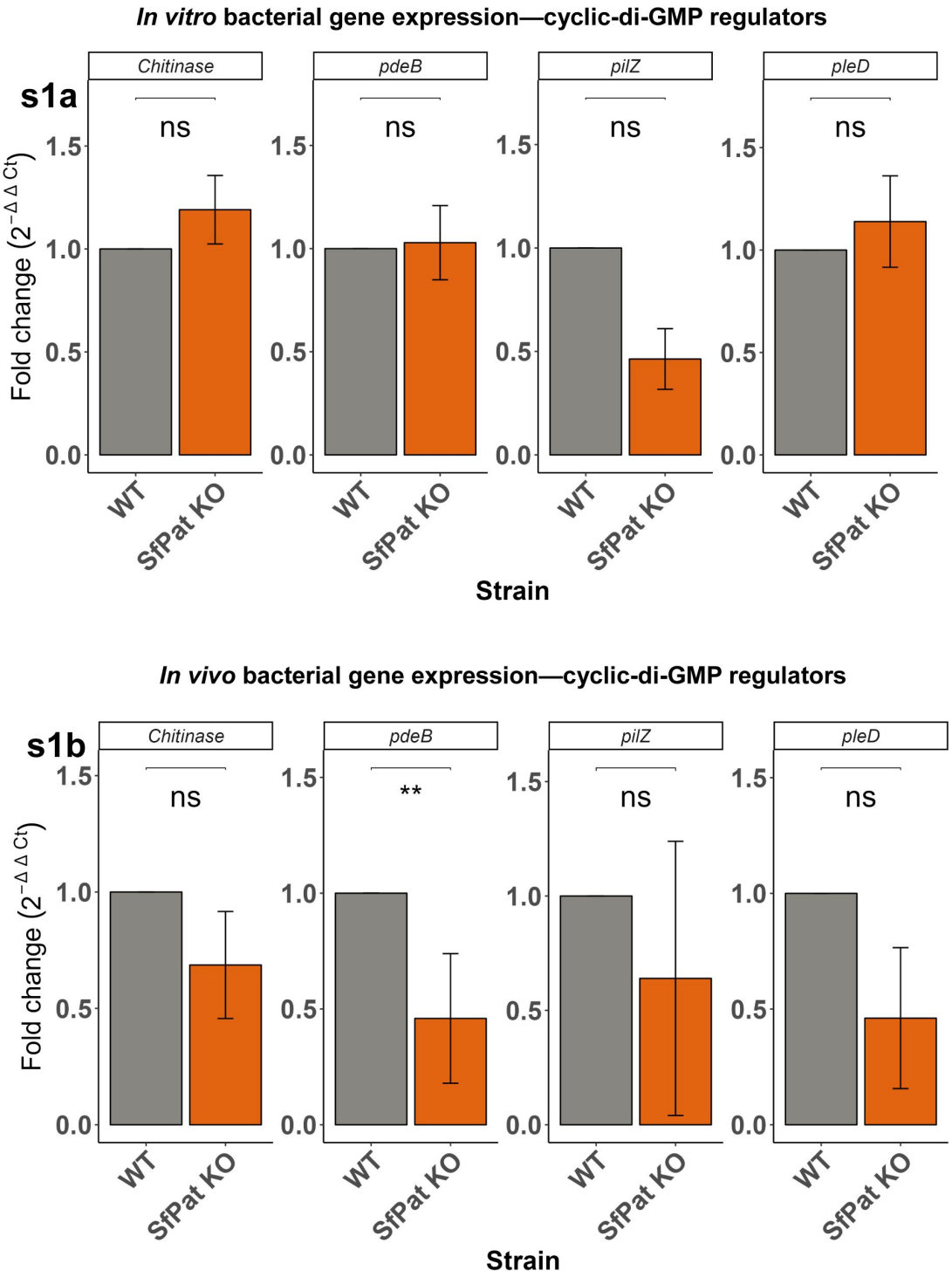

Figure 2—figure supplement 1.

qPCR showing relative fold change gene expression with Rho as an endogenous control.

(a) Cyclic-di-GMP regulators expression in WT and ΔSfPat *in vitro* after 24 hours of exposure (n=4), (b) Cyclic-di-GMP regulators expression in WT and ΔSfPat *in vivo* of *Ciona* MS4 after 24 hours of exposure (n=4). (ns= not significant)

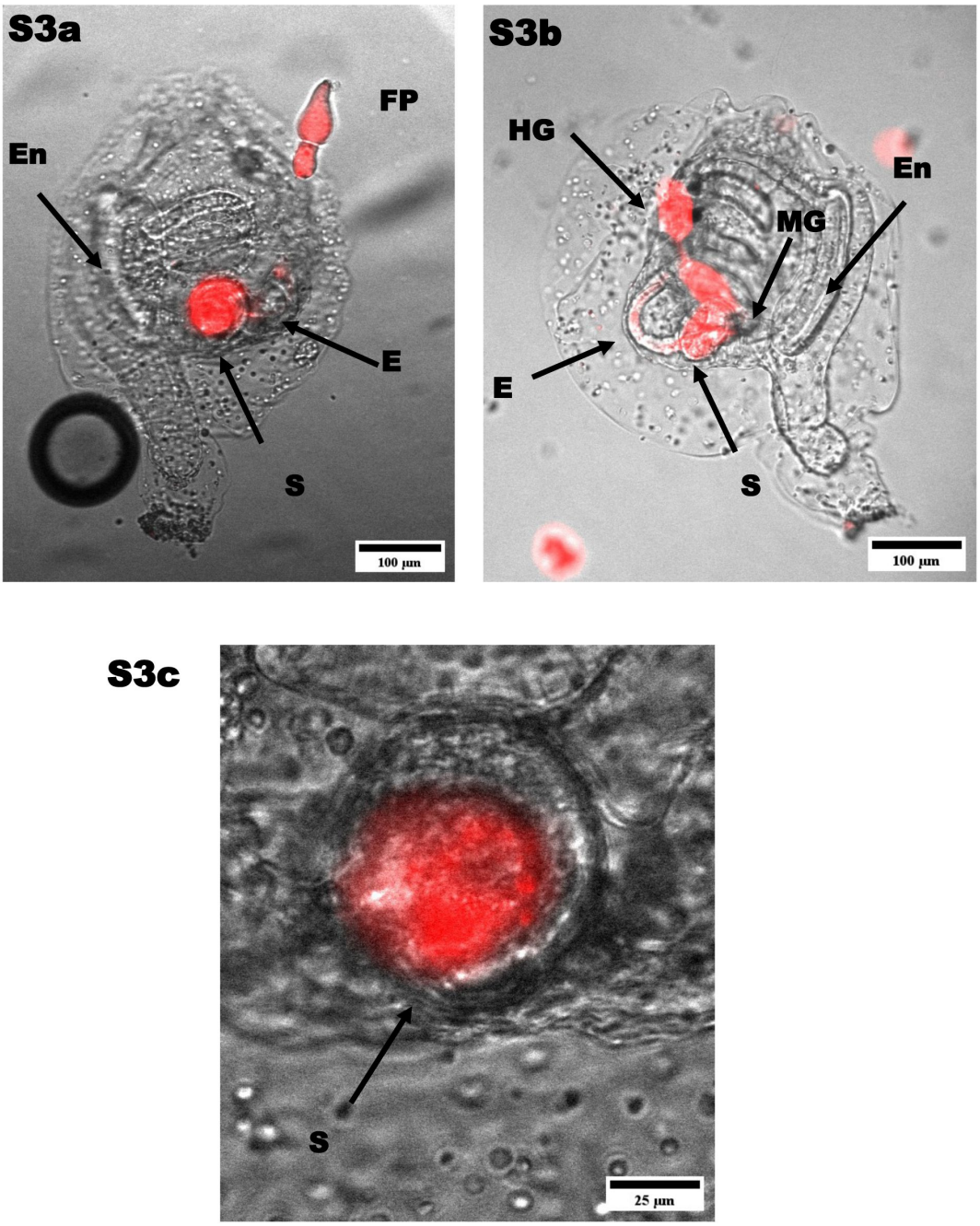

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Figure 3—figure supplement 1.

WT stained with BacLight Red exposed to *Ciona* MS4 for one hour.

(a) WT found in esophagus (E), stomach (S), and fecal pellet (FP). (b) WT also found to occupy the hind gut (HG). (c) WT is retained in the center of the stomach but not the stomach walls.

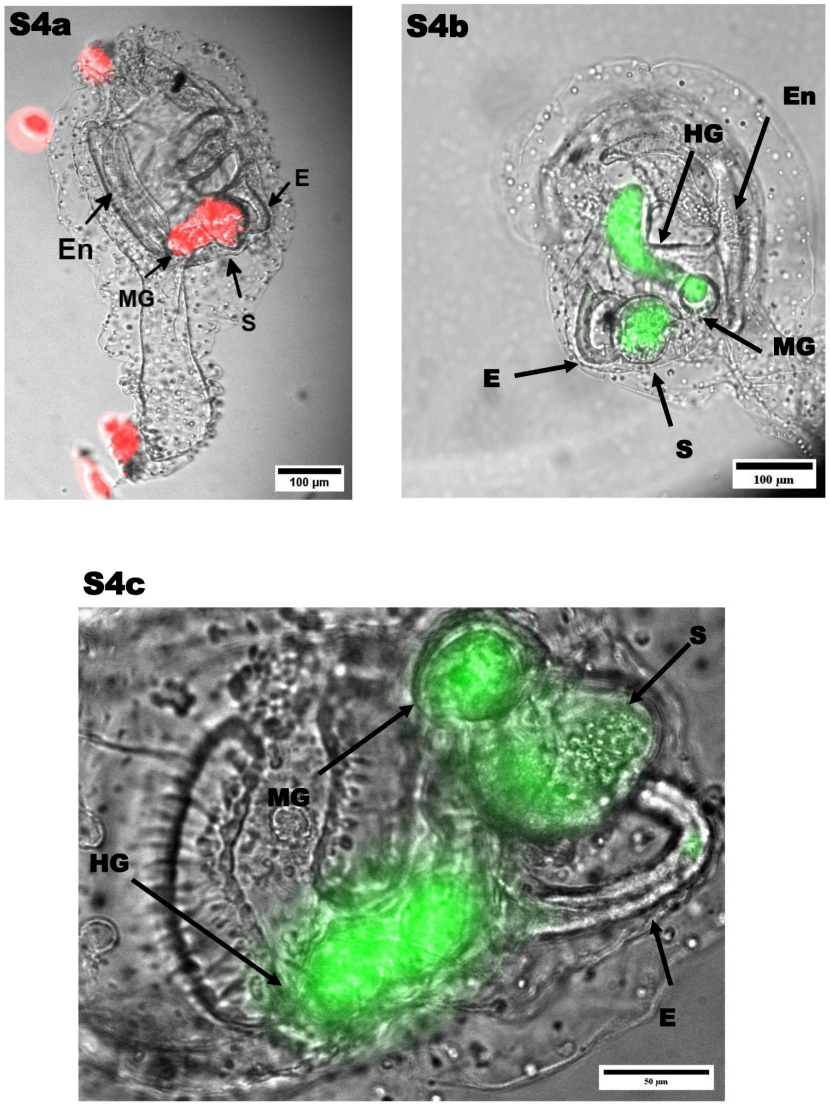

Figure 3—figure supplement 2.

ΔSfPat localization in *Ciona* MS4 after one hour exposure.

(a) Stained with BacLight Red, ΔSfPat is found adhered to the stomach (S) and mid gut (MG). (b) Stained with BacLight Green, ΔSfPat is found in the mid gut and hind gut (HG). (c) ΔSfPat is more localized in the stomach fold than the center of the stomach, with presence in the mid gut.

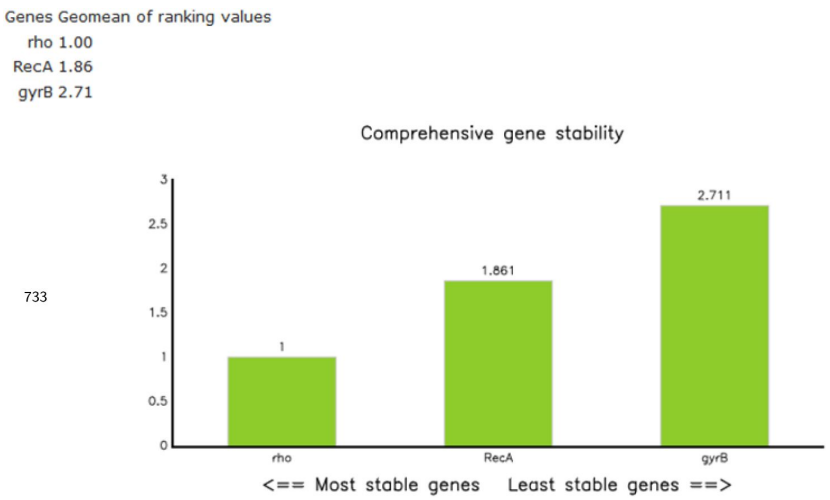

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Figure 5—figure supplement 1.

Rho is the most stable gene across strains when tested.

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Reviewer #1 (Public review):

Summary:

The manuscript aims to elucidate the impact of a prophage within the genome of Shewanella fidelis on its interaction with the marine tunicate Ciona robusta. The authors made a deletion mutant of S. fidelis that lacks one of its two prophages. This mutant exhibited an enhanced biofilm phenotype, as assessed through crystal violet staining, and showed reduced motility. The authors examined the effect of prophage deletion on several genes that could modulate cyclic-diGMP levels. While no significant changes were observed under in vitro conditions,

the gene for one protein potentially involved in cyclic-diGMP hydrolysis was overexpressed during microbe-host interactions. The mutant was retained more effectively within a one-hour timeframe, whereas the wild-type (WT) strain became more abundant after 24 hours. Fluorescence microscopy was used to visualize the localization patterns of the two strains, which appeared to differ. Additionally, a significant difference in the expression of one immune protein was noted after one hour, but this difference was not evident after 23 hours. An effect of VCBC-C addition on the expression of one prophage gene was also observed.

Strengths:

I appreciate how the authors integrate diverse expertise and methods to address questions regarding the impact of prophages on gut microbiome-host interactions. The chosen model system is appropriate, as it allows for high-throughput experimentation and the application of simple imaging techniques.

Weaknesses:

My primary concern is that the manuscript primarily describes observations without providing insight into the molecular mechanisms underlying the observed differences. It is particularly unclear how the presence of the prophage leads to the phenotypic changes related to bacterial physiology and host-microbe interactions. Which specific prophage genes are critical, or is the insertion at a specific site in the bacterial genome the key factor? While significant effects on bacterial physiology are reported under in vitro conditions, there is no clear attribution to particular enzymes or proteins. In contrast, when the system is expanded to include the tunicate, differences in the expression of a cyclic-diGMP hydrolase become apparent. Why do we not observe such differences under in vitro conditions, despite noting variations in biofilm formation and motility? Furthermore, given that the bacterial strain possesses two prophages, I am curious as to why the authors chose to target only one and not both.

Regarding the microbe-host interaction, it is not clear why the increased retention ability of the prophage deletion strain did not lead to greater cell retention after 24 hours, especially since no differences in the immune response were observed at that time point.

Concerning the methodological approach, I am puzzled as to why the authors opted for qPCR instead of transcriptomics or proteomics. The latter approaches could have provided a broader understanding of the prophage's impact on both the microbe and the host.

Comments on revisions:

While the authors were able to solve some of my issues, I see that other questions were not tackled.

https://doi.org/10.7554/eLife.103107.2.sa3

Reviewer #2 (Public review):

Summary:

In the manuscript, "Prophage regulation of Shewanella fidelis 3313 motility and biofilm formation: implications for gut colonization dynamics in Ciona robusta", the authors are experimentally investigating the idea that integrated viruses (prophages) within a bacterial colonizer of the host Ciona robusta affect both the colonizer and the host. They found a prophage within the Ciona robusta colonizing bacterium Shewanella fidelis 3313, which affected both the bacteria and host. This prophage does so by regulating the phosphodiesterase gene pdeB in the bacterium when the bacterium has colonized the host.

The prophage also regulates the activity of the host immune gene VCBP-C during early bacterial colonization. Prophage effects on both these genes affect the precise localization of the colonizing bacterium, motility of the bacterium, and bacterial biofilm formation on the host. Interestingly, VCBP-C expression also suppressed a prophage structural protein, creating a tripartite feedback loop in this symbiosis. This is exciting research that adds to the emerging body of evidence that prophages can have beneficial effects not only on their host bacteria but also on how that bacteria interacts in its environment. This study establishes the evolutionary conservation of this concept with intriguing implications of prophage effects on tripartite interactions.

Strengths:

This research effectively shows that a prophage within a bacterium colonizing a model ascidian affects both the bacterium and the host in vivo. These data establish the prophage effects on bacterial activity and expand these effects to the natural interactions within the host animal. The effects of the prophage through deletion on a suite of host genes are a strength, as shown by striking microscopy.

Weaknesses:

Unfortunately, global transcriptomics of the bacteria and the host during colonization by the prophage-containing and prophage-deleted bacteria (1 hour and 24 hours) would be suggested to better understand the tripartite interactions.

Impact:

The authors are correct to speculate that this research can have a significant impact on many animal microbiome studies, since bacterial lysogens are prevalent in most microbiomes. Screening for prophages, determining whether they are active, and "curing" the host bacteria of active prophages are effective tools for understanding the effects these mobile elements have on microbiomes. There are many potential effects of these elements in vivo, both positive and negative, this research is a good example of why this research should be explored.

Context:

The research area of prophage effects on host bacteria in vitro has been studied for decades, while these interactions in combination with animal hosts in vivo have been recent. The significance of this research shows that there could be divergent effects based on whether the study is conducted in vitro or in vivo. The in vivo results were striking. This is particularly so with the microscopy images. The benefit of using Ciona is that it has a translucent body which allows for following microbial localization. This is in contrast to mammalian studies where following microbial localization would either be difficult or near impossible.

Comments on revisions:

I am satisfied with the great amount of work that went into the comments provided by the reviewers. The figure presentations are more compelling for the story, and this latest revision is a very interesting read that should be considered for future microbiome studies.

https://doi.org/10.7554/eLife.103107.2.sa2

Reviewer #3 (Public review):

In this manuscript, Natarajan and colleagues report on the role of a prophage, termed SfPat, in the regulation of motility and biofilm formation by the marine bacterium Shewanella fidelis. The authors investigate the in vivo relevance of prophage carriage by studying the gut

occupation patterns of Shewanella fidelis wild-type and an isogenic SfPat- mutant derivative in a model organism, juveniles of the marine tunicate Ciona robusta. The role of bacterial prophages in regulating bacterial lifestyle adaptation and niche occupation is a relatively underexplored field, and efforts in this direction are appreciated.

Comments on revisions:

The authors have addressed my main concerns. While some responses remain somewhat ambiguous or defer key clarifications to future studies, I appreciate that not everything can be resolved within a single manuscript.

https://doi.org/10.7554/eLife.103107.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The manuscript aims to elucidate the impact of a prophage within the genome of Shewanella fidelis on its interaction with the marine tunicate Ciona robusta. The authors made a deletion mutant of S. fidelis that lacks one of its two prophages. This mutant exhibited an enhanced biofilm phenotype, as assessed through crystal violet staining, and showed reduced motility. The authors examined the effect of prophage deletion on several genes that could modulate cyclic-diGMP levels. While no significant changes were observed under in vitro conditions, the gene for one protein potentially involved in cyclic-diGMP hydrolysis was overexpressed during microbe-host interactions. The mutant was retained more effectively within a one-hour timeframe, whereas the wild-type (WT) strain became more abundant after 24 hours. Fluorescence microscopy was used to visualize the localization patterns of the two strains, which appeared to differ. Additionally, a significant difference in the expression of one immune protein was noted after one hour, but this difference was not evident after 23 hours. An effect of VCBC-C addition on the expression of one prophage gene was also observed.*
>
> *Strengths:*
>
> *I appreciate how the authors integrate diverse expertise and methods to address questions regarding the impact of prophages on gut microbiome-host interactions. The chosen model system is appropriate, as it allows for high-throughput experimentation and the application of simple imaging techniques.*
>
> *Weaknesses:*
>
> *My primary concern is that the manuscript primarily describes observations without providing insight into the molecular mechanisms underlying the observed differences. It is particularly unclear how the presence of the prophage leads to the phenotypic changes related to bacterial physiology and host-microbe interactions.*

We appreciate the overall, enthusiastic reviewer feedback. The current manuscript presents experimental evidence of the biological impact of the deletion of a stably integrated prophage in the genome of Shewanella fidelis 3313. The molecular mechanisms responsible for these biological effects are currently unknown but based on the limited genetic insight of some predicted gene regions, we can speculate on prophage-mediated influences impacting swimming behaviors. Below, we address additional concerns raised by the reviewer.

> *Which specific prophage genes are critical, or is the insertion at a specific site in the bacterial genome the key factor? While significant effects on bacterial physiology are reported under in vitro conditions, there is no clear attribution to particular enzymes or proteins.*

In this particular case, it is not entirely clear, as most ORFs within the prophage region have unknown functions, i.e., predicted as hypothetical proteins. In addition, the original insertion site does not appear to interrupt any specific gene but may impact adjacent genes/pathways (Fig 1b). Enhanced annotations, along with future targeted deletion methods for distinct prophage segments, will help us better investigate which predicted gene regions influence the observed traits. This will deepen our understanding of the mechanisms that regulate prophage influence on these traits.

> *In contrast, when the system is expanded to include the tunicate, differences in the expression of a cyclic-diGMP hydrolase become apparent. Why do we not observe such differences under in vitro conditions, despite noting variations in biofilm formation and motility? Furthermore, given that the bacterial strain possesses two prophages, I am curious as to why the authors chose to target only one and not both.*

Differences in expression patterns of c-di-GMP regulators were also noted in vitro, but they just missed the statistical significance threshold when rho was used as a bacterial reference gene. The expression pattern of pdeB was consistent among each biological replicate, however. In full transparency, pdeB qPCR was originally performed with recA as a reference standard (bioRxiv preprint, ver 1). Here, significant changes in pdeB expression were observed in the in vitro assays comparing WT and ΔSfPat. These results prompted us to study changes in pdeB expression during in vivo colonization experiments, which also revealed significant changes. However, there was a concern that a potential SOS response would also activate recA, despite our preliminary data suggesting SOS was not involved. As a precautionary, we repeated the experiments with rho as a reference gene after it was identified as a stable reference. However, with rho as a reference gene, statistically significant responses were noted during in vivo colonization, but not in the in vitro assays.

In the current manuscript, one prophage was targeted based on preliminary findings indicating that the SfPat prophage region influences behaviors likely to impact colonization of the Ciona robusta gut. A separate genetic segment was also previously targeted for deletion as a misidentified prophage-like region, but that strain is not included in the current description. The currently presented data indicate that the observed phenomena can be attributed to the SfPat prophage.

> *Regarding the microbe-host interaction, it is not clear why the increased retention ability of the prophage deletion strain did not lead to greater cell retention after 24 hours, especially since no differences in the immune response were observed at that time point.*

A predominantly adherent (non-motile) phenotype would likely facilitate elimination within fecal strings. There is substantial evidence from multiple model systems that strong swimming ability enhances the exploration and colonization of mucosal surfaces. Swimming helps with the penetration of mucus layers, chemotaxis toward epithelial surfaces, and overall “decision-making” in terms of shifting from a free-swimming (planktonic) state in the lumen within dietary material to a more sessile, adherent phenotype at the mucosal surface.

> *Concerning the methodological approach, I am puzzled as to why the authors opted for qPCR instead of transcriptomics or proteomics. The latter approaches could have*

> *provided a broader understanding of the prophage's impact on both the microbe and the host.*

We agree with the reviewer that a transcriptomics approach would provide a broader understanding of the prophage's impact on the microbe and animal host. Future studies will include a full multi-omic evaluation of this interaction.

> ***Reviewer #1 (Recommendations for the authors):***
>
> *Besides my above mentioned issues, I have a few more mini things:*
>
> *(A) what makes S. fidelis being a persistant member of the host microbiome? Please elaborate more on quantitive studies in this respect. –*

Shewanella species are stable members of the Ciona gut, and previous efforts (Dishaw et al, 2016) revealed that chitin and/or secreted host effectors could influence biofilm formation. The Ciona gut produces copious amounts of endogenous chitin-rich mucus, and a variety of bacteria have been identified that thrive under these conditions. In addition, versatile bacteria like Shewanella sp. likely expand the metabolic potential of filter-feeders like Ciona. Thus, our subsequent studies began to focus on these and other microbes isolated from the Ciona gut that appear to be stable residents. Identical strains have been recovered numerous times (since 2011) from this wild population of Ciona robusta.

> *(B) The authors use the word inter kingdom and refer to phage, bacterium and animal. As phages are not part of the three kingdoms of life I believe the terminology is wrong.*

Thank you for bringing this to our attention. In this context, we were referring to bacteria+phage as a unit and their interkingdom interaction with the animal host. But we recognize that this term can be misleading. Another, more appropriate term is 'tripartite,' and we have changed interkingdom to tripartite as appropriate, e.g., the abstract.

> *(C) I like lines 55-61 and was expecting to see in the manuscript what of those things would be true for the chosen prophage.*

We looked at the coding region annotations within the prophage and the adjacent regions. The prophage coding regions are mostly annotated as unknown or predicted proteins, and a few as known phage-related components. We intend to reanalyze future and improved annotations and conduct deletion experiments targeting specific open reading frames (ORFs).

> *(D) In line 76 the authors mention a Gödecke reference for Pseudomonas. I believe that this paper only deals with S. oneidensis.*

The inadvertent Gödecke reference has been removed.

> *(E) All figures: The captions are too short to understand what the figures are showing and everything is too small and hard to read or see. Along these lines it is often unclear what the many datapoints show. Biological replicates, technical replicates....Overall figure 1 does not seem to contain much information.*

Figures and captions have been improved as suggested. Thank you for bringing this to our attention.

> *(F) Figure 3 what are a and b showing?*

Figure and descriptive legend have been improved.

> *(G) Figure 4: Why did the author check expression only for one gene after 1 h but several genes after 24 h?*

Since we observed that in vitro VCBP-C alters biofilms of S. fidelis 3313 (Dishaw et al 2016), we hypothesized that the bacteria may alter host VCBP-C expression and that the influence of integrated prophages may further modulate gene expression. Since VCBP-C is endogenously expressed in the gut of Ciona, we expected that early exposure/colonization (one hour) would be crucial for the bacterial-VCBP interactions. Hence, the VCBP-C was our primary target. We then tested multiple immune response genes at 24 hours to get a more detailed understanding of the maturing immune responses. Future studies will expand our efforts using global transcriptomics to understand better the immune response during bacterial exposure and colonization events.

> *(H) Do the authors mean stationary or localised?*

We are not sure about the context of the reviewer's question here but we think our modifications have addressed these concerns.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *In the manuscript, "Prophage regulation of Shewanella fidelis 3313 motility and biofilm formation: implications for gut colonization dynamics in Ciona robusta", the authors are experimentally investigating the idea that integrated viruses (prophages) within a bacterial colonizer of the host Ciona robusta affect both the colonizer and the host. They found a prophage within the Ciona robusta colonizing bacterium Shewanella fidelis 3313, which affected both the bacteria and host. This prophage does so by regulating the phosphodiesterase gene pdeB in the bacterium when the bacterium has colonized the host. The prophage also regulates the activity of the host immune gene VCBP-C during early bacterial colonization. Prophage effects on both these genes affect the precise localization of the colonizing bacterium, motility of the bacterium, and bacterial biofilm formation on the host. Interestingly, VCBP-C expression also suppressed a prophage structural protein, creating a tripartite feedback loop in this symbiosis. This is exciting research that adds to the emerging body of evidence that prophages can have beneficial effects not only on their host bacteria but also on how that bacteria interacts in its environment. This study establishes the evolutionary conservation of this concept with intriguing implications of prophage effects on tripartite interactions.*
>
> *Strengths:*
>
> *This research effectively shows that a prophage within a bacterium colonizing a model ascidian affects both the bacterium and the host in vivo. These data establish the prophage effects on bacterial activity and expand these effects to the natural interactions within the host animal. The effects of the prophage through deletion on a suite of host genes are a strength, as shown by striking microscopy.*
>
> *Weaknesses:*
>
> *Unfortunately, there are abundant negative data that cast some limitations on the interpretation of the data. That is, examining specific gene expression has its limitations, which could be avoided by global transcriptomics of the bacteria and the host during colonization by the prophage-containing and prophage-deleted bacteria (1 hour and 24 hours). In this way, the tripartite interactions leading to mechanism could be better established.*

We thank the reviewer for their comments and recognize this important limitation. As a follow-up to the current study, we plan to perform more comprehensive global meta-transcriptomics analyses to better understand differentially expressed genes across both the host and microbe during colonization.

> *Impact:*
>
> *The authors are correct to speculate that this research can have a significant impact on many animal microbiome studies, since bacterial lysogens are prevalent in most microbiomes. Screening for prophages, determining whether they are active, and "curing" the host bacteria of active prophages are effective tools for understanding the effects these mobile elements have on microbiomes. There are many potential effects of these elements in vivo, both positive and negative, this research is a good example of why this research should be explored.*
>
> *Context:*
>
> *The research area of prophage effects on host bacteria in vitro has been studied for decades, while these interactions in combination with animal hosts in vivo have been recent. The significance of this research shows that there could be divergent effects based on whether the study is conducted in vitro or in vivo. The in vivo results were striking. This is particularly so with the microscopy images. The benefit of using Ciona is that it has a translucent body which allows for following microbial localization. This is in contrast to mammalian studies where following microbial localization would either be difficult or near impossible.*
>
> ***Reviewer #2 (Recommendations for the authors):***
>
> *In general, I found that the research shown in this manuscript is solid, and the manuscript is well-written. I have no specific comments about the writing of the manuscript that would be of benefit.*
>
> *Figure 1 would benefit from the shrinking of white space between panels a and b. Also, in panel b, it is very difficult to read the x-axis, the number of basepairs. It is suggested to increase this font size.*

Figure 1 has been improved as suggested.

> *Figure 2 is fine, however, what do three asterisks (***) in panel a signify? It is not described in the legend. One minor point that affects data understanding as presented, the wildtype (WT) change in expression is normalized to itself, therefore always equaling 1.0. This method of presentation muddies the variation in gene expression in the presence of the prophage. This is not an issue in Figure 2, but does have an effect on understanding Figure 2 - figure supplement 1.*
>
> *Figure 2 - figure supplement 1, as stated above, the normalization of the WT change in gene expression to 1.0 makes it difficult to understand the results. Why is pilZ change in gene expression not significant in panel s1a? It seems the median change is 50%, or whatever averaging is done, it's unclear whether this is the median and whether the error bars are standard deviation or some other metric.*
>
> *These should be defined in the statistical analysis section of the methods or in the legend itself. Further, in panel s1b, why is the reduction in gene expression of pdeB statistically significant, while a similar reduction in gene expression of pleD is not statistically significant?*

RQ values were calculated from 2^{-ddCt}. The error bars in the figures were calculated by adding or subtracting the standard error from RQ. Since WT was used as a reference value for qPCR, the RQ value was normalized as 1 for all replicates and nonparametric tests were used to calculate the statistical significance. The values for pilZ were very close to significant; a value of 0.063 was derived via the Wilcoxon test. Only the changes in expression of pdeB were determined to be statistically significant, via the Wilcoxon test.

> *Figure 3 panels a and b would be helped by having the same y-axis for each. It is impressive the amount of WT bacterial colonization takes place in 24 hours, particularly in the absence of the prophage, but it does not appear as impressive when the axes are changed between panels. Similar axes should be considered for every comparative graph.*
>
> *Figure 3 - figure supplement 1 legend would benefit from the same description of the animal's digestive locations as in the legend in Figure 3.*

We appreciate these suggestions and have made these changes accordingly. We have remade and combined Figure 3 a and b

> *Figure 4, while it is unfortunate that none of the immune genes evaluated had a response to the deletion of the SfPat prophage in S. fidelis 3313 at 24 hours, did any of these genes have an effect at 1 hour of evaluation as VCBP-C did?*

The expression of this expanded gene set was not evaluated at one hour. This time point will, however, be included in our global evaluation of gene expression in our future transcriptome sequencing effort.

> *Figure 5, the only question I have with these data is whether or not there is a dose-dependent effect of VCBP-C on SfPat P5 expression?*

Prior studies have found VCBP-C can impact biofilm formation in Shewanella sp. in a dose-dependent manner (some of the data appears in Dishaw et al, 2016). However, we have not yet considered whether VCBP-C impacts the expression of SfPat P5 (a phage capsid component) in a dose-dependent manner. We will consider this in future experimental designs.

> *It is mentioned in the introduction (and data shown in the preprint) that there is more than one active prophage in Shewanella fidelis 3313. The preprint data shows that the Mu prophages had little effect on the studies. It may be worth discussing the presence and lack of effects of these Mu prophages. It also may lead to some discussion about the complexities of polylysogeny (as discussed by Silpe, et al, Nature, 2023).*

A full-length, inducible, Mu-like prophage region has been identified in the genome that has not been targeted for deletion, but will be included in follow-up studies. An earlier incomplete genome assembly contributed to the incorrect targeting and deletion of a prior Mu-like region, which was discussed in an earlier preprint version. Discussion and references to that strain have been removed from the more recent preprint versions. For clarity, the current manuscript describes strains that remain focused on the SfPat prophage, noting its contribution to the observed behavioral changes / traits.

> *Is there any spontaneous induction of SfPat in vitro or in vivo with temperature change (prophages have been induced with heat stress), excessive UV exposure, or mitomycin C treatment?*

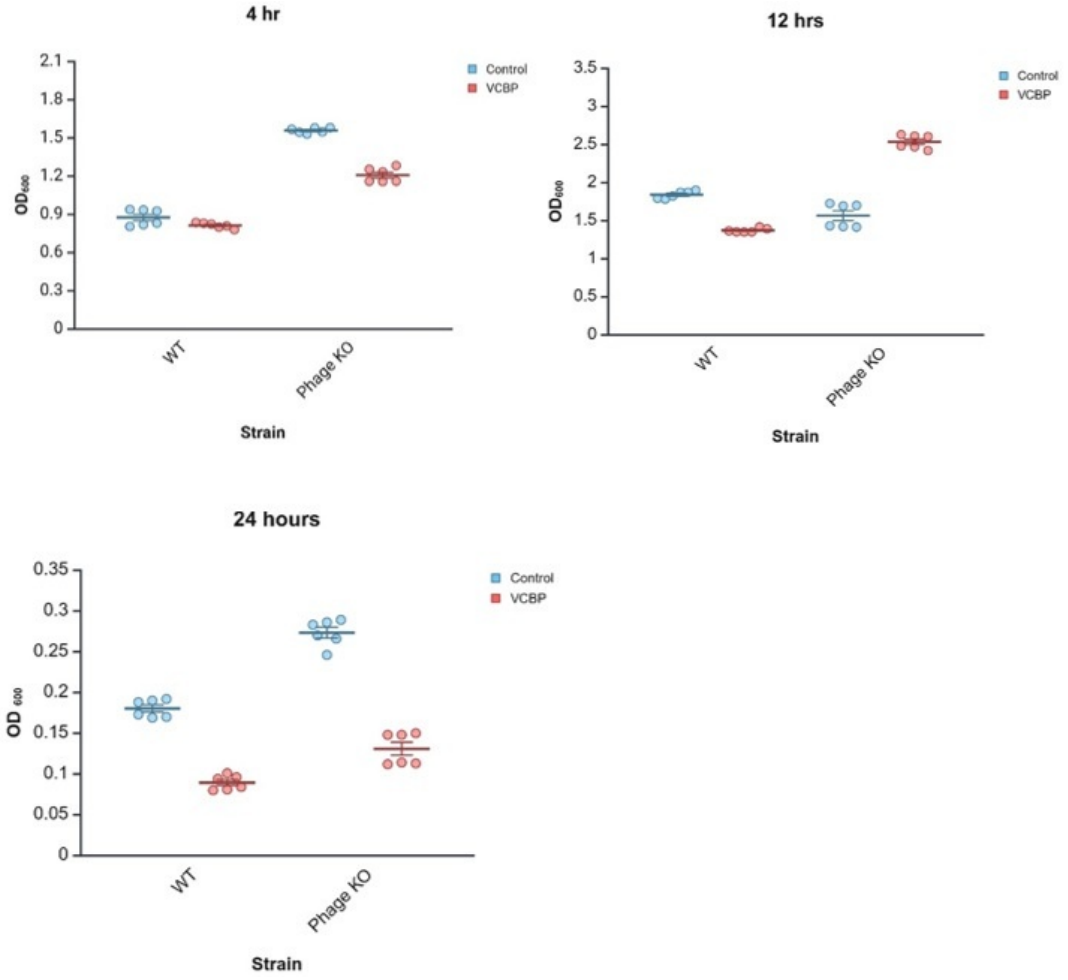
Preliminary induction studies using UV, mitomycin C, and temperature have been completed, but remain inconclusive with SfPat due to inconsistent induction patterns.

> *Could you speculate, or perhaps do the experiment, as to whether the addition of VCBP-C to S. fidelis 3313 cultures affects biofilm production? The deletion of SfPat leads to greater biofilm production in vitro, while exogenously added VCBP-C represses SfPat P5 expression, would VCPB-C addition lead to greater biofilm production? Lastly, and this may be a failure of my understanding, is VCBP-C able to bind to S. fidelis? If so, does the prophage alter the bacteria and, consequently, the ability of VCBP-C to bind to the bacteria?*

Our lab is actively working to better understand the physical interactions of VCBP-C and bacteria, particularly lysogenic bacteria. Deletion mutants are helping us better understand the potential influence of the bacterial accessory genome on interactions with host immune mediators. Biofilm assays have been done in the context of VCBP-C (Dishaw et al, 2016). Subsequently, we tested the influence of 50 μg/ml VCBP-C on WT and prophage KO-strains, which include SfPat KO along with neutral (control) regions of the genome. We found that the presence of VCBP-C reduced biofilm formation in WT and phage KO variants at 4 hrs and 24 hrs. However, at 12 hrs, VCBP-C treatment appears to increase biofilm formation in the phage-KO strain. While the role (if any) of SfMu is remains unclear, these preliminary data imply the existence of a feedback circuit (influenced by time) where immune effector binding and prophage influence on host gene expression together shape retention outcomes in the gut microbiome. This hypothesis remains to be tested further.

Author response image 1.

WT S. fidelis 3313 was exposed in vitro to 50 μg/ml VCBP-C in stationary cultures. Biofilms were observed for 24hrs. At 12 hrs, the presence of VCBP-C increased the amount of biofilms, whereas reduced biofilms were observed at 4 and 24hrs. Our findings (manuscript Fig 2a) reveal that SfPat contributes to biofilm formation, exposure to SfPat deletion mutants increases host VCBP-C expression (manuscript Fig. 4a), and VCBP-C binding to WT S. fidelis 3313 reduces the expression of SfPat P5 capsid protein (manuscript Fig. 5). These findings suggest that in vivo exposure/ colonization assays benefit from detailed time-course observations to be further explored in follow-up, future experiments.



Reviewer #3 (Public review):

In this manuscript, Natarajan and colleagues report on the role of a prophage, termed SfPat, in the regulation of motility and biofilm formation by the marine bacterium Shewanella fidelis. The authors investigate the in vivo relevance of prophage carriage by studying the gut occupation patterns of Shewanella fidelis wild-type and an isogenic SfPat- mutant derivative in a model organism, juveniles of the marine tunicate Ciona robusta. The role of bacterial prophages in regulating bacterial lifestyle adaptation and niche occupation is a relatively underexplored field, and efforts in this direction are appreciated.

While the research question is interesting, the work presented lacks clarity in its support for several major claims, and, at times, the authors do not adequately explain their data.

Major concerns:

(1) Prophage deletion renders the SfPat- mutant derivative substantially less motile and with a higher biofilm formation capacity than the WT (Fig. 2a-b). The authors claim the mutant is otherwise isogenic to the WT strain upon sequence comparison of draft genome sequences (I'll take the opportunity to comment here that GenBank accessions are preferable to BioSample accessions in Table 1). Even in the absence of secondary mutations, complementation is needed to validate functional associations (i.e., phenotype restoration). A strategy for this could be phage reintegration into the mutant strain (PMID: 19005496).

We are currently investigating complementation strategies. However, there have been some challenges in re-infecting and/or reintegrating the prophage into the genome. A preferred integration site may be damaged due to the deletion approach. While the SfPat prophage has mostly predicted genes of unknown function or significance, we have begun prioritizing the deletion of distinct segments to help identify functional relevance.

> *(2) The authors claim that the downshift in motility (concomitant with an upshift in biofilm formation) is likely mediated by the activity of c-di-GMP turnover proteins. Specifically, the authors point to the c-di-GMP-specific phosphodiesterase PdeB as a key mediator, after finding lower transcript levels for its coding gene in vivo (lines 148-151, Fig. 2c), and suggesting higher activity of this protein in live animals (!)(line 229). I have several concerns here:*
>
> *(2.1) Findings shown in Fig. 2a-b are in vitro, yet no altered transcript levels for pdeB were recorded (Fig. 2c). Why do the authors base their inferences only on in vivo data?*
>
> *(2.2) Somewhat altered transcript levels alone are insufficient for making associations, let alone solid statements. Often, the activity of c-di-GMP turnover proteins is local and/or depends on the activation of specific sensory modules - in the case of PdeB, a PAS domain and a periplasmic sensor domain (PMID: 35501424). This has not been explored in the manuscript, i.e., specific activation vs. global alterations of cellular c-di-GMP pools (or involvement of other proteins, please see below). Additional experiments are needed to confirm the involvement of PdeB. Gaining such mechanistic insights would greatly enhance the impact of this study.*
>
> *(2.3) What is the rationale behind selecting only four genes to probe the influence of the prophage on Ciona gut colonization by determining their transcript levels in vitro and in vivo? If the authors attribute the distinct behavior of the mutant to altered c-di-GMP homeostasis, as may be plausible, why did the authors choose those four genes specifically and not, for example, the many other c-di-GMP turnover protein-coding genes or c-di-GMP effectors present in the S. fidelis genome? This methodological approach seems inadequate to me, and the conclusions on the potential implication of PdeB are premature.*

We chose to study genes that were shown previously to influence biofilms and motility in a cyclic-di-GMP dependent manner in a Shewanella spp (Chao et al 2013, S Rakshe 2011). Future transcriptomic efforts and targeted deletion approaches will further define the specific influence of prophages.

> *(3) The behavior of the WT strain and the prophage deletion mutant is insufficiently characterized. For instance, how do the authors know that the higher retention capacity reported for the WT strain with respect to the mutant (Fig. 3b) is not merely a consequence of, e.g., a higher growth rate? It would be worth investigating this further, ideally under conditions reflecting the host environment.*

To clarify the method, in vitro growth curves did not suggest any significant difference in growth rate between the WT and the deletion mutant strains. Subsequently, for the in vivo experiments, bacterial cultures were pelleted and resuspended in sterile, nutrient-free artificial seawater. This limits growth until the bacterial strains are introduced to the animals.

> *(4) Related to the above, sometimes the authors refer to "retention" (e.g., line 162) and at other instances to "colonization" (e.g., line 161), or even adhesion (line 225). These are distinct processes. The authors have only tracked the presence of bacteria by*

> *fluorescence labeling; adhesion or colonization has not been assessed or demonstrated in vivo. Please revise.*

We thank the reviewer for this feedback; the manuscript has been revised accordingly. While we refer to our assays as 'colonization assays,' we report results of 'retention' of various bacterial strains in the 'exposed' animals. Furthermore, when fluorescent staining is utilized, we report retention in defined niches. Since colonization is likely a two-step process, i.e., 1) retention and 2) colonization or long-term establishment of these microbial communities, using these terms correctly is warranted. In separate (unpublished) surveys of adult animals taken from the field, identical strains have been recovered numerous times over a twelve-year period.

> *(5) The higher CFU numbers for the WT after 24 h (line 161) might also indicate a role of motility for successful niche occupation or dissemination in vivo. The authors could test this hypothesis by examining the behavior of, e.g., flagellar mutants in their in vivo model.*

Interestingly, we find numerous flagellar/motility-associated protein coding genes like Flg, Fli and Fle present within the S. fidelis genome possessing an EAL domain, implicating them in the regulation of cyclic-di-GMP. Hence, a future global transcriptomic approach will help improve our understanding of the roles of these regulatory pathways.

> *(6) The endpoint of experiments with a mixed WT-mutant inoculum (assumedly 1:1? Please specify) was set to 1 h, I assume because of the differences observed in CFU counts after 24 h. In vivo findings shown in Fig. 3c-e are, prima facie, somewhat contradictory. The authors report preferential occupation of the esophagus by the WT (line 223), which seems proficient from evidence shown in Fig. S3. Yet, there is marginal presence of the WT in the esophagus in experiments with a mixed inoculum (Fig. 3d) or none at all (Fig. 3e). Likewise, the authors claim preferential "adhesion to stomach folds" by the mutant strain (line 225), but this is not evident from Fig. 3e. In fact, the occupation patterns by the WT and mutant strain in the stomach in panel 3e appear to differ from what is shown in panel 3d. The same holds true for the claimed "preferential localization of the WT in the pyloric cecum," with Fig. 3d showing a yellow signal that indicates the coexistence of WT and mutant.*

The results section is reworded to improve clarity. The WT and KO are mixed 1:1 to achieve the 10^7 cfu count.

> *(7) In general, and especially for in vivo data, there is considerable variability that precludes drawing conclusions beyond mere trends. One could attribute such variability in vivo to the employed model organism (which is not germ-free), differences between individuals, and other factors. This should be discussed more openly in the main text and presented as a limitation of the study.*

Yes, a salient feature of this model is that we can leverage genetic diversity in our experimental design, but it can introduce experimental variability.

> *Even with such intrinsic factors affecting in vivo measurements, certain in vitro experiments, which are expected, in principle, to yield more reproducible results, also show high variability (e.g., Fig. 5). What do the authors attribute this variability to?*

For experiments involving VCBP-C protein, we can use affinity-purified protein recovered from live animals, or recombinant protein that we synthesize in-house (Dishaw et al 2011, 2016). In the latter, we often observe slight lot-to-lot variation in affinity for the target (the

bacterial surface). To account for this variation and to ensure the observations are robust despite it, production lots can be mixed in additional biological replicates. As such, slight variability in the in vitro assays can be due to this batch effect.

> *(8) Line 198-199: Why not look for potential prophage excision directly rather than relying on indirect, presumptive evidence based on qPCR?*

The decision to rely on qPCR of prophage structural genes was based on preliminary data, in particular among lysogens possessing more than one prophage. Neither the plaque assay nor SYBR Gold staining could distinguish among the particles, and TEM imaging was not sufficiently qualitative. Since these prophages do not exclusively produce particles when induced, qPCR targeting structural proteins was found to be most informative.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *Other major comments:*
>
> *Line 137 (and Fig. 2 legend): The authors did not test chemotaxis towards any specific chemoeffector, only motility. Please correct and see below my comments about motility assays.*

The reviewer is correct; we have modified our descriptors.

> *Lines 142-144: The authors conflate quorum sensing with c-di-GMP metabolism. If the authors measured the expression of genes "regulating cyclic di-GMP," it is likely because c-di-GMP is known to regulate the switch between planktonic and sessile lifestyles. However, whether this is mediated by quorum sensing is a separate issue that was not explored in this work. Please revise.*

Thank you; these changes were made accordingly.

> *Line 150: c-di-GMP is not a quorum sensing signal; please correct.*

Yes, we corrected the inadvertent yet misleading statement.

> *Line 193: Please clarify "RNA was extracted from the biofilms." If S. fidelis was grown on "MA [Marine Agar] for 24 h in the presence or absence of 50 µg/ml VCBP-C" (lines 192-193), was RNA isolated from colonies growing on the plates? Was VCBP-C added to the agar? This is also unclear in the Methods section (lines 381-384), where it seems the authors conducted this experiment using broth cultures in multiwell plates, removing the supernatant, and extracting RNA from the biofilms (i.e., cells adhered to the walls and bottom of the wells?). Why only biofilm cells?*

Thank you for bringing this to our attention. We have rewritten the appropriate sections and methods to improve clarity. Following our initial studies, which revealed differential bacterial phenotypes (biofilm formation and motility assays), we decided to target and investigate gene expression in the biofilms. This way, the sessile cells that were not part of the biofilm do not obfuscate the data.

> *Lines 204-205: The authors should refer to the behavior of the mutant, since they did not test what happens upon prophage integration, but after prophage deletion.*

The wording has been changed accordingly.

> *Lines 206-207: Please explain why the authors state that "these different bacterial phenotypes" (referring to altered biofilm formation and motility) "influence host immune responses in a manner consistent with influences on gut colonization dynamics". What specific relationship are the authors suggesting between these processes, and in what way is this "consistent"?*

We previously demonstrated (Dishaw et al 2016) that copious amounts of VCBP-C protein are present under normal conditions in the gut and mostly found tethered to chitin-rich mucus lining the gut epithelium. The up-regulation of VCBP-C within one hour of exposure to the SfPat mutant relative to the WT S. fidelis is consistent with a role for VCBP-C in modulating bacterial settlement dynamics (Dishaw et al 2016). The mutant phenotype of reduced swimming and increased biofilm production is a likely trigger for the increased production of this secreted immune effector that may influence the retention of this bacterial variant, relative to the WT.

> *Line 229: Apart from what I noted above about the authors' claim regarding PdeB activity, I believe the figure referred to here should be Fig. 2, not Fig. 5.*

Thank you for catching that oversight. It has been corrected.

> *Figure 1: Was hypothetical protein 2 included in the deletion?*

Yes, the hypothetical protein 2 was included in the deletion

> *Figure 3a-b: It is challenging to interpret data on plots using so many colors - including what appears to be a white circle (?) in Fig. 3a. How many replicates are represented here? Is it indeed n=3 in Fig. 3a and n=6 in Fig. 3b?*

Figure 3a is a bee swarm plot. Each color represents biological replicates, and the smaller circles represent technical replicates. It facilitates showing ALL the data, including the spread of the data. Regarding the number replicates, 3a and 3b are different experiments, with 3a representing a biofilm assay with three biological replicates and 3b a motility assay with six biological replicates.

> *Figure 3: An explanation for the abbreviation "FP" is missing.*

Thank you for catching this oversight. The abbreviation has been defined.

> *Figure S3: FP, which is proficiently occupied by the WT strain (Fig. S3a), is not labeled in the images provided for the mutant (Fig. S3c-d). It would be helpful to show it for comparison.*

Those other images did not have fecal pellets to label; however, Figure 3c does show a fecal pellet for an animal exposed to both WT and the SfPat mutant.

> *Questions and comments regarding methods:*
>
> *Lines 290-291, 307: Please indicate an approximate range for "room temperature."*

The information has been added to the revised manuscript.

> *Lines 292, 302: Why use hybrid LB/MB broth and agar? And strictly speaking, which LB formula (Lennox/Luria/Miller)?*

The hybrid broth reduces the concentration of salts that can interfere in some assays. The LB formula was Luria, and it is now included in the manuscript.

> *Lines 300-302: The conjugation procedure is poorly described. It seems the authors conducted conjugal transfer by biparental mating in broth culture by inoculating a single colony of S. fidelis 3313 into an already grown culture of the E. coli donor strain?*

The biparental mating was done on plates; the manuscript has been clarified.

> *Motility assay concerns:*
>
> *Swimming motility is generally assayed in soft agar (0.25-0.3% w/v). Why did the authors use 0.5% low-melt agarose? Usually, agar is employed instead of agarose, and such a high concentration of solidifying agent typically prevents proper swimming (see e.g. Kearns 2010).*

Our laboratory uses low-melt agarose for phage propagation and other assays. We continued using it because we observed robust and reproducible results in the swarming and swimming motility assays. In addition, 0.5% agarose is less dense than 0.5% agar, and its consistency is similar to that of the lower percentage soft agar.

> *Lines 316-317: Please clarify: what is the "overlay motility assay" that was carried out "overnight at RT and then inoculated onto the center of soft agar"? Was this a two-step experiment? How were bacteria inoculated (stabbed, injected)? If injected, what volume and cell density were used?*

Thank you for bringing this to our attention. The methods section has been revised for clarity.

> *Line 319: Each variable tested in duplicate? From what I understand, the only variable measured in this test is the diameter of the swimming halos. Do the authors mean they used two biological replicates? If so, please indicate the number of technical replicates as well.*

Multiple biological replicates were performed, each time with two technical replicates. Two perpendicular measurements (of diameter) for each technical replicate was recorded to avoid bias. The methods section has been edited to improve clarity.

> *Line 320: Were the swimming halos asymmetrical, hence the need to take two perpendicular measurements? If that was the case, it could indicate an excessive amount of solidifying agent.*

The halos were sometimes asymmetric, but to avoid variation across datasets, it became standard practice to measure perpendicular distances as stated above.

> *Regarding qPCR experiments:*
>
> *Please clarify how normalization of transcript levels was performed.*
>
> *It seems the authors conducted a double normalization, first with respect to the calibrator (rho), and again using the wild-type as a baseline reference for fold-change calculations (absence of error bars for WT data). If so, please specify on the vertical axes of the figures and in the Methods/figure legends.*
>
> *Since, in addition to rho, the authors assessed the expression stability of the "housekeeping" genes gyrB and recA, please also include the primers used for these*

> *genes.*

The appropriate manuscript sections have been updated for clarity. The bacterial qPCR was normalized to an internal standard, and then relative expression differences between SfPat and the WT were determined. The missing primer sequences have also been added.

> *Observations:*
>
> *Figure 2a-b: It is intriguing that the remarkable reduction in motility of the mutant is not associated with a comparably significant increase in biofilm formation.*

A statistically significant increase in biofilm was observed, along with a decrease in motility. As is common in crystal violet assays, some of the tertiary structures were not very stable and likely washed out during processing.

> *Additionally, it is noteworthy that data for the mutant in panel 2a exhibit minimal variability, with all OD570 recordings being around 3.0. Did the authors dilute the crystal violet elution solution after adding acetic acid, or might they have reached the saturation limit of the spectrophotometer?*

The eluted acetic acid was not diluted further, and significant changes were observed. If the solution had been further diluted, the observed changes might have been more pronounced.

> *Minor comments and recommendations:*

All the suggested changes below have been incorporated

• Line 55: "Antibiotic resistance determinants" might be preferable to "genes" to avoid using "genes" twice in the same sentence.

• Line 75-76: Italicize Pseudomonas aeruginosa.

• Line 134: Instead of "at least," specify the average fold-change.

• Line 141: In the heading, refer to the influence of the "prophage" (singular) rather than "prophages" (plural).

• Discussion (style): Consider using past tense for phrases like "we utilize..." (line 202); "we find..." (line 204), etc.

• Line 365 and elsewhere: Consider "mRNA levels" or "transcript levels" instead of "gene expression".

• Table 3: UQ950 is a strain, not a plasmid. I assume the plasmid carried by UQ950 is pSMV3.

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