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Acidic pH promotes lipopolysaccharide modification and alters colonization in a bacteria–animal mutualism

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Summary

Environmental pH can be an important cue for symbiotic bacteria as they colonize their eukaryotic hosts. Using the model mutualism between the marine bacterium *Vibrio fischeri* and the Hawaiian bobtail squid, we characterized the bacterial transcriptional response to acidic pH experienced during the shift from planktonic to host-associated lifestyles. We found several genes involved in outer membrane structure were differentially expressed based on pH, indicating alterations in membrane physiology as *V. fischeri* initiates its symbiotic program. Exposure to host-like pH increased the resistance of *V. fischeri* to the cationic antimicrobial peptide polymixin B, which resembles antibacterial molecules that are produced by the squid to select *V. fischeri* from the ocean microbiota. Using a forward genetic screen, we identified a homolog of *eptA*, a predicted phosphoethanolamine transferase, as critical for antimicrobial defense. We used MALDI-MS to verify *eptA* as an ethanolamine transferase for the lipid-A portion of *V. fischeri* lipopolysaccharide. We then used a DNA pulldown approach to discover that *eptA* transcription is activated by the global regulator H-NS. Finally, we revealed that *eptA* promotes successful squid colonization by *V. fischeri*, supporting its potential role in initiation of this highly specific symbiosis.

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Introduction

The host environment provides a landscape of informative chemical cues for symbiotic microbes (Wang and Ruby, 2011; Li *et al.*, 2016). These cues often induce protective responses against host innate defenses, increasing symbiont fitness. While sub-lethal exposure to a toxic compound can cue a protective response (Merrell and Camilli, 2002), an unrelated environmental cue from the host (Palmer *et al.*, 2007), other microbes (Ramsey and Whiteley, 2009), or the symbiont population itself (Goo *et al.*, 2012) can also induce a protective response. Such anticipatory switching can evolve when the factors that microbes experience are predictably correlated (Mitchell *et al.*, 2009; Brunke and Hube, 2014; Schwartzman and Ruby, 2016a; Cao and Goodrich-Blair, 2017), and predictive of each other, or where the cue predictably precedes the stress. In both examples, the ability of microbes to predict their future environments requires a period of entrainment and adaptive change. Foreseeable aspects of host biology, such as circadian regulation of inflammatory responses (Tognini *et al.*, 2017), and conserved environmental features at sites of host–microbe interaction provide such a predictable landscape. In this way, the anticipatory regulation of protective responses by symbiotic microbes can be used to prepare for the characteristic chemistry of the host tissue environment.

In metazoans, the mucus matrix is an ecological filter that 'winnows' symbionts from the surrounding environment (Nyholm and McFall-Ngai, 2004; Fraune and Bosch, 2007). A particularly well-studied example of host–microbe interactions at the mucus interface is that of the bobtail squid *Euprymna scolopes* and its bioluminescent symbiont *Vibrio (Aliivibrio) fischeri.* The first contact between host and microbe occurs in mucus that covers the symbiotic organ in this species-specific association (Schwartzman and Ruby, 2016b). The mildly acidic pH of the squid mucus is one of several initial cues presented to symbiotic cells during their transition from the planktonic state to host association (Wang and Ruby, 2011; Kremer

et al., 2013; Tischler et al., 2019). Because *V. fischeri* cells aggregate in this mucus immediately before encountering a multitude of chemical assaults from the squid, we hypothesized that acidic pH serves as an important cue to induce defensive responses from *V. fischeri* as it establishes its exclusive relationship with the host.

In this work, we describe an asymmetrical anticipatory switch in *V. fischeri* related to protection against cationic antimicrobial peptides like polymyxin B (PMB). Specifically, we show that in response to mild acidity, such as is encountered in the surface mucus of the host, *V. fischeri* alters the structure of its lipid A by attaching positively charged ethanolamine residues. We find that the modification of lipid A by *V. fischeri* is catalyzed by a homolog of the ethanolamine transferase, *eptA*, and is sufficient to confer acid-induced PMB resistance. Ethanolamine transferase-catalyzed lipid A modification is a conserved mechanism for resisting host innate immunity derived cationic antimicrobial peptides (CAPs) among diverse pathogenic and mutualistic microbes, including *Vibrio spp.* (Beceiro *et al.*, 2011; Chen and Groisman, 2013; Cullen *et al.*, 2015; Herrera *et al.*, 2017). Moreover, we show that ethanolamine transferase activity increases the infectivity of *V. fischeri.*

Results

pH drives differential expression of several V. fischeri *genes*

To examine the role of acidic pH in *V. fischeri* gene regulation during light organ colonization of the newly hatched *E. scolopes* juvenile (Fig. 1A), we measured the global transcriptional profile of bacteria exposed to planktonic (seawater) or host-associated pHs (*i.e.*, pH 8.0 and 6.5 respectively) (Fig. 1B, Table S1). We measured the pH that *V. fischeri* encounters in the mucus around the light organ pores using the pH sensitive fluorescent dye SNARF and found that this pH is approximately 6.4, consistent with previous findings (Kremer *et al.*, 2013). To better mimic conditions experienced by the bacteria as they first contact the acidic regions of the squid's light organ surface, we included soluble mucins in both treatments, simulating the acidified mucus produced by the ciliated fields of the squid light organ. Under these growth conditions, we identified a total of 77 genes that were differentially expressed based on pH; 36 of these were upregulated at a host-like acidic pH (Fig. 1B, Table S1). Acid-induced genes included those that encode several predicted outer membrane porins, including the major outer membrane proteins, OmpU and OmpC, as well as the homolog of a phosphoethanolamine transferase, EptA (also known as PmrC, or polymyxin resistance protein C). EptA homologs have previously been shown to modify the structure of the lipid A portion of lipopolysaccharide (LPS)

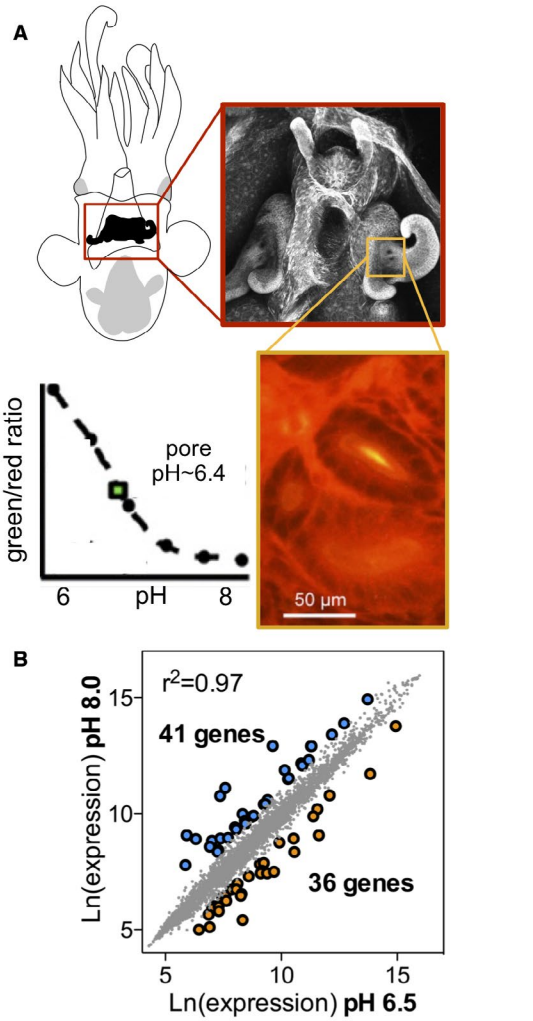

Fig. 1. *V. fischeri* differentially regulates many genes after exposure to marine or host-like pH.
A. Drawing (upper left) and confocal micrograph (red box) of the *E. scolopes* light organ. Orange box displays pH measurement of the region surrounding the light organ pore using the pH-sensitive dual emission dye SNARF. Comparison of the emission ratio at green and red wavelengths gives a ratiometric measurement of tissue pH, relative to a calibration curve (inset graph). Plotting the tissue signal on the graph shows that the pore is a more acidic pH (yellow in the magnified image) than surrounding tissues.
B. Correlation between expression levels of *V. fischeri* transcripts after exposing cells to pH 8.0 or pH 6.5. Sets of genes were differentially expressed at high (blue) or low (orange) pH values.

in Gram-negative bacteria like *Salmonella typhimurium* and certain strains of *Vibrio cholerae* (Lee *et al.*, 2004; Herrera *et al.*, 2017). We verified the differential transcription of *eptA* and *ompC (VF_1795)* at different growth pHs using qRT-PCR (Fig. S1).

Acid exposure increases V. fischeri*'s resistance to CAPs*

Because pH had a strong effect on the expression of *eptA* and other genes predicted to be involved in membrane

function, we predicted that acidic conditions would lead to a shift in the cell's sensitivity to certain membrane-targeting antimicrobials. Many eukaryotes, including the squid, produce CAPs that prevent growth of susceptible microbial partners (Krasity *et al*., 2011; Chen *et al*., 2017), and we reasoned that such a response might serve as an acidity-cued, symbiont-selection mechanism. To test this hypothesis, we exposed *V. fischeri* cells grown in either acidic or alkaline growth conditions to serial dilutions of PMB, a collection of CAPs that associates with negatively charged regions of the lipid A portion of LPS. This association eventually creates pores in the outer membrane, killing the cells (Orwa *et al*., 2001). We found that upon exposure to acidic conditions, the symbiotic *V. fischeri* strain ES114 became sixfold more resistant to PMB killing (Fig. 2A). In contrast, sensitivity to sodium dodecyl sulfate (SDS) treatment increased under these conditions (Fig. 2B), unlike with previously described mutations that altered PMB sensitivity (DeLoney *et al*., 2002), indicating that the response does not represent a general enhancement of membrane integrity.

Acid exposure increases resistance to CAPs across different species of the Vibrionaceae

To determine how common this pH-altered sensitivity to PMB was, we grew several *V. fischeri* isolates, as well as other *Vibrio* and non-*Vibrio* species of marine bacteria, under the same set of acidic and alkaline conditions, before testing their resistance to PMB killing. The *V. fischeri* isolates were light organ symbionts from several squid and fish hosts, as well as a single environmental seawater isolate (Table 1). We found that the increased resistance to PMB in cells grown under acidic conditions was shared across the *V. fischeri* clade (Fig. 2C), even in presumably non-symbiotic strains like 9CS99. This effect was more variable elsewhere in *Vibrionaceae* lineage: the tested strains of *V. harveyi*, and *V. cholerae* showed high basal resistance to PMB in the absence of the acidic pH cue, while *Vibrio azureus* and *Photobacterium leiognathi* showed intermediate levels of resistance in both pH environments (Fig. 2C). Similarly, growth under acidic conditions did not induce PMB resistance in the Gram-positive marine bacteria *Exiguobacterium aestuarii* (Fig. 2C).

eptA *increases the PMB resistance of* V. fischeri

We conducted a forward genetic screen to identify genes that might contribute to PMB resistance in *V. fischeri*. Using a previously generated transposon mutant library (Brennan *et al*., 2013), we screened>13,000 insertions for increased pH-dependent susceptibility to PMB. We also assayed the response of these mutants to SDS exposure so that we could exclude those isolates with an increased sensitivity to both membrane-perturbing agents as mutants with broadly compromised membranes. As a result, we identified 12 mutants with diminished resistance to PMB, but not SDS, relative to wild-type *V. fischeri* ES114 (Table S2). Of these, only one – *eptA* – was also differentially expressed based

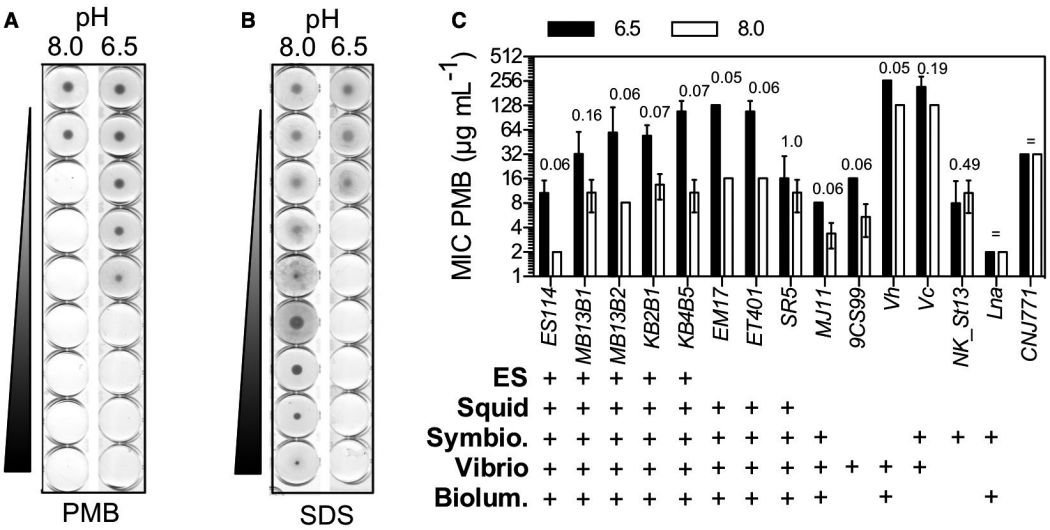

Fig. 2. *V. fischeri* isolates become more resistant to cationic antimicrobial peptides after exposure to low pH.
A. Representative PMB MIC assay of *V. fischeri* ES114 after exposure to seawater (pH 8.0) or host-associated (pH 6.5) conditions.
B. SDS MIC assay of strain ES114, grown under the same conditions as (A).
C. pH-mediated PMB MIC values of other marine bacteria. 'ES', symbiont isolated from *Euprymna scolopes* light organ; 'Squid', light organ isolate from *Euprymna* or *Sepiola* squid species; 'Symbio.', host-associated strains; 'Vibrio', a species of the genus *Vibrio*; 'Biolum.', isolate producing bioluminescence. See Table 1 for strain descriptions. *p* values are reported according to a Mann–Whitney test of three replicates. The '=' symbol indicates that all measured MIC values are equal in both conditions and, therefore, the *p* value was not calculated.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Description/relevant genotype	Reference
V. fischeri		
ES114	Wild-type *Euprymna scolopes* light-organ isolate	Boettcher and Ruby (1990)
MJ11	Wild-type isolate from *Monocentris japonica* light organ	Ruby and Nealson (1976)
MB13B1	Wild-type *E. scolopes* light-organ isolate	Wollenberg and Ruby (2012)
MB13B2	Wild-type *E. scolopes* light-organ isolate	Wollenberg and Ruby (2012)
KB2B1	Wild-type *E. scolopes* light-organ isolate	((Wollenberg and Ruby, 2012)
KB4B5	Wild-type *E. scolopes* light-organ isolate	Wollenberg and Ruby (2012)
SR5	Wild-type *Sepiola robusta* light-organ isolate	Wollenberg and Ruby (2012)
EM17	Wild-type *E. morsei* light-organ isolate	(Wollenberg and Ruby, 2012)
ET401	Wild-type *E. tasmanica* light-organ isolate	Wollenberg and Ruby (2012)
9CS99	Wild-type planktonic isolate from North Atlantic Ocean	Wollenberg *et al.* (2012)
JAS340	Δ*eptA* (*VF_A0210*) deletion in ES114	This work
MB06256	*VF_A0176::*Tn*erm* in ES114	This work
MB13215	*VF_2503::*Tn*erm* in ES114	This work
MB13278	*VF_2503::*Tn*erm* in ES114	This work
MB13555	*VF_2503::*Tn*erm* in ES114	This work
SLV15	*hns::*Tn*erm* (*VF_1631*) in ES114	Lyell *et al.* (2010)
MB06859	*waaL::*Tn*erm* (*VF_0151*) in ES114	Post *et al.* (2012)
AMJ2	Δ*arcA* (*VF_2120*) deletion in ES114	Bose *et al.* (2007)
Seawater isolates		
NK_St13	*Vibrio azureus*-like	Kremer *et al.* (2014)
Lna	*Photobacterium leiognathi*	Dunlap (1985)
CNJ-771	*Exiguobacterium aestuartii*-like	Gontang *et al.* (2007)
0395-N1	*Vibrio cholerae* isolate (*ctxAB* mutant)	Mekalanos *et al.* (1983)
B392	*Vibrio harveyi* isolate	Reichelt and Baumann (1973)
E. coli		
DH5α-λpir	*deoR hsdR17 endA1 gyrA96 pir*	Hanahan (1983)
β-3914	*RP4-2 gyrA462 zei298::*Tn10 Δ*dapA*::(*erm-pir*)	Le Roux *et al.* (2007)
WM3064	*RP4-1360 rpsL hsdS* Δ*dapA1341*::(*erm-pir*)	Saltikov and Newman (2003)
Plasmids		
pVSV105	Cm^r, *lacZ*α-(SphI, SalI/HincII, XbaI, SmaI/XmaI, KpnI, SacI)	Dunn *et al.* (2006)
pVSV104	Kn^r, *lacZ*α-(SphI, SalI/HincII, XbaI, SmaI/XmaI, KpnI, SacI)	Dunn *et al.* (2006)
pEVS104	R6Kori RP4 *oriT trb tra* Kn^r	Stabb and Ruby (2002)
pKV363	Mobilizable suicide vector; Cm^r; pSW7848 + MCS	Shibata *et al.* (2012)
pTM267	Two-color fluorescent reporter, *tetA'::mCherry*, *GFP* downstream of a multiple cloning site, Cm^r	Miyashiro *et al.* (2010)
pJAS340	Δ*eptA*::pKV363	This work
pJAS341	*eptA::*pVSV105	This work
pJAS342	*eptA'::GFP* in pTM267	This work
pJBL301	*tetA'::eptA* in pVSV104H	This work

on pH. These results led us to predict that this gene was responsible for pH-mediated antimicrobial resistance. We deleted the entire *eptA* open reading frame, and confirmed that the gene product was necessary to confer acid-induced PMB resistance (Fig. 3A). An *in trans* complementation of *eptA,* including its native promoter region, restored the pH-induced activity, further supporting this finding (Fig. 3A). Using qRT-PCR to measure transcript levels, we found that low pH activated *eptA* expression (Fig. 3B). This transcriptional increase occurred within 15 min of low pH exposure, and plateaued after approximately 30 min (Fig. 3B). The outer membrane protein OmpU has been shown to confer CAP resistance in *V. cholerae* (Mathur and Waldor, 2004; Mathur *et al.*, 2007), but when we tested a *V. fischeri* Δ*ompU* mutant for PMB sensitivity, we observed no difference between this strain and its isogenic parent (Fig. S2). In contrast, a *V. fischeri waaL* mutant had 2- to 4-fold increase in sensitivity to PMB that was pH-independent (Fig. S2). WaaL homologs ligate the O-antigen to LPS, and mutation of this gene in *V. fischeri* also conferred sensitivity to SDS (Table S2). Thus, we concluded that, while there are several activities that promote cellular resistance to PMB, *eptA* is the main gene responsible for pH-induced PMB resistance in *V. fischeri.*

eptA *is essential for the addition of a ethanolamine residue to* V. fischeri *lipid A*

The *eptA* homologs in *Salmonella typhimurium* and *Vibrio cholerae* El Tor encode a protein that acts as a phosphoethanolamine transferase, altering the charge on the bacterial outer membrane and increasing CAP resistance (Lee *et al.*, 2004; Needham and Trent, 2013; Herrera *et al.*, 2017). Heterologous expression experiments have also demonstrated that the EptA homolog in

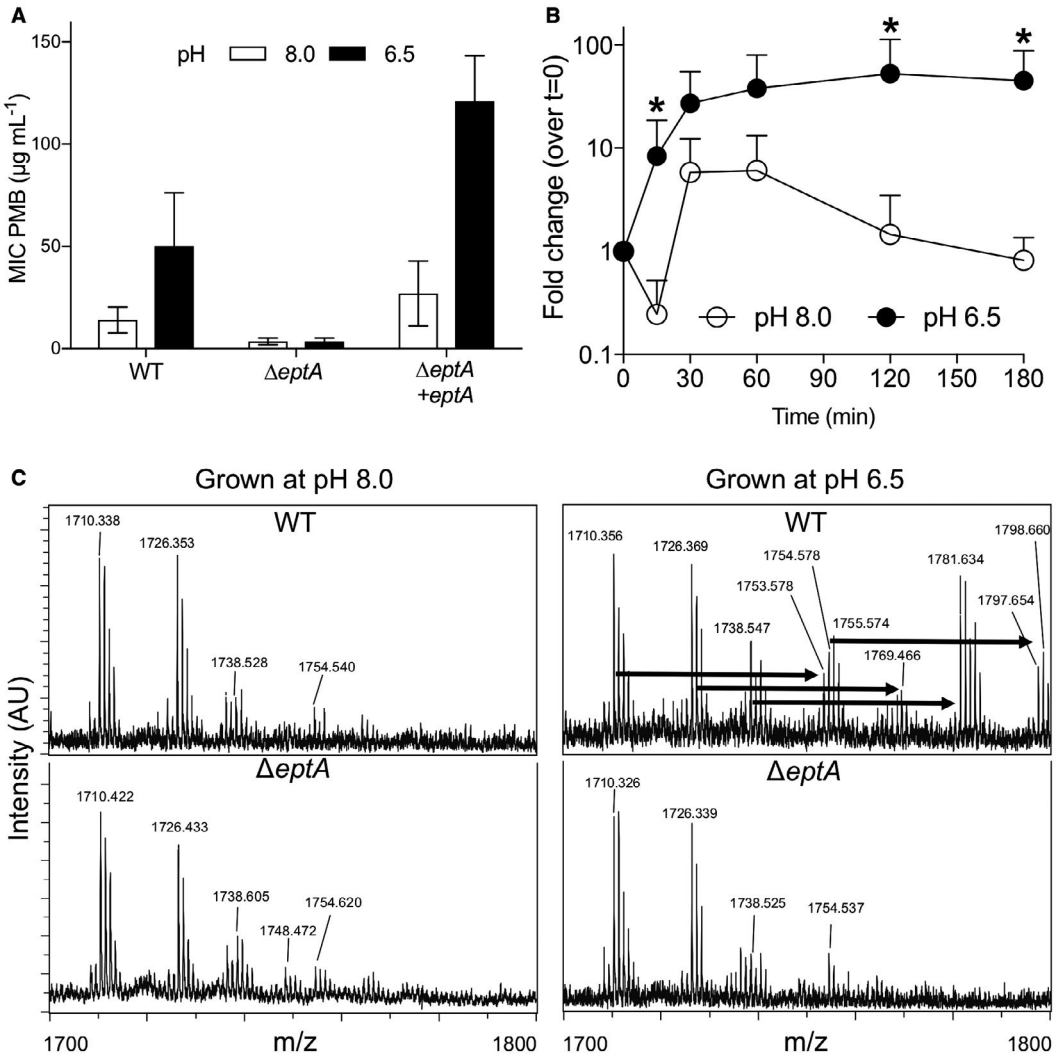

Fig. 3. The *eptA* homolog of *V. fischeri* (*VF_A0210*) is differentially expressed under acidic conditions and acts as a lipid A ethanolamine transferase.
A. pH-dependency of PMB MIC assay performed on wild-type *V. fischeri* ES114 (WT) and its derivatives, with or without the *eptA* gene. Bars represent mean with 95% confidence intervals (CI).
B. Transcription of *eptA* over time after exposure to acidic or basic pH. Points represent mean + SD. Data represent at least three biological replicates, $*p \leq 0.1$ according to Mann–Whitney test.
C. Representative mass spectrometry spectra of lipid A extracts isolated from either wild-type *V. fischeri* or its Δ*eptA* derivative, grown at pH 6.5 or 8.0. Arrows indicate 43 m/z shifts, consistent with a dehydration addition of an ethanolamine residue.

V. fischeri is functional (Herrera *et al*., 2017). To confirm the biochemical role of EptA in *V. fischeri*, we performed a mass spectroscopy analysis of purified lipid A from wild-type and Δ*eptA V. fischeri*, grown at different pHs (Fig. 3C). At pH 8.0, signals with m/z corresponding to previously identified lipid A species were observed in both wild type and mutant extracts (Phillips *et al*., 2011). When grown at pH 6.5, but not 8.0, we identified mass shifts of ~43 Da in wild-type lipid A that corresponded to the dehydration attachment of ethanolamine (Fig. 3C, top). These shifts were missing in the Δ*eptA* mutant (Fig. 3C, bottom) at both pHs, consistent with an acidic pH-dependent and EptA-catalyzed addition of this moiety to lipid A. Together, these data support the conclusion that EptA acts as an acid-induced ethanolamine transferase in *V. fischeri*.

eptA *expression is promoted by the dual regulator H-NS*

We next sought out regulators of *eptA* expression that might connect pH variation to CAP resistance by *V. fischeri*. In *S. typhimurium*, *eptA* is transcriptionally regulated by PhoP in a magnesium- and PmrD-dependent manner (Chen and Groisman, 2013). In addition, our transposon

screen for PMB-sensitivity identified several predicted transcriptional regulators that were potentially involved in *V. fischeri eptA* expression (Table S2). To clarify the roles of these candidates, we determined the relative levels of *eptA* transcription in mutants lacking two *phoP* homologs (*VF_0526* and *VF_1396*) (Hussa *et al*., 2007) and two of our predicted regulators of *eptA* (*VF_2503* and *VF_A0176*) in *V. fischeri*. We found no effect of the absence of any of these candidates on the activity of the *eptA* promoter (data not shown). We reasoned that regulators of *eptA* may have been missed in a loss-of-function screen either if they were required for *in vitro* growth of *V. fischeri*, or if pH-dependent transcription was mediated by a repressor. To address these issues, we performed a DNA-pulldown with the presumed promoter of *eptA* as bait to discover potential regulatory proteins of *eptA* transcription (Table 2, Table S3; see Methods). We identified eight non-ribosomal target genes, and tested strains with mutations in a subset of these genes for defects in *eptA* promoter activity at acidic and alkaline pHs. Mutation of three of the eight candidates (*nagC*, *arcA* and *VF_1195*) showed no significant alteration in *eptA* expression (data not shown). We also found that ToxR, a master regulator of *V. cholerae* host-associated physiology, did not alter *eptA* expression at either pH (Fig. 4A). However, a mutant of *hns* (Lyell *et al*., 2010), displayed significantly decreased *eptA* expression at both low and high pH (Fig. 4A). We tested this *hns* mutant for pH-dependent PMB sensitivity, and found that it showed significantly diminished resistance to PMB (Fig. 4B). This strain was equally sensitive to PMB at low and high pH, similar to the Δ*eptA* mutant, indicating that it had lost its pH-mediated resistance. We were able to complement the PMB sensitivity defect by driving *eptA* expression off of the *tetA* promoter, showing that the increased sensitivity of the *hns* mutation could be overridden *in trans* (Fig. 4B). Interestingly, although we have previously found the TetA promoter to be insensitive to our experimental conditions (data not shown), this complemented strain still showed a pH-influenced effect, indicating that additional post-transcription regulation (*e.g.*, acting either on mRNA or protein expression, or directly on EptA activity) may also be pH-dependent.

Table 2. Most abundant potential regulators of *eptA*, identified through promoter pulldown.†

Locus Tag	Gene name	Predicted function
VF_2423	*tufA*	Elongation factor Tu
VF_0806	*nagC*	DNA-binding transcriptional dual regulator, repressor of *N*-acetylglucosamine
VF_0815	*seqA*	Negative modulator of initiation of replication
VF_0252	*rplF*	50S ribosomal protein L6
VF_0658	*rimK*	Probable alpha-L-glutamate ligase
VF_1951	*fabZ*	3-hydroxyacyl-[acyl-carrier-protein] dehydratase
VF_1962	*rpsB*	30S ribosomal protein S2
VF_2566	*atpA*	ATP synthase subunit alpha
VF_A0001		Uncharacterized protein
VF_1631	*hns*	Global DNA-binding transcriptional dual regulator

†Ten most abundant proteins listed. For complete list of proteins pulled down with the *eptA* promoter region, see Table S1. Genes listed in descending order of #PSM in pulldown analysis.

eptA *is important for colonization initiation*

The tissues of *E. scolopes* present several chemical and physical challenges to bacteria transitioning from

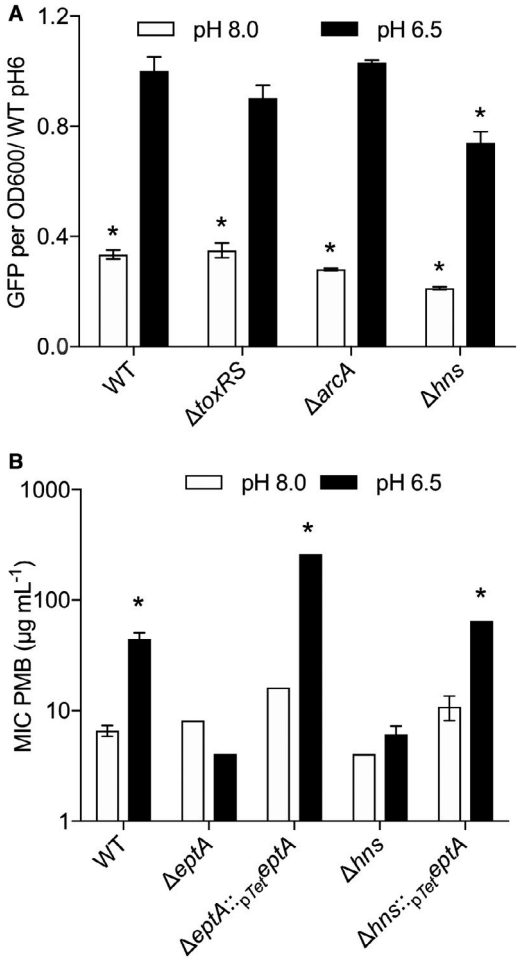

Fig. 4. The *V. fischeri eptA* is regulated by H-NS.
A. *eptA* reporter expression in mutants strains of *V. fischeri* after exposure to alkaline or acidic pH.
B. PMB MIC assay of *eptA* and *hns* mutants. The *eptA* mutation is complemented by a plasmid borne copy of *eptA* under the control of the *tetA* promoter. In (A), *indicates significant ($p < 0.05$) difference from WT at pH 6 by Dunnett's test; in (B) *indicates significant $p < 0.05$ difference from WT at pH 8.

the ambient seawater to the surface of the symbiotic organ, where the cell must then traverse these assaults after exposure to the squid's acidic mucus. We hypothesized that *eptA* is important for surviving these challenges and efficiently initiating colonization. To test this idea, hatchling squid were exposed for 3 h to a relatively low inoculum of either wild-type or Δ*eptA V. fischeri* cells, and the fraction of squid successfully colonized was determined. Although the data are spread, the Δ*eptA* mutant was markedly less likely to colonize squid under these conditions (Fig. 5A), consistent with our prediction. We then tested whether the colonization defect of the Δ*eptA* mutant could be overcome by increasing the concentration of *V. fischeri* that the squid were exposed to. In fact, the Δ*eptA* mutant could successfully colonize the squid with an efficiency similar to wild type, but it required a higher inoculum to do so; mutating *eptA* resulted in a>2-fold increase in ID_{50}, from 2,500 to 5,700 CFU ml^{-1} (Fig. 5B). Significantly, animals successfully colonized under any of these conditions had similar bacterial loads in the light organ after 24 h (data not shown), and there was no difference in the competitive index of Δ*eptA* when co-colonized with wild-type *V. fischeri* at an initial cell density greater than the ID_{50}. Together, these results indicate that the effect of the Δ*eptA* mutation was restricted to the early initiation events of colonization, and that the carriage of *eptA* enhances the colonization efficiency of *V. fischeri.*

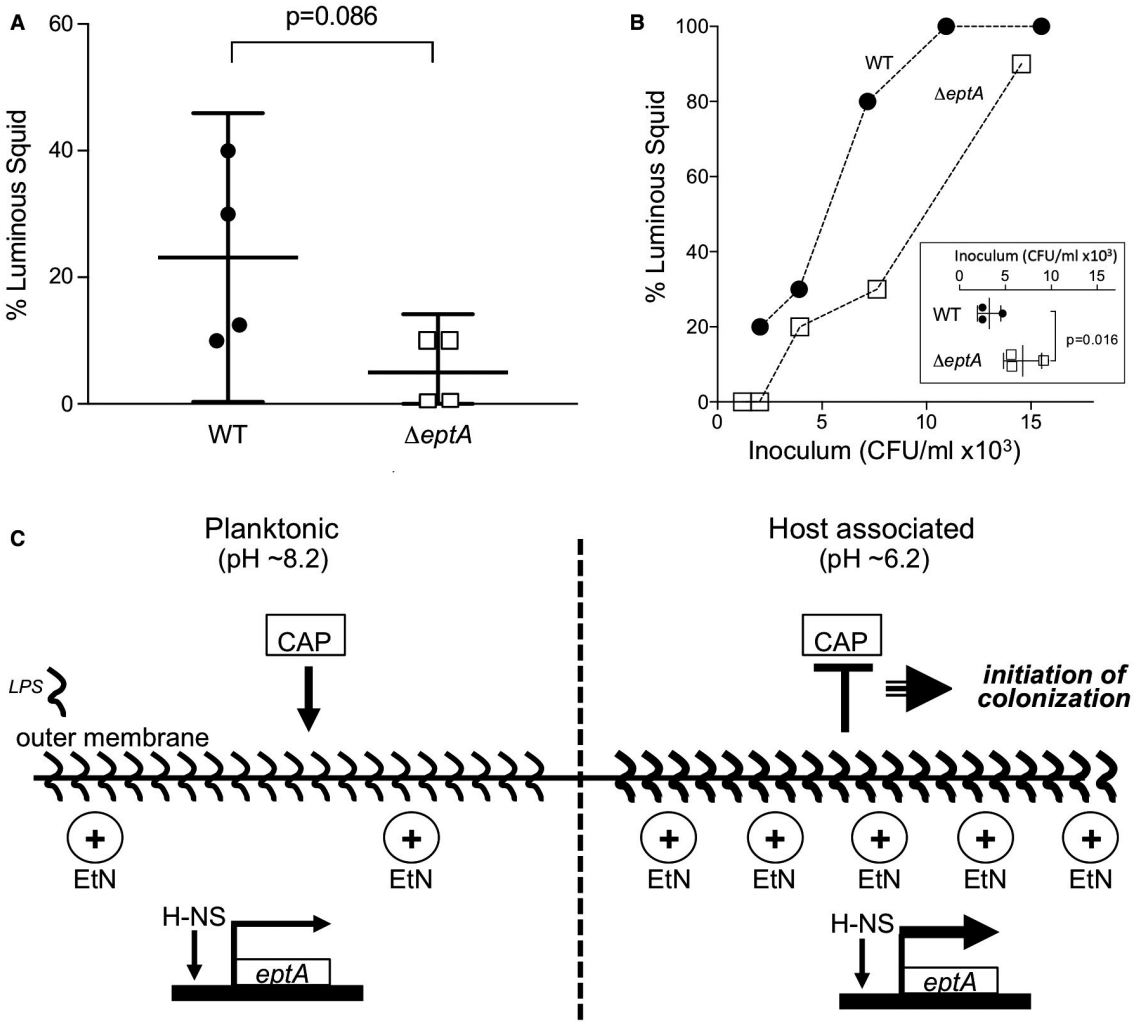

Fig. 5. *eptA* is required for efficient squid colonization by *V. fischeri*.
A. Percentage of luminous squid after a 3 h exposure to 800 CFU ml^{-1} of *V. fischeri*. The means, with 95% CI, are indicated.
B. Fraction of colonized squid as a function of *V. fischeri* inoculum size. Inset: calculated ID_{50} for WT and the *eptA* mutant *V. fischeri*.
C. Schematic model of *eptA* regulation and its role in cationic antimicrobial peptide (CAP) resistance in *V. fischeri* by LPS modification with ethanolamine (EtN)

Discussion

In this study, we show that an acid-cued switch in *V. fischeri* physiology is an anticipatory modification that allows *V. fischeri* to effectively colonize its squid host. Our molecular characterization of acid-induced PMB resistance (Figs. 3 and 4), together with the requirement of this protective response for efficient initiation of symbiosis (Fig. 5B), contributes to an understanding of the mechanisms by which the host's environmental filter promotes colonization of its tissues. Specifically, an H-NS-dependent transcriptional activation of *eptA* governs the decoration of *V. fischeri* LPS with ethanolamine (Fig. 5C). Interestingly, this addition differs from the predicted function of *eptA* homologs, in that we described lipid A mass shifts consistent with an addition of ethanolamine, not phosphoethanolamine as has been previously described (Herrera *et al*., 2017), potentially owing to the unique lipid A structure of *V. fischeri* (Phillips *et al*., 2011). This remodeling of the outer membrane increases the resistance of *V. fischeri* to host antimicrobial peptides, some of which are thought to be enhanced by charge–charge interactions, similar to PMB (Heath-Heckman *et al*., 2014; Chen *et al*., 2017). Additionally, the *E. scolopes* genome (Belcaid *et al*., 2019) has revealed a large set of potential homologs for known antimicrobials that may rely on similar interactions to either control *V. fischeri* populations or selectively allow *V. fischeri* to prosper in the light organ. By inducing *eptA* expression before the symbionts enter the antimicrobial environment of the light organ ducts (Nyholm and Graf, 2012; McFall-Ngai, 2014), the acidic pH encountered by *V. fischeri* cells in the>2 h they spend aggregating outside the light organ (Kremer *et al*., 2013) provides a cue and enough time for an anticipatory switch to a symbiosis-competent physiological state (Figs. 3B and 5C). As a result, the timely expression of *eptA* potentiates colonization of squid by *V. fischeri*, even at the low cell concentrations characteristic of its natural environment (Lee and Ruby, 1994).

Despite the fact that acidic pH and antimicrobial peptides are common motifs of metazoan epithelial tissue (Wesley *et al*., 1985), pathogenic and mutualistic bacteria are likely to experience the host tissue environment differently. A comparison between the PMB resistance mechanisms and acid-induced transcriptional responses described in *V. fischeri* and in the related gastrointestinal pathogen *V. cholerae* hints at these differences. Unlike *V. fischeri, V. cholerae* encodes an additional LPS-modifying system that confers PMB resistance: the aminoacyl lipid modifying enzymes AlmEFG. In contrast to the acid-induced modification described here, transcription of *almEFG* is repressed by exposure to acid via the LeuO-ToxRS-CarRS/VprAB cascade of transcription factors (Herrera *et al*., 2014; Ante *et al*., 2015; Bilecen *et al*., 2015; Bina *et al*., 2016). The *V. cholerae* PMB resistance phenotype is also regulated by the envelope stress response through the alternative sigma factor RpoE and the outer membrane protein OmpU (Mathur *et al*., 2007; Shaw, 2012). Several lines of evidence suggest that this architecture of the envelope stress response pathway is not conserved between the *V. cholerae* and *V. fischeri*: (i) our transposon screen for PMB-sensitive mutants identified neither *ompU* nor *rpoE* (Table S2), (ii) there was no evidence that any of the four *V. fischeri* RpoE homologs bound upstream of *eptA* (Table S1) (Mandel *et al*., 2008), and (iii) deletion of *ompU* did not increase sensitivity to PMB (Fig. S1). Like *V. fischeri*, the *El Tor* biotype of *V. cholerae* expresses EptA in response to an acid cue (Herrera *et al*., 2017); however, *El Tor* encodes the acid-responsive regulator AphB, which lacks a homolog in *V. fischeri.* The AphB regulated genes *pepA*, *tcpPH* or the *cadABC*, all of which are components of the acid-inducible response in *V. cholerae* (Merrell and Camilli, 2000; Behari *et al*., 2001), but are not induced by *V. fischeri* under our experimental conditions (Table S1). Taken together, the additional AlmEFG-dependent mechanism of acid-independent PMB resistance, the integration of PMB resistance into the envelope stress response, and the existence of a dedicated acid stress response all suggest that *V. cholerae El Tor* encounters conserved, but differently presented, chemical cues than *V. fischeri* in their respective host tissue environments. Future comparative studies of the regulation of *eptA* in diverse symbiotic microbes will provide further insight into the range of adaptations by which pathogenic and mutualistic microbes sense and respond to a host environment.

Our finding that H-NS plays an activating role in *eptA* expression through interaction with the promoter region was surprising given H-NS's typical role as a repressor; however, it is consistent with previous findings where H-NS promotes expression by outcompeting other repressors or organizing the genome to coordinate positive regulation (Ayala *et al*., 2017). In our case, it appears as though H-NS is involved in activity of the putative promoter region upstream of *eptA*, but that additional pH-sensitive regulation of the *eptA*-driven membrane modification occurs (Fig. 4B). H-NS activity has been found to be pH-sensitive (Liu *et al*., 2010), but as we found the *hns* mutant to decrease $_{p}$*eptA* activity at both tested pHs, we believe additional factors are involved in pH-mediated *V. fischeri* membrane structure. Specifically, H-NS may drive overall expression of *eptA*, while something else facilitates pH-mediated expression. Future work may explore additional interactions between H-NS and other regulators at the interface of the *eptA* promoter region, as well as discovering other actors that guide *V. fischeri*'s physiologic shifts at acidic pH.

Bacteria are highly effective at sensing changes in their environment, and rapidly adjusting their physiology and biochemistry to heighten the cell's fitness under the new

conditions. Typically, the connection between the environmental cue and the response is direct; for example, when planktonic *V. fischeri* cells first encounter the nascent light organ, they respond to the presence of host-derived NO by inducing the detoxifying enzyme Hmp (Wang *et al*., 2010), and to the host's release of chitobiose increasing their ability to chemotax toward that sugar (Kremer *et al*., 2013). In both of these examples, the response is initially primed as the bacteria pause in an aggregate just outside the organ (McFall-Ngai, 2014), and failure to adapt results in a colonization defect. Here we have described a different strategy, called anticipatory adaptation, by which *V. fischeri* 'bets' that coming across an acidity cue means they have contacted host tissue, and that inducing an LPS-modifying enzyme in response will help protect them from an inevitable encounter with host-derived cationic antimicrobial peptides. This example is likely to be only the first evidence of adaptive switching in the squid-vibrio symbiosis: the NO-protective enzyme Hcr (VF_A0863) (Wang *et al*., 2016) also appears to be induced by an acidity cue (Table 1).

While the work presented here establishes that EptA is a significant colonization factor for symbiotic *V. fischeri*, it remains possible that there are context-specific costs to *eptA* expression that select for the ability to differentially, rather than constitutively, express this gene. Regardless, EptA restructures *V. fischeri* LPS in a fashion that is similar to that of several other animal-associated microorganisms, yet is regulated in a manner that has not been previously described. This difference may be related to lineage and lifestyle related factors, and highlights the benefit of comparing co-association strategies across diverse animal-microbe symbioses.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* was cultured at 37°C with shaking at 250 rpm in Luria-Bertani medium (LB) (Sambrook *et al*., 1989), with (per ml) 150 µg erythromycin (Erm), 25 µg chloramphenicol (Cam) or 50 µg kanamycin (Kan), as needed. Unless otherwise noted, *V. fischeri* was grown in Luria-Bertani salt medium (LBS) shaking at 225 rpm at 28°C, with (per ml) 5 µg Erm, 5 µg Cam or 100 µg Kan where indicated. All *Vibrio fischeri* mutant strains were derived from the light organ isolate ES114 (Boettcher and Ruby, 1990).

Sample collection, RNA extraction and transcriptional analysis

To assess the transcription of *V. fischeri* in an environment approximating the squid's mucus, strain ES114 was cultured in an artificial seawater medium (ASW: 300 mM NaCl, 50 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM KCl) containing 50 mM of PIPES buffer and 0.1% of hog gastric mucin (HGM, w/v; 1% bound sialic acid, Sigma). The medium was buffered at pH 6.5 or pH 8.0 to characterize transcription in response to acidic pH. Triplicate overnight cultures of ES114 in LBS were diluted 1:100 into 25 ml high osmolarity seawater tryptone medium (SWTO, [Stabb *et al*., 2004]) in 125 ml flasks, and grown with shaking and 28°C. When cultures reached 0.60 ± 0.05 optical density at 600 nm (OD_{600}, 1 cm path length), they were centrifuged for 8 min at 6000 × RCF and 4°C to pellet the cells. The supernatants were aspirated, and pellets were resuspended in 25 ml of 50 mM PIPES (pH 8.0) ASW in a 125 ml flask. Cultures were incubated for 1 h and were centrifuged again, and the pellet was resuspended in 1 ml of PIPES ASW. One-half milliliter of this resuspension was used to inoculate 15 ml of PIPES ASW/HGM (0.1%), buffered to either pH 6.5 or at pH 8.0. Cultures were incubated for 2 h, then centrifuged, and the pellet was resuspended in 4 ml RNAlater (Life Technologies). This final suspension contained 2.5×10^8 CFU ml^{-1} (±5%).

The cells suspended in RNAlater were pelleted by centrifugation at 5000 × RCF and 4°C for 5 min. An RNeasy mini kit (Qiagen) was used to extract RNA, according to the manufacturer's instructions. Samples were eluted in RNase-free water, and treated with RQ-DNase (Promega), according to the manufacturer's instructions. An on-column sample cleanup was performed following the protocol outlined in the kit. Samples were at least 1 µg RNA μL^{-1}, with absorbance ratios at 260 nm:280 nm and at 260 nm:230 nm of>2.1. Samples were submitted to the University of Wisconsin-Madison Gene Expression Center for further quality assessment using an Agilent RNA NanoChip. Ten micrograms of total RNA was used for cDNA synthesis following protocols defined by NimbleGen array analysis (Roche NimbleGen). cDNA was submitted to NimbleGen for prokaryotic gene expression microarray analysis. The expression values were processed to yield the fold change in expression between experimental conditions using ArrayStar software (DNAStar). Differential regulation was defined as>2-fold difference in gene expression between the two conditions with at least 95% confidence, which allowed us to detect potentially subtle physiological responses to each condition. The linear correlation in signal intensities between the two conditions was 0.97. BLAST2GO software (BioBam Bioinformatics Solutions) was used to perform functional enrichment analysis of the dataset based on gene ontology classification.

Quantitative RT-PCR procedures were conducted in accordance with the MIQE protocol guidelines (Bustin *et al*., 2009). Primers were designed by OligoAnalyzer software (Integrated DNA Technologies) with a 60°C annealing temperature. Primer pair efficiencies were between 95% and 105%. Candidate gene expression was normalized using the geometric mean of *polA*, a previously defined endogenous control gene (Wang *et al*., 2010), whose expression was confirmed to be invariant between the conditions compared in this experiment. Mann–Whitney test, were used to compare measurements across conditions.

Minimum inhibitory concentration assay

Minimum inhibitory concentration (MIC) assays were performed with *V. fischeri* based on previously described

protocols (DeLoney *et al*., 2002; Kremer *et al*., 2014). To assay for pH-dependent resistance to various stressors, MIC assays were set up in pH 6.5 or pH 8.0 PIPES MIC buffer containing 10 mM PIPES (pH 6.5 or 8.0) in ASW. Twofold serial dilutions of stressors of interest (starting concentrations: 0.26 mg ml^{-1} PMB or 1% SDS) were set up in 96-well plates containing 90 µL total volume. Bacterial cultures were started in SWT medium (Boettcher and Ruby, 1990) at 28°C, and grown to an OD_{600} of 0.10 ± 0.02. Cultures were diluted 1:100 into 3 ml of PIPES (pH 8.0) in ASW, and incubated for 3 h at 28°C with shaking. Another 1:100 dilution into SWT was performed upon completion of the 3 h incubation, and 10 µl of the bacterial culture was added to 90 µl of a MIC buffer (pH 6.5 or 8.0) plus stressor. Plates were incubated under static conditions at 28°C and checked at 24 h for pellet formation at the bottom of the plate as well as any turbidity in the wells. Inoculation levels that fell between 10 and 100 CFUs were found to produce consistent MIC values.

Molecular techniques

In-frame deletions of *V. fischeri* genes were constructed by CcdB-toxin mediated double homologous recombination, using a *V. fischeri*-specific suicide vector pKV363 as previously described (Stabb and Ruby, 2002; Le Roux *et al*., 2007; Shibata *et al*., 2012). Briefly, segments upstream and downstream of the coding region were amplified, and ligated together using a unique restriction enzyme site. The resulting chimeric DNA was ligated directionally into the pKV363 backbone at the multiple cloning site (MCS). Conjugative transfer was used to introduce the plasmid into *V. fischeri* ES114, and the double crossover of the construct was selected by arabinose induction of the CcdB toxin encoded on the pKV363 plasmid backbone. Deletions were confirmed by sequencing around the target locus.

To complement *eptA* under control of its native promoter, the intact locus was amplified from wild-type *V. fischeri*, and cloned into the plasmid pVSV105: a shuttle vector carrying an origin of replication derived from a *V. fischeri*-specific plasmid pES213 (Dunn *et al*., 2006),) Cloning was carried out in the *E. coli* strain DH5α-λ *pir*. The resultant vector was introduced into *V. fischeri* by pEVS104-assisted conjugation (Stabb and Ruby, 2002). For complementation of *eptA* off a non-native, constitutive promoter, the *tetA* promoter from plasmid pTM267 was fused to the upstream region of *eptA*, and the resulting construct cloned into the MCS of pVSV104 by Golden Gate cloning. This plasmid construct was transformed into *E. coli* WM3064, and conjugated into *V. fischeri.*

Construction of two-color fluorescent reporters: The regions 400 bp upstream and 200 bp downstream of the predicted translational start sites of the *eptA* gene were amplified, and cloned into XmaI and XbaI sites of the of plasmid pTM267. This plasmid encodes a constitutively activated copy of the red fluorescent protein mCherry, as well as the gene (*gfp*) encoding a green-fluorescent protein downstream of a MCS (Miyashiro *et al*., 2010). The insertion of the putative promoter region into the MCS of pTM267 resulted in the promoter-dependent transcription of *gfp*. The reporter plasmid is maintained at ~10 copies per cell.

Screening of an arrayed transposon mutant library for genetic components of PMB resistance

We screened 13,686 mutants of an existing 23,904 strain library (Brennan *et al*., 2013), for a loss of resistance to PMB. Briefly, a pin replicator was used to inoculate 96-well plates containing 100 µL of LBS-Erm from frozen glycerol stocks of the first-generation plates created in the original screen. After overnight growth, the pin replicator was used to inoculate fresh 96-well plates containing 100 µL pH 6.5 SWTO with the LBS-Erm overnight cultures. The plates were placed on a shaker for 3 h at 28°C prior to being used to inoculate 100 µL MIC buffer (pH 6.5) containing 10% of SWT medium, with and without 8 µg PMB ml^{-1}, in a round-bottomed 96-well plate. Plates were incubated under static conditions overnight at 28°C, prior to assessment of growth in the presence and absence of PMB. Five microliters of culture material from strains of interest was combined with 95 µL of elution buffer (Qiagen), and frozen at –20°C to create template mixes for arbitrarily primed PCR. Arbitrarily primed PCR was performed as described previously (Brennan *et al*., 2013), with the addition of ExoSAP-IT treatments (Affymetrix), following the manufacturer's instructions between successive rounds of PCR to remove excess single-strand oligonucleotide primers. Amplified reactions were sent for Sanger sequencing at the University of Wisconsin-Madison Gene Expression Center.

Lipid A analysis by MALDI-TOF mass spectrometry

V. fischeri lipid A was purified as previously described (Phillips *et al*., 2011). Overnight cultures of wild-type and Δ*eptA V. fischeri* grown in LBS were diluted 1:100 into 600 ml SWTO in 2 L flasks, then grown with shaking at 28°C. When cultures reached OD_{600} 0.60 ± 0.05, they were centrifuged for 20 min at 6000 × RCF and 4°C to pellet the cells. Supernatants were aspirated, and pellets were resuspended in 600 ml of 50 mM PIPES (pH 8.0) ASW in a 2 L flask. Cultures were incubated for 1 h and were centrifuged again, and the pellets were resuspended in 10 ml of PIPES ASW. For each strain, 5 ml of this resuspension was used to inoculate 250 ml of PIPES ASW/HGM (0.1%), buffered to either pH 6.5 or pH 8.0. Cultures were incubated for 2 h, then centrifuged, and the pellets were stored at –20°C for subsequent lipid A purification. Each lipid A pellet was then extracted twice with $CHCl_3$/MeOH/H_2O (10:5:6, v/v/v), and the pooled extract was concentrated under a gentle stream of N_2. Mass spectral analysis was performed in negative ion mode using a Bruker UltraFlex III MALDI-TOF/TOF mass spectrometer equipped with a 337 nm wavelength, 50 Hz N_2 laser (pulse energy: 150 µJ; 3 ns pulse width). Mass spectra were acquired and analyzed with FlexControl 3.3 and FlexAnalysis 3.3 software. Samples were dissolved in $CHCl_3$/CH_3OH (3:1, v/v) at approximately 1–1.3 µg $µl^{-1}$. A 0.5 µl aliquot of the sample was mixed with an equal volume of 20 mg ml^{-1} matrix (5-chloro-2-mercaptobenzothiazole (Sigma-Aldrich) in $CHCl_3$/MeOH/H_2O (4:4:1, v/v/v) with 50 mM ammonium citrate on a stainless steel target, and allowed to dry at room temperature. Approximately 1500 laser shots were recorded for each sample. Calibration was performed with a mixture of angiotensin II, renin substrate tetradecapeptide and insulin chain B (Sigma-Aldrich).

DNA pulldown and proteomic analysis

DNA pulldowns using the presumptive *eptA* promoter region were performed as previously described (Lynch, *in prep*). Briefly, 250 bp upstream of the *eptA* coding region were PCR amplified using biotinylated primers (Integrated DNA Technologies). This probe was then added in excess to *V. fischeri* cell lysates and incubated at room temperature. Probe-protein complexes were precipitated with streptavidin-coupled DynaBeads (Thermo-Fisher), and proteins were eluted with increasing NaCl concentrations (100–700 mM). Proteins were processed and identified proteomically using LC-MS/MS at the University of Vermont VGN Proteomics Facility, and aligned against the predicted *V. fischeri* ES114 proteome (UniProt, ID: UP000000537).

Effects of regulators on eptA *activity*

To monitor promoter activity in response to pH and other environmental cues, strains carrying reporter plasmids were grown to an OD_{600} of 0.60 ± 0.05 in LBS with appropriate antibiotic selection. One milliliter of this culture was centrifuged for 1 min at 10,000 × RCF to pellet the cells, and the cell pellet was resuspended in 0.5 ml of PIPES MIC buffer, modified by adjusting pH or adding stressors, as indicated in the text. Duplicate 200 µL aliquots of the cell suspensions were added to a 96-well plate, and the plate was incubated with shaking for 3 h at 28°C. A Tecan Genios Pro plate reader was used to monitor the accumulation of GFP (485 and 535 nm excitation/emission filter sets) mCherry (535 and 612 nm filter sets), and OD_{600} in the pTM267-based transcriptional reporter constructs. Alternatively, cells were grown to equivalent OD_{600} values in the defined media (50 mM PIPES, 60 mM glucose, 2% casamino acids, 300 mM NaCl, 100 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM KCl, 287 µM Na_2PO_4, 33 µM K_2HPO_4, 25 µM NaH_2PO_4 and 20 µM $FeSO_4$) at the noted pH, and GFP levels were normalized to OD_{600}. Non-fluorescent ES114 strain was used as a control for autofluorescence, and emission values obtained from this sample were subtracted from experimental measurements.

Squid colonization

Adult *Euprymna scolopes* were collected in near-shore Oahu, Hawaii, USA. Juveniles were hatched in captivity in either filter sterilized ocean water or artificial seawater, and exposed to the indicated inoculum of wild-type or Δ*eptA V. fischeri* ES114 for three hours, rinsed and maintained on a normal 12 h:12 h light: dark schedule. After 24 h, squid were individually checked for luminescence>10 AU in a TD 20/20 luminometer (Turner Designs).

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.