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

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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***); ***Bolling 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 sur-

face 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 phage-mediated 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 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 Red-stained 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 eval-

uated 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 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 por-

tion 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.

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

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).

Table 2. Primer sequences on *S. flidelis* 3313 used for generating Δ SfPat deletion suicide vector and for deletion verification

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

Table 3. Plasmids and strains used in the study

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

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 incu-

bated 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 (OD_{570}) after the biofilm-bound 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™ in an in-house sump-aquarium 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 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 $\Delta\Delta$Ct method ***Pfaffl*** (***2001***) and the ABI7500 software suite. For $\Delta\Delta$Ct 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 $\Delta\Delta$Ct 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

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

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	Chitin utilization gene	NF027718.3	CAGTGTAGCTAAGTCGTCATC	This study
Chitinase-Rev			CGCCAACCAGTGCTTTATTG	
pilZ-Fwd	Type IV Pilus control protein	NZ_JADX01000014.1	TGGCAAGGTCGTTTGGATTA	This study
pilZ-Rev			AGGCAAGCTCACTGGAAAG	
pdeB-Fwd	Phosphodiesterase	NF012772.3	GCATCAGGGCTCTTACCAATAG	This study
pdeB-Rev			GAGGCGGTGATCCTTACAGATA	
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	

was used to calculate the significance of non-parametric data.

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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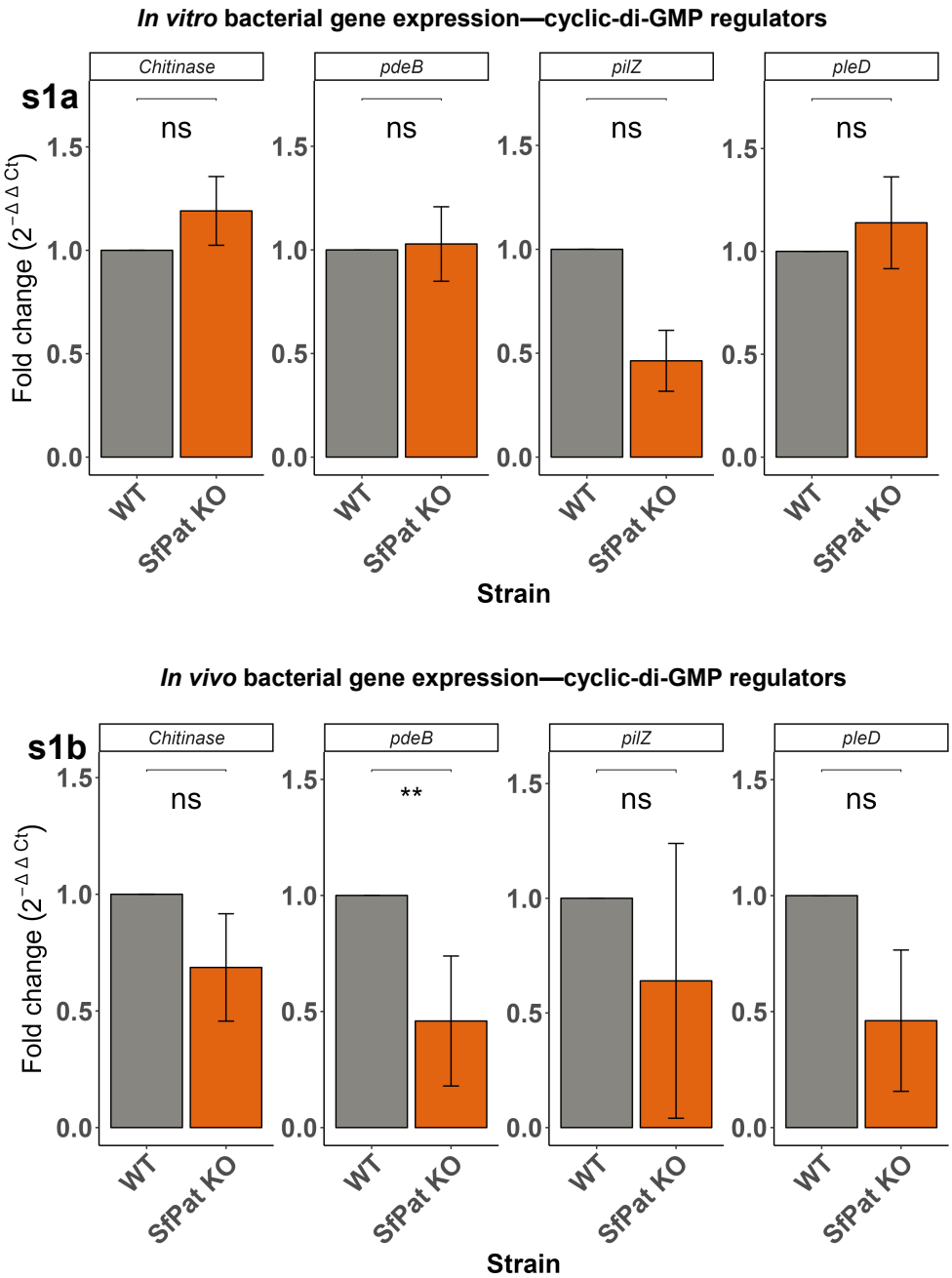

Figure 2—figure supplement 1. qPCR showing relative fold change gene expression with Rho as an endogenous control (a) Cyclic-di-GMP regulators expression in WT and ΔSfPat *in vitro* after 24 hours of exposure (n=4), (b) Cyclic-di-GMP regulators expression in WT and ΔSfPat *in vivo* of *Ciona* MS4 after 24 hours of exposure (n=4). (ns= not significant)

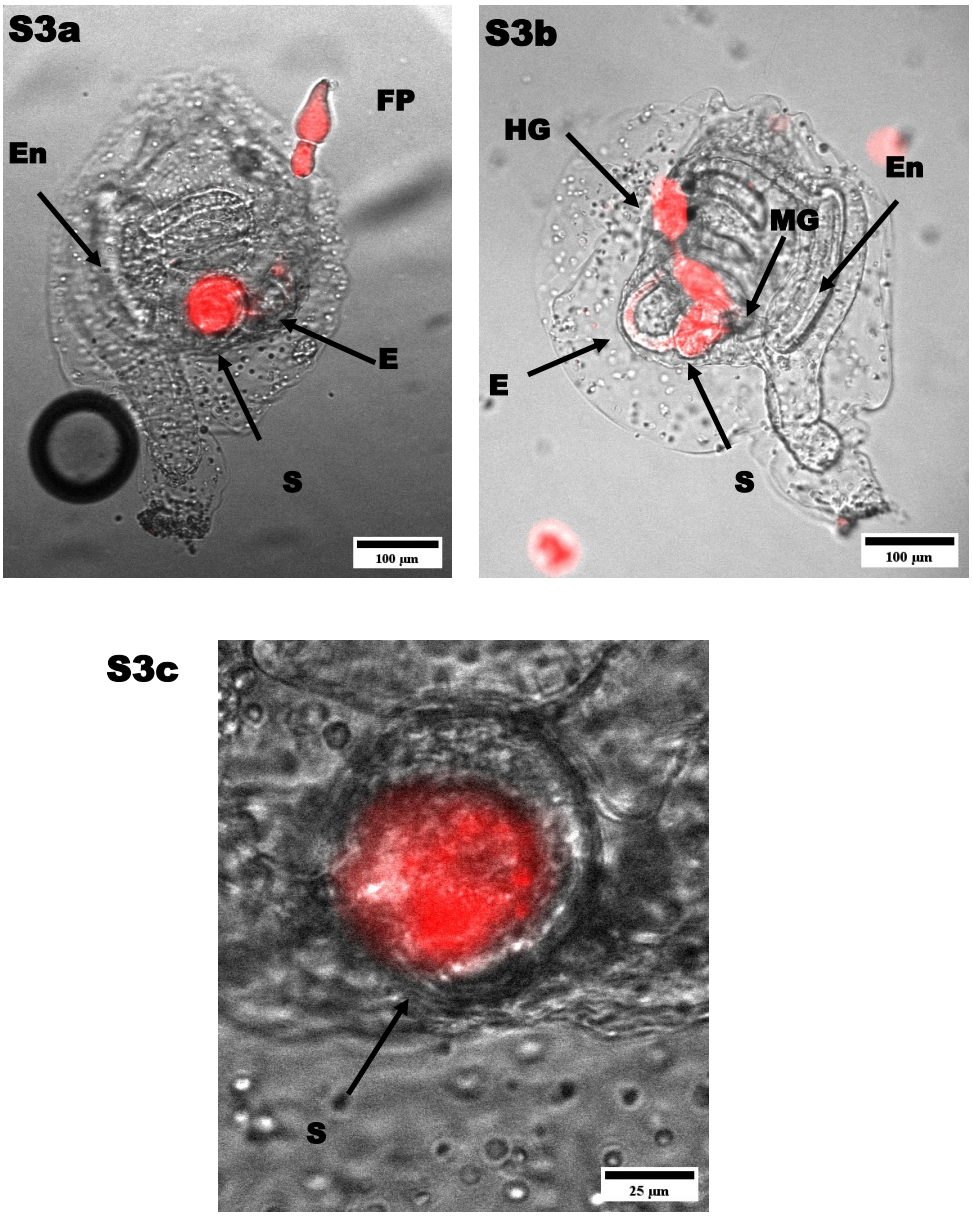

Figure 3—figure supplement 1. WT stained with BacLight Red exposed to *Ciona* MS4 for one hour. (a) WT found in esophagus (E), stomach (S), and fecal pellet (FP). (b) WT also found to occupy the hind gut (HG). (c) WT is retained in the center of the stomach but not the stomach walls.

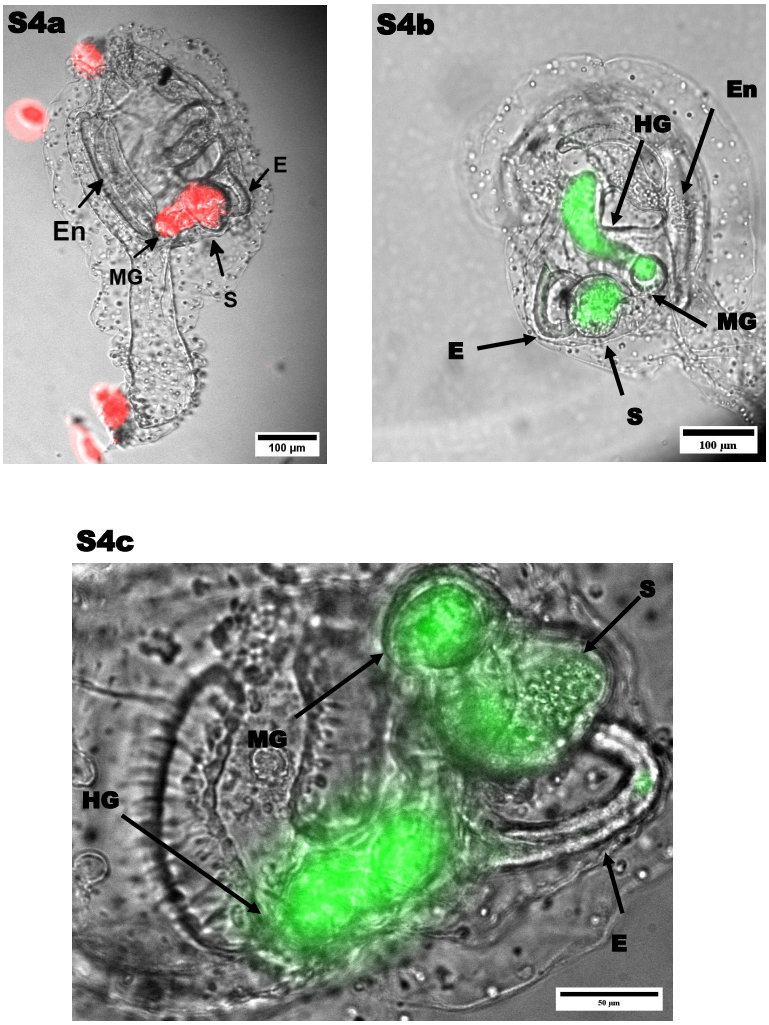

Figure 3—figure supplement 2. ΔSfPat localization in *Ciona* MS4 after one hour exposure. (a) Stained with BacLight Red, ΔSfPat is found adhered to the stomach (S) and mid gut (MG). (b) Stained with BacLight Green, ΔSfPat is found in the mid gut and hind gut (HG). (c) ΔSfPat is more localized in the stomach fold than the center of the stomach, with presence in the mid gut.

Genes Geomean of ranking values
rho 1.00
RecA 1.86
gyrB 2.71

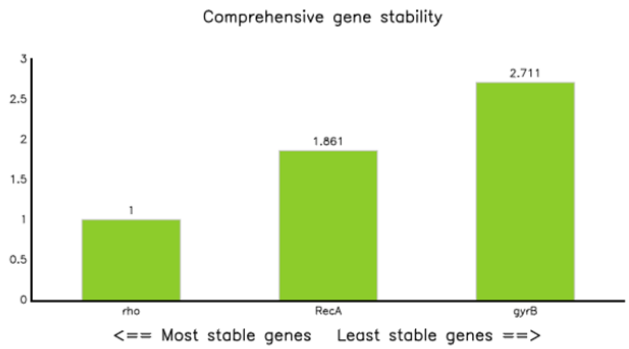

Figure 5—figure supplement 1. Rho is the most stable gene across strains when tested *in vitro* (n=3)

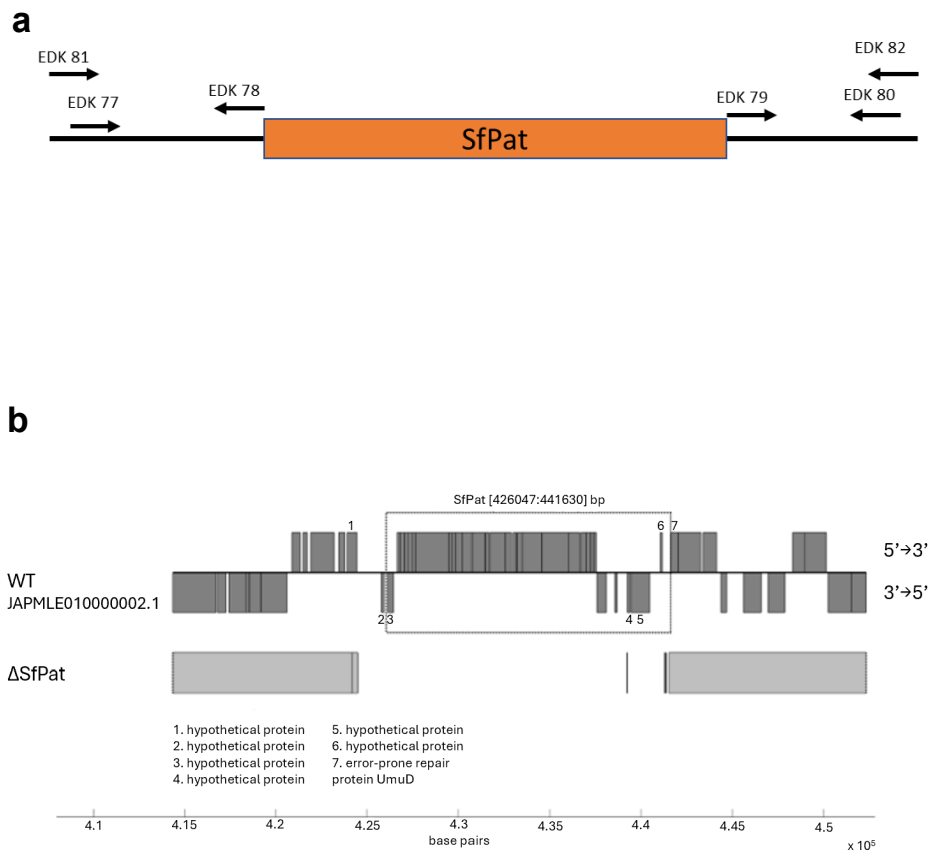

Figure 1. General prophage deletion scheme. (a) Location of upstream, downstream, and flanking primers used in the deletion of SfPat, primer orientation shown with respect to the prophage. (b) Deletion of SfPat from *S. fidelis* 3313 identified after assembling Illumina (short-read) sequenced genomes 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.

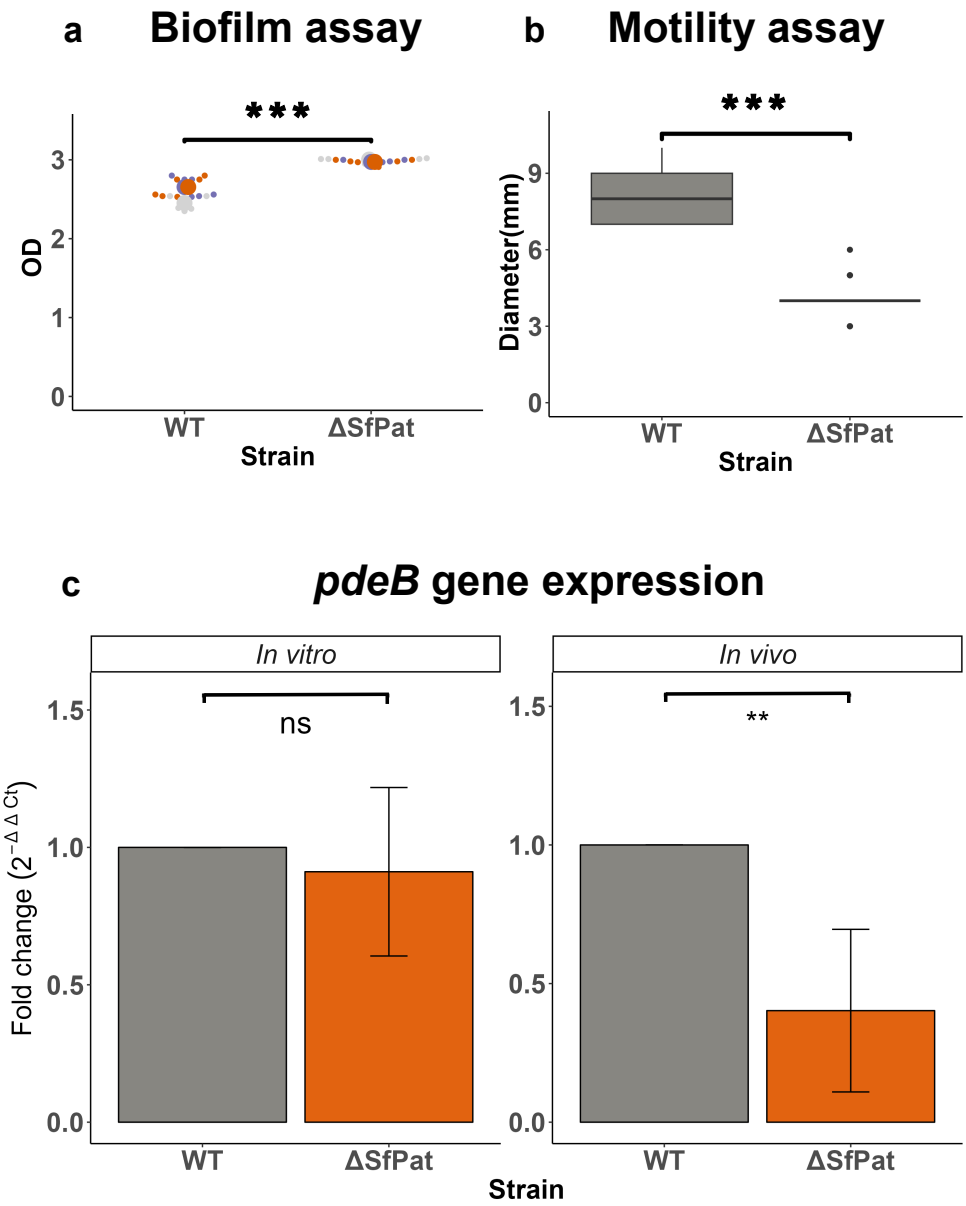

Figure 2. Effects of prophages on biofilm and swimming in *S. fidelis* 3313. (a) Influence of prophages on *in vitro* biofilm formation over 24 hours quantified with crystal violet assay (n=3), (b) role of prophages in swimming quantified as the diameter of spread on soft agar after 24-hours (n=6), and (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 2—figure supplement 1. Fig S2

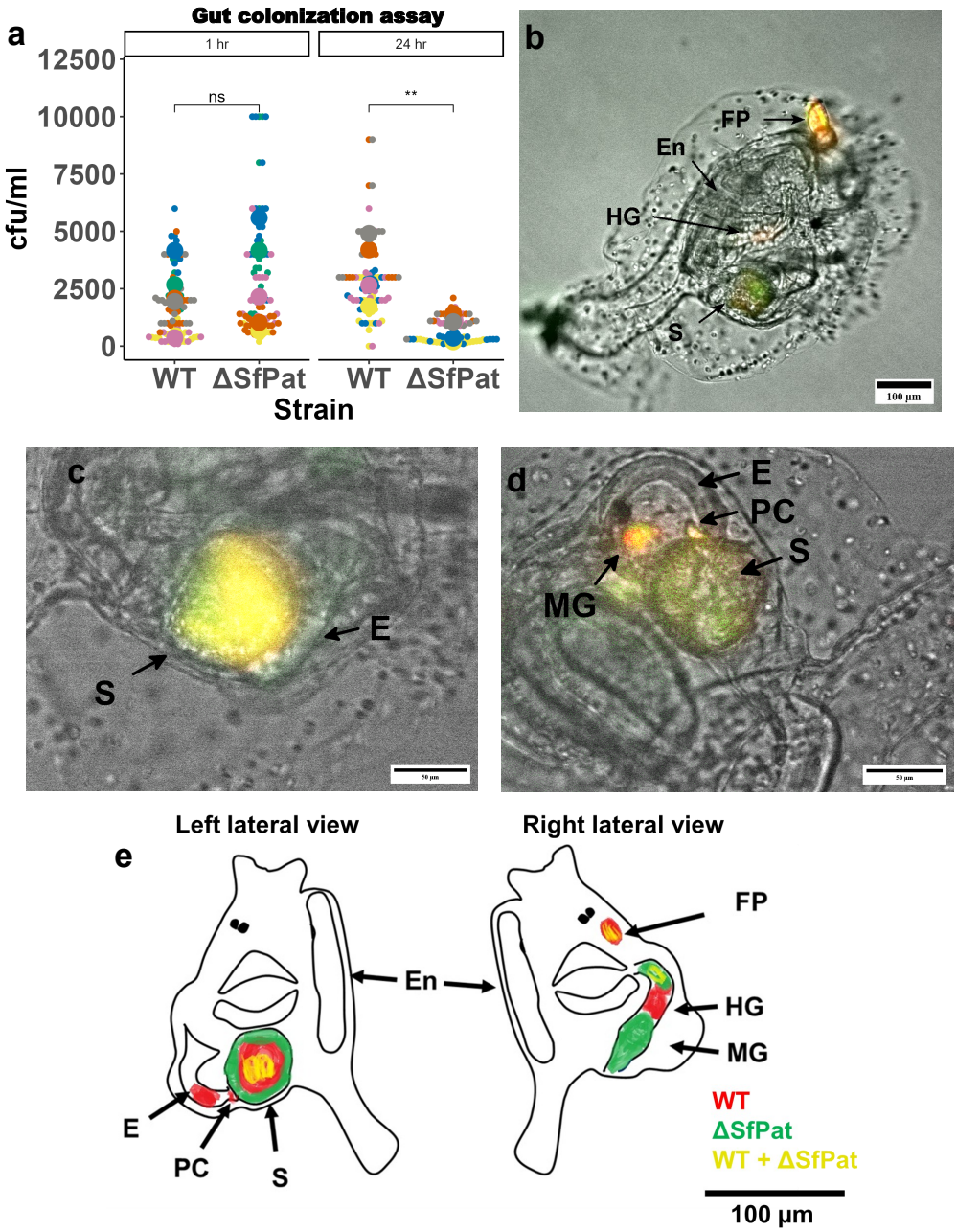

Figure 3. The influence of SfPat prophage on gut colonization in *Ciona*. (a) Results of six biological replicates (n=6, each replicate being a pool of ten juvenile tunicates) of the experimental exposure of *Ciona* MS4 juveniles to either WT or ΔSfPat strains for 1 hr and 24 hrs; retention quantification 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)

Figure 3—figure supplement 1. Figure 3—figure supplement 1

Figure 3—figure supplement 2. Figure 3—figure supplement 2

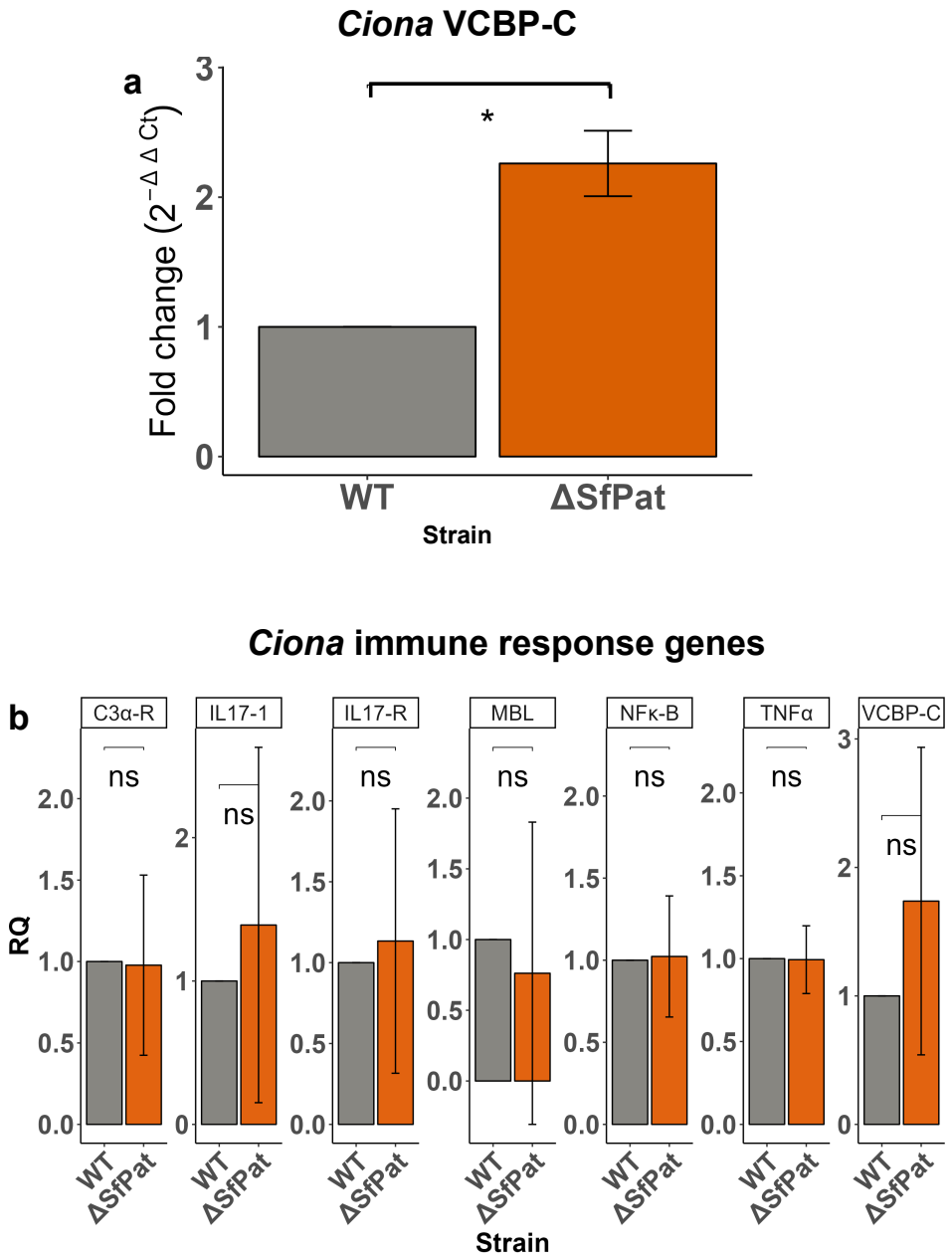

Figure 4. The influence of prophages on host gene expression. (a) VCBP-C gene expression in MS4 juveniles after one hour of exposure to *S. fidelis* 3313 strains (n=4), (b) Survey of additional innate immune gene expression in MS4 juveniles after 24-hour exposure to WT or ΔSfPat mutant strains (n=3).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

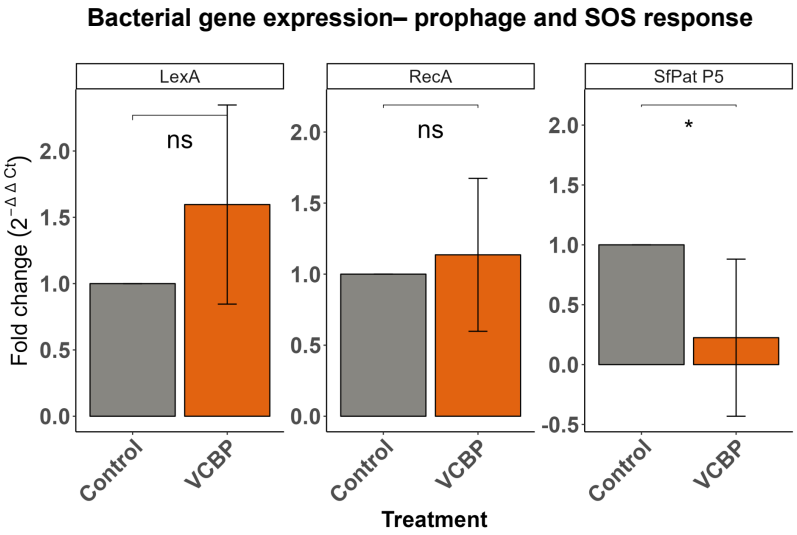

Figure 5. Lysogen gene expression in response to host immune effector binding. Gene expression of SfPat structural protein p5, *recA* and *lexA* of WT strain grown as a 24-hour biofilm while exposed to 50 μg/ml VCBP-C. Rho is the internal control (n=4). (*= p-value<0.05, ns = not significant).

Figure 5—figure supplement 1. Figure 5 supplement