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RESEARCH ARTICLE

Ambient pH Alters the Protein Content of Outer Membrane Vesicles, Driving Host Development in a Beneficial Symbiosis

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ABSTRACT Outer membrane vesicles (OMVs) are continuously produced by Gram-negative bacteria and are increasingly recognized as ubiquitous mediators of bacterial physiology. In particular, OMVs are powerful effectors in interorganismal interactions, driven largely by their molecular contents. These impacts have been studied extensively in bacterial pathogenesis but have not been well documented within the context of mutualism. Here, we examined the proteomic composition of OMVs from the marine bacterium *Vibrio fischeri*, which forms a specific mutualism with the Hawaiian bobtail squid, *Euprymna scolopes*. We found that *V. fischeri* upregulates transcription of its major outer membrane protein, OmpU, during growth at an acidic pH, which *V. fischeri* experiences when it transitions from its environmental reservoir to host tissues. We used comparative genomics and DNA pulldown analyses to search for regulators of *ompU* and found that differential expression of *ompU* is governed by the OmpR, H-NS, and ToxR proteins. This transcriptional control combines with nutritional conditions to govern OmpU levels in OMVs. Under a host-encountered acidic pH, *V. fischeri* OMVs become more potent stimulators of symbiotic host development in an OmpU-dependent manner. Finally, we found that symbiotic development could be stimulated by OMVs containing a homolog of OmpU from the pathogenic species *Vibrio cholerae*, connecting the role of a well-described virulence factor with a mutualistic element. This work explores the symbiotic effects of OMV variation, identifies regulatory machinery shared between pathogenic and mutualistic bacteria, and provides evidence of the role that OMVs play in animal-bacterium mutualism.

IMPORTANCE Beneficial bacteria communicate with their hosts through a variety of means. These communications are often carried out by a combination of molecules that stimulate responses from the host and are necessary for development of the relationship between these organisms. Naturally produced bacterial outer membrane vesicles (OMVs) contain many of those molecules and can stimulate a wide range of responses from recipient organisms. Here, we describe how a marine bacterium, *Vibrio fischeri*, changes the makeup of its OMVs under conditions that it experiences as it goes from its free-living lifestyle to associating with its natural host, the Hawaiian bobtail squid. This work improves our understanding of how bacteria change their signaling profile as they begin to associate with their beneficial partner animals.

KEYWORDS *Vibrio fischeri*, squid-*Vibrio* symbiosis, outer membrane vesicles

Horizontally transferred beneficial bacteria have evolved a number of mechanisms by which to communicate with their hosts to properly establish and maintain their partnerships (1–7). Additionally, these bacteria often modify this communication as they move from environmental reservoirs to their host (8), as they transit between

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different hosts (9), or as the relationship with their partners develops over time (10, 11). Such signaling modifications are often associated with broader physiological alterations stimulated by changes in environmental conditions such as nutrient content or pH (12–14).

The species-specific mutualism between the Hawaiian bobtail squid, *Euprymna scolopes*, and the marine luminescent gammaproteobacterium *Vibrio fischeri* is a powerful system for studying host-microbe communication (15, 16). *V. fischeri* transits from its planktonic reservoir and colonizes the host's symbiotic bioluminescent organ, also known as the light organ, soon after the juvenile squid hatches (17). *V. fischeri* cells detect cues, such as reactive nitrogen species and chemoattractants, that drive the initiation of symbiosis immediately before they colonize the light organ. The bacterium physiologically adapts to and survives in the newly encountered host environment while simultaneously triggering developmental phenotypes in the squid (18–24).

Study of this binary partnership has led to the discovery of several bacterial envelope molecules that promote symbiotic development, including peptidoglycan (PGN) fragments (1), lipopolysaccharide (LPS) components (25, 26), and outer membrane proteins (27). In turn, the squid provides amino acids (28, 29), chitin sugars (13, 30, 31), and other nutrients (32) to the bacteria, while also producing a swath of immune defenses that *V. fischeri* is uniquely adapted to navigate, creating a highly privileged niche for this species (33–37). The ultimate outcome of these signaling interactions is the dramatic reshaping and maturation of the light organ that will house the symbionts for the duration of the squid's life. Among these developmental events are (i) infiltration of the squid's immune cells, or hemocytes, into the sinuses of the nascent light organ's surface appendages (20) and (ii) programmed apoptosis of the epithelial cells of these appendages (18).

Recently, we discovered that bacterial outer membrane vesicles (OMVs) can stimulate several of these developmental phenotypes, even in the absence of *V. fischeri* cells (38, 39). OMVs are continuously produced from the surfaces of essentially all Gram-negative bacteria under a range of growth conditions and are powerful governors of intra- and interbacterial physiology (40–46). These vesicles have also been identified as powerful modulators of host-microbe interactions, leading to the investigation of their role in pathogenesis (46–48). Such effects are often conveyed by virulence factors packaged in the OMVs' cargo or by the delivery of immunostimulatory microbe-associated molecular patterns (MAMPs) such as LPS (49–52). The latter characteristic of OMVs has made them attractive vaccine candidates (49, 53–56).

Within the *Vibrio* clade, OMVs have been reported to traffic several toxins, including cholera toxin, cytolysins, and hemolysin (57–60). Additionally, OMVs from several *Vibrio* species carry OmpU, a major *Vibrio* outer membrane protein (OMP). OmpU is annotated as a size-selective porin (27, 61–64) that can act as a virulence factor (65–69), inducing a variety of innate immune responses in mammalian and invertebrate hosts (37, 39, 70–73).

V. fischeri OMVs were first characterized as components of biofilms (74). It was later discovered that this bacterium's flagellar activity is a critical productive force for its OMVs, likely due to the unique architecture of its outer membrane-enveloped, or sheathed, flagella and the membrane dynamics needed to facilitate flagellar rotation (39, 75, 76). The OmpU homolog of *V. fischeri* is critical for these host responses, and OMVs from a mutant lacking *ompU* displayed diminished symbiotic signaling (39).

OMVs have been characterized *in vitro*, and several studies have provided a molecular description of these vesicles produced during culture in rich media. While this approach has yielded important basic information concerning their biochemical potential, it has recently become clear that the composition and components of OMVs can vary substantially based on the conditions in which they are produced (77–80). In some cases, researchers have exploited this variation to isolate OMVs with enriched compositions more suited to a desired goal (81).

We hypothesized that environmental signals alter the communication between bacteria and their symbiotic hosts via changes in OMV cargo molecules. Here we

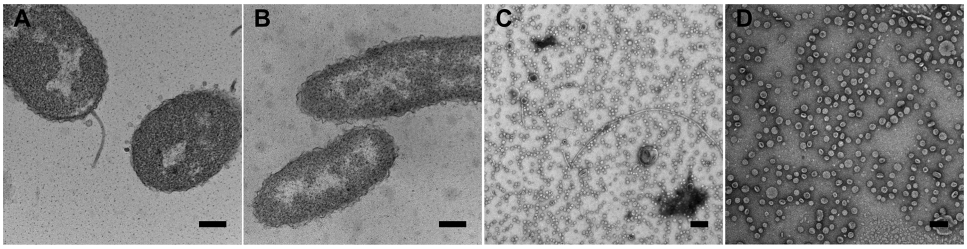

FIG 1 *V. fischeri* releases OMVs during log-phase growth. (A) TEM image of log-phase *V. fischeri* cells producing OMVs around the soma of the cell. (B) TEM image of stationary-phase *V. fischeri* cells. (C and D) Purified OMVs before (C) and after (D) sucrose gradient purification. Samples were negatively stained with osmium tetroxide before imaging. Scale bars, 200 μm (A and B) and 100 μm (C and D).

identify how cue-driven modifications contribute to the capacity of OMVs to trigger normal host development. Using the squid-*Vibrio* model, we first defined the proteome of *V. fischeri* OMVs and identified ambient pH as a meaningful cue for outer membrane modifications, resulting in differential levels of OmpU in OMVs through either passive or active means. We then measured the effects that these variations had on the ability of OMVs to stimulate symbiotic phenotypes in the squid, finding that OMVs produced under conditions found in host tissues were stronger potentiators of symbiotic development. We next identified ToxR, H-NS, and OmpR as regulators of *ompU* expression in *V. fischeri* and characterized the role of OmpU itself in promoting light organ development in the squid. These studies expand our understanding of OMP regulation in *V. fischeri*, compare these mechanisms to those of other *Vibrio* species such as *V. cholerae*, and define a connection between environmental variation, bacterial physiology, and bacterium-host communication.

RESULTS

***Vibrio fischeri* cells generate OMVs during active growth.** Transmission electron micrographs (TEMs) of *V. fischeri* cells grown in rich medium revealed a high number of budding OMVs on the cells' surfaces during log phase (Fig. 1A). Cells in stationary phase displayed lower levels of vesiculation (Fig. 1B), suggesting that active growth promotes OMV production in *V. fischeri*. We purified these vesicles using ultracentrifugation and sucrose gradient separation (Fig. 1C and D). After gradient purification, flagellar filaments were no longer observed in the preparations (Fig. 1D).

***V. fischeri* OMVs contain several potential effector molecules.** To characterize their total (soluble and membrane-associated) protein content, we performed proteomic analysis on OMVs from *V. fischeri* strain ES114 grown in liquid culture. This proteome contained previously identified symbiotic effectors, such as the dominant OMP of *V. fischeri*, OmpU (Table 1; see Fig. S1 [OMV SDS-PAGE gel] in the supplemental material) (39). Cell envelope-modifying proteins were the most common functional category in the proteome (15.6% of identified OMV proteins, compared to 6.4% of the total ES114 proteome), but additional groups, such as energy production, (9.0% of OMV proteins, compared to 5.0% of total ES114 proteome) were also enriched (Fig. 2). Other abundant proteins included flagellins and various components of the flagellar apparatus, despite low apparent contamination from flagellar filaments in our samples (Fig. 1D). In OMVs from the divergent fish light organ symbiont MJ11 (82), the relative fractions of proteins from different functional categories were similar (Fig. 2), supporting a broad-level conservation of OMV composition across *V. fischeri* isolates. Similarly, both OMV proteomes contained comparable proportions of proteins categorized as "unknown," indicating that the reference databases of the *V. fischeri* strains are similarly annotated.

***V. fischeri* OMVs have different levels of OmpU depending on culture pH.** Previous transcriptional analyses discovered that several predicted membrane components of *V. fischeri* cells were differentially expressed at distinct pHs (126). We hypothesized that the pH shifts characteristic of the transition that *V. fischeri* cells experience

TABLE 1 Most-abundant proteins identified in the symbiotic *V. fischeri* ES114 OMV proteome[a]

ES114 rank[b]	Gene name	Predicted function[c]	MJ11 rank[d]
1	*ushA*	Bifunctional UDP-sugar hydrolase/5′-nucleotidase	3
2	*flgK*	Flagellar hook-associated protein 1	2
3	*fliD*	Flagellar hook-associated protein 2	4
4	*VF_0542*	Peptidase family M16	5
5	*lpoA*	Penicillin-binding protein activator	7
6	*VF_0697*	Lipoprotein, putative	1
7	*btuB*	Vitamin B_{12} transporter	6
8	*VF_1812*	Long-chain fatty acid transport protein	16
9	*VF_1569*	Uncharacterized protein	20
10	*yfeN*	Conserved outer membrane protein	27
11	*ompU*	Outer membrane protein U, porin	23
12	*VF_1011*	Uncharacterized protein	103
13	*tolB*	Protein TolB	10
14	*VF_A0612*	9-Hexadecenoic acid *cis-trans* isomerase	17
15	*flgL*	Flagellar hook-associated protein 3	15

[a]OMVs were produced by cells grown at in LBS medium at pH 7.5.
[b]Proteins are listed in rank order of number of peptide spectrum matches (PSM).
[c]Predicted functions are from the UniProt database.
[d]PSM rank order of homologs in the proteome of *V. fischeri* strain MJ11 OMVs.

between planktonic and host-associated lifestyles would induce changes in the molecular composition of their OMVs as well and that such changes would affect the ability of these vesicles to trigger developmental responses in the newly colonized juvenile squid.

To test this hypothesis, we compared the relative levels of the major protein subunits in *V. fischeri* OMVs during growth in a defined medium set to a pH that was either alkaline (8.0; seawater-like) or acidic (6.5; host mucus-like). While there is no loss

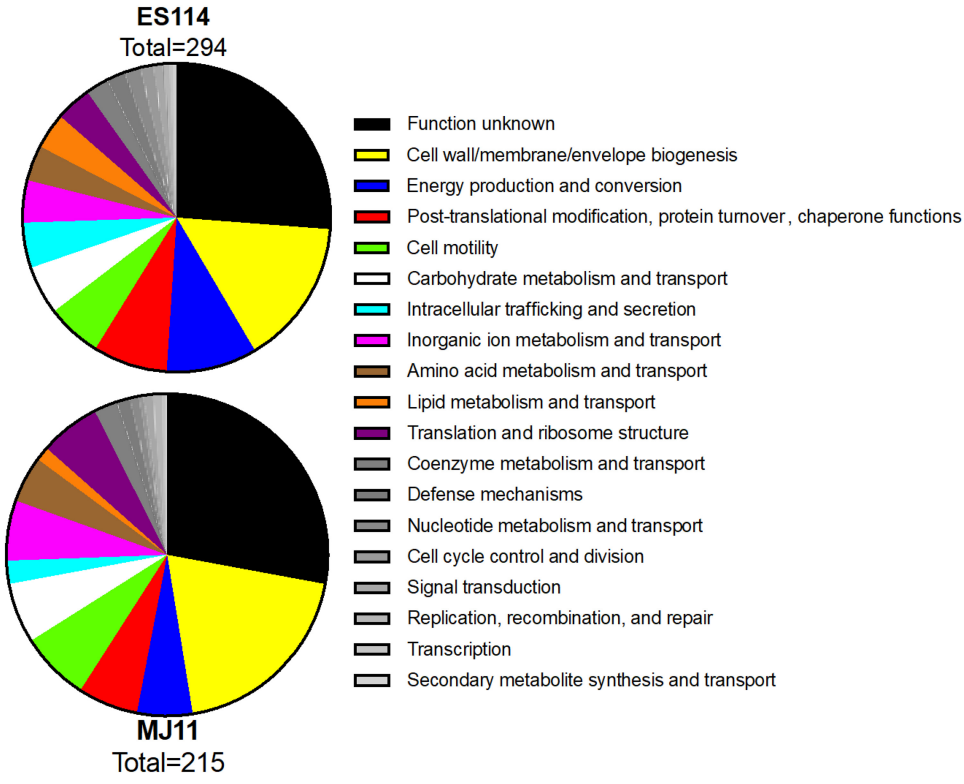

FIG 2 *V. fischeri* OMVs contain many proteins associated with envelope remodeling. Relative proportions of COG protein functional groups identified in the OMVs of symbiotic *V. fischeri* strains ES114 and MJ11 grown in LBS medium at pH 7.5 are shown.

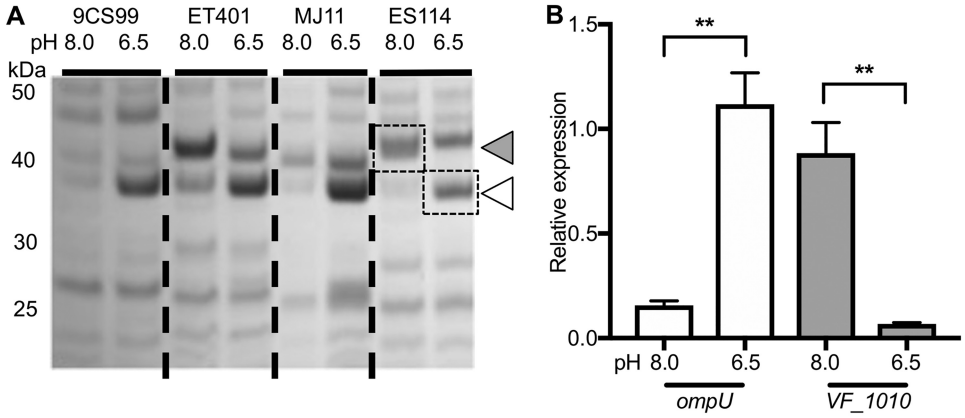

FIG 3 Low pH induces an increase in OmpU levels in *V. fischeri* OMVs and transcriptional upregulation of *ompU*. (A) Coomassie blue-stained SDS-PAGE protein gel of *V. fischeri* OMVs from cells grown in minimal medium at either pH 6.5 or 8.0. the *V. fischeri* strains shown were isolated from North Atlantic Ocean seawater (9CS99) (124) or the light organs of the Australian bobtail squid (ET401) (82), the Japanese pinecone fish (MJ11) (125), or the Hawaiian bobtail squid (ES114). The dashed boxes indicate bands cut out and proteomically analyzed. The white arrowhead and dashed box indicate band 1 (OmpU; enriched at pH 6.5), and the gray arrowhead and dashed box indicate band 2 (VF1010/VF1011, enriched at pH 8.0); the bands were isolated for proteomic analysis (for an expanded image of the gel, see Fig. S3 in the supplemental material; for identification of proteins in specific bands, see Table S2 in the supplemental material). (B) RT-qPCR quantification of relative transcript expression in *V. fischeri* ES114 for *ompU* and *VF_1010*, another predicted OMP gene. **, $P < 0.005$.

of viability, the growth rate of *V. fischeri* is reduced in the acidic medium (see Fig. S2 in the supplemental material), which is consistent with the low growth rate of the bacteria under acidic conditions in the light organ (36) (see Discussion). We found that at acidic pH, *V. fischeri* released OMVs that contained significantly larger amounts of OmpU protein (Fig. 3A; see Table S2 in the supplemental material). We confirmed upregulation of *ompU* transcripts under acidic conditions using reverse transcription-quantitative PCR (RT-qPCR) (Fig. 3B). This increase was not the result of a general upturn in outer membrane protein expression, as the predicted porin VF_1010 was downregulated under acidic conditions (Fig. 3B). Increased amounts of OmpU in OMVs are conserved across *V. fischeri* isolates from different habitats, niches, and geographic locations (Fig. 3A). Notably, OMVs from cells grown at pH 8.0 were enriched in two proteins (VF_1010 and VF_1011) that are currently annotated as uncharacterized porins, as well as several predicted iron uptake gene products (see Fig. S3 and Table S2 in the supplemental material).

***ompU* is transcriptionally regulated by H-NS, ToxR, and OmpR.** *V. fischeri* possesses homologs of several genes reported to control OMP expression in other organisms, including homologs of *hns*, *toxR*, and *ompR*. H-NS has been identified as a wide-ranging transcriptional regulator in *V. cholerae* (83), where it interacts antagonistically with the virulence regulator ToxR (84, 85), which promotes *ompU* expression in *V. cholerae* (86, 87). Similarly, in *Escherichia coli*, OmpR controls differential expression of two major OMP genes, *ompC* and *ompF* (88). To further characterize the role of these and other transcriptional modulators of *V. fischeri ompU*, we took an unbiased approach to identifying potential *ompU* regulators in *V. fischeri* by performing a DNA pulldown using the 250-bp region upstream of *ompU* as the bait. Employing this method, we proteomically identified several potential regulators of *ompU* transcription, including H-NS (Table 2). We deleted *toxR* (along with its cognate chaperone *toxS*) plus two nonessential genes identified by the pulldown, *VF_1195* and *nagC*. We also obtained previously generated *ompR* and *hns* insertional mutants (89, 90). The expression of *ompU* in these mutants, grown at both alkaline (8.0) and acidic (6.0) pHs, was measured using a green fluorescent protein (GFP) reporter driven by the *ompU* promoter (*ompUp*) (Fig. 4A). A mutation in *toxR* greatly decreased *ompU* expression at both pHs, whereas mutations in *hns* or *ompR* each increased *ompU* expression, but only at pH 6.0 (Fig. 4A

TABLE 2 Potential *ompU* regulators identified by pulldown of the predicted promoter region

Gene name	Predicted function[a]
rimK	Probable alpha-L-glutamate ligase
lon	Lon protease
hupB	HU, DNA-binding transcriptional regulator, beta subunit
nagC	DNA-binding transcriptional dual regulator, repressor of *N*-acetylglucosamine
seqA	Negative modulator of initiation of replication
VF_1195	Uncharacterized protein
ihfA	Integration host factor subunit alpha
hns	Global DNA-binding transcriptional dual regulator
ihfB	Integration host factor subunit beta
fabZ	3-Hydroxyacyl-(acyl-carrier-protein) dehydratase
degP	Periplasmic serine endoprotease
VF_A0001	Uncharacterized protein
VF_A0085	Transporter

[a]Identified proteins are listed in the order of their locus tag. Only sequences with a score of >1 and a positive +biotin/−biotin score are reported.

and B), indicating that the proteins encoded by the latter two genes may act as repressors when the cells are experiencing acidic conditions.

We then deleted *toxRS* in the *hns* and *ompR* mutant backgrounds to measure the combinatorial effects of these regulators. We found that *ompU* promoter activity did not return to wild-type levels in these double mutant backgrounds (Fig. 4C and D), supporting the conclusion that ToxR, OmpR, and H-NS do not act epistatically to regulate *ompU* (i.e., ToxR does not primarily regulate *ompU* transcription by repressing OmpR and/or H-NS). However, the slight recovery of *ompUp* activity in the double mutants indicates that there might be other undiscovered factors that are regulated by a combination of these genes that also govern *ompU* expression.

Transcriptional regulation is coupled with OmpU load in OMVs. To determine whether *ompU* transcription was directly coupled to OmpU levels in OMVs, we measured OmpU levels in OMVs from *V. fischeri* strains carrying mutations in the regulators described above. In a defined minimal medium, the *toxRS* mutant loaded significantly less OmpU into OMVs at pH 6.0 but had levels comparable to those of the wild type at pH 8.0 (Fig. 5A). In this minimal medium, the *hns* mutant also showed a decreased amount of OmpU in OMVs at pH 6.0 that approached statistical significance (Fig. 5A), a result opposite to the effect of *hns* disruption on *ompUp* activity. We found that in a rich medium, this pattern reversed, and an *hns* disruption led to an increase in OmpU levels in OMVs compared to those in the wild type (Fig. 5B). However, under these growth conditions, there was no appreciable decrease in OmpU levels in the *toxRS* mutant, consistent with previous findings (27). These results indicate that in our defined minimal medium, ToxRS is a pH-dependent transcriptional activator of *ompU* that also increases OmpU levels in OMVs, while H-NS and OmpR repress *ompU* transcription but have little effect on OmpU levels in OMVs. In our rich media, ToxRS does not impact OMV OmpU levels, but H-NS reduces levels of OmpU in OMVs. These combined findings suggest that in addition to pH, nutritional status, transcriptional regulation, and other regulatory factors contribute to the amount of OmpU present in *V. fischeri* OMVs.

pH-induced OmpU variation in OMVs is sufficient to increase symbiotic signaling. Several developmental steps in the normal symbiont-induced morphogenesis of the squid light organ, including apoptosis of its ciliated fields and hemocyte trafficking into its ciliated appendages, are triggered when *V. fischeri* initiates colonization (91). These effects can be recapitulated simply by exposing uninoculated squid to OMVs (38, 39). To test whether growth at different pHs affects this symbiotic stimulation of squid development, we measured the level of epithelial apoptosis 24 h after exposure of aposymbiotic juveniles to OMVs isolated from bacteria grown under either acidic or alkaline conditions. We found that wild-type OMVs generated under growth at pH 6.0 stimulated significantly more developmental apoptosis in the light organ appendages than OMVs produced during growth at pH 8.0 (Fig. 6A).

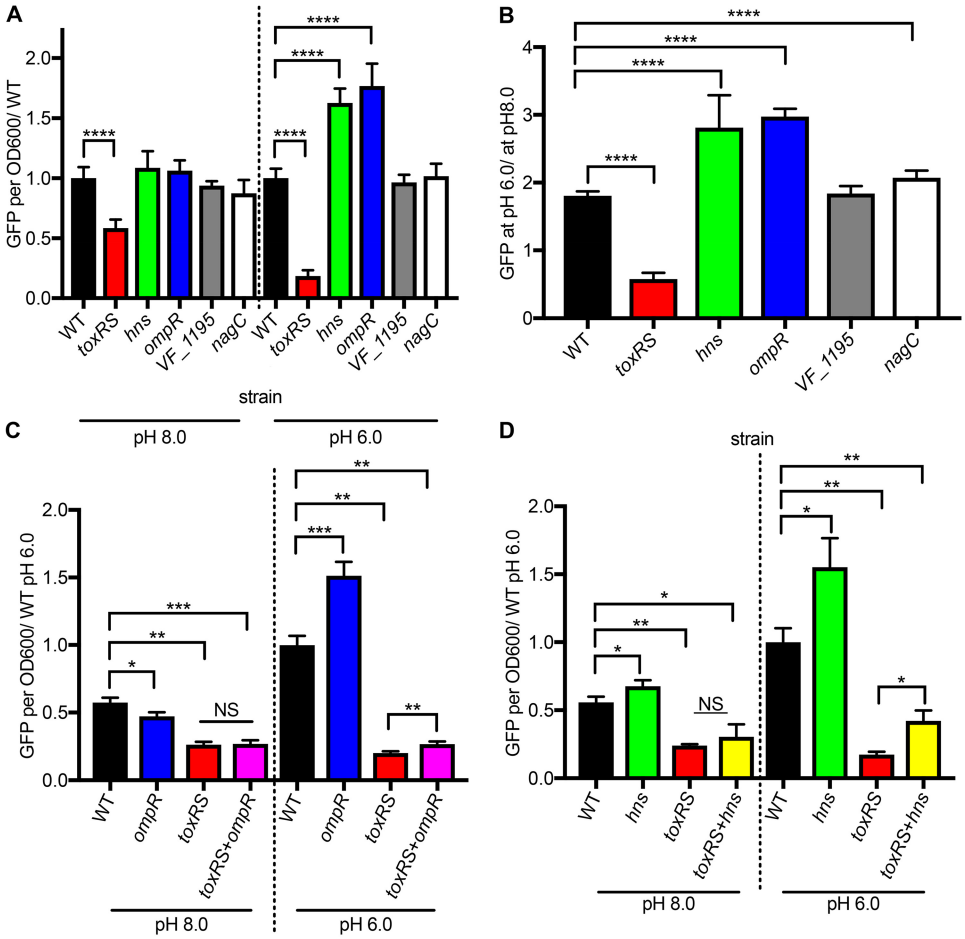

FIG 4 ToxRS, H-NS, and OmpR govern *ompU* expression and pH regulation. A GFP-based reporter was used to measure *ompU* promoter activity in *V. fischeri* strains with the indicated genetic backgrounds. (A) Expression of the *ompU* reporter at two different pHs in different *V. fischeri* mutant backgrounds. Results are normalized to wild-type (WT) expression levels at the specified pH. (B) Ratio of *ompU* reporter expression at pH 6.0 relative to 8.0. (C) *ompU* reporter expression in *ompR* and *toxRS* mutant backgrounds. (D) *ompU* reporter expression in *hns* and *toxRS* mutant backgrounds. The results in panels C and D are normalized to reporter expression in the WT at pH 6.0. "+" indicates that the strain carries mutations in both genes. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$; NS, not statistically significant at a P value of <0.05.

Similarly, OMVs from pH 6.0-grown cells stimulated more hemocyte trafficking into the light organ appendages at 24 h than pH 8.0-grown OMVs (Fig. 6B). This effect was dependent on the presence of OmpU; OMVs produced by an *ompU* mutant at either pH were unable to stimulate hemocyte trafficking, indicating that OmpU presence in the OMVs is the driver of this response (Fig. 6C). This effect is independent of large-scale reorganization of the OMV proteome in response to mutation of *ompU* (39). Importantly, we were able to complement this effector activity by expressing *ompU* from the strong *nrdR* promoter (*nrdRp*) (92) in the *ompU* deletion background (Fig. 6C). With this strain, we not only could restore hemocyte trafficking with acid-grown OMVs, but we also could stimulate similar levels of hemocyte trafficking with pH 8.0-grown OMVs, indicating that the difference in OmpU levels stimulated by the pH cue is sufficient to induce this response (Fig. 6C).

Another reported host response involving OmpU is an avoidance of hemocyte binding and phagocytosis by *V. fischeri* cells (37). This avoidance requires the presence of *ompU*, which led us to propose that OmpU might mediate some physical interaction with the hemocytes and that adding OmpU-presenting OMVs might rescue the altered binding of hemocytes to an OmpU-deficient *V. fischeri* mutant. However, the presence

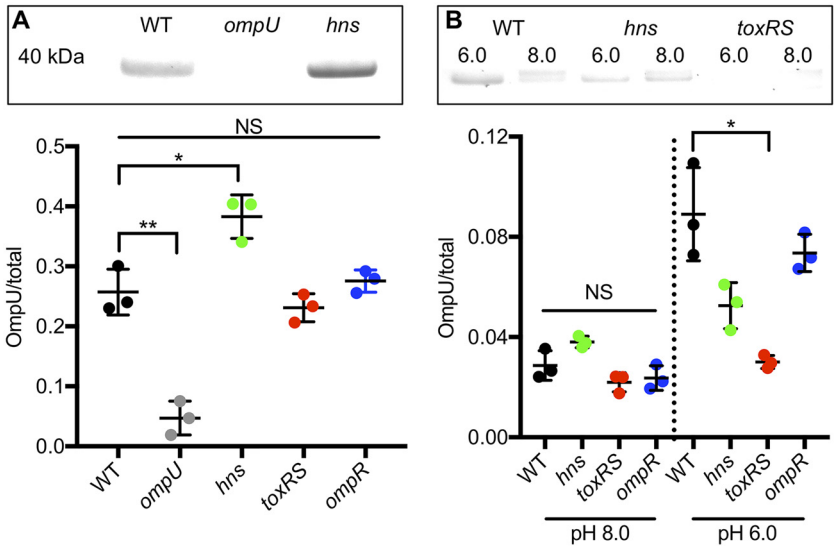

FIG 5 OmpU abundance in OMVs is altered by pH in a medium- and regulator-dependent manner. (A) Top, example of an OmpU band from a Coomassie blue-stained SDS-PAGE gel of OMVs from *V. fischeri* strains grown in LBS medium. Bottom, OmpU quantification of an SDS-PAGE gel of OMVs from *V. fischeri* cells grown in LBS medium (pH 7.5). (B) Top, example of an OmpU band from a Coomassie blue-stained SDS-PAGE gel of OMVs from *V. fischeri* strains grown in defined minimal medium at the indicated pH. Bottom, quantification of a Coomassie blue-stained SDS-PAGE gel of OMVs from *V. fischeri* cells grown in a defined minimal medium at the indicated pH. OmpU levels were quantified relative to total protein using gel densitometry. *, $P < 0.05$; **, $P < 0.005$; NS, not statistically significant at a P value of <0.05.

of wild-type OMVs did not directly alter hemocyte-binding activity toward *V. fischeri* cells that lacked OmpU (see Fig. S4 in the supplemental material).

The surface-exposed domain of OmpU is not specifically required for symbiotic stimulation of host development. The OmpU homolog in *V. cholerae* ($OmpU_{Vc}$) has been described as a size-selective porin (61, 93). Because the OmpU homologs of *V. fischeri* ($OmpU_{Vf}$) and *V. cholerae* share a high level of conservation through their predicted channel regions (69.2% identity in this region overall, with 16/16 predicted

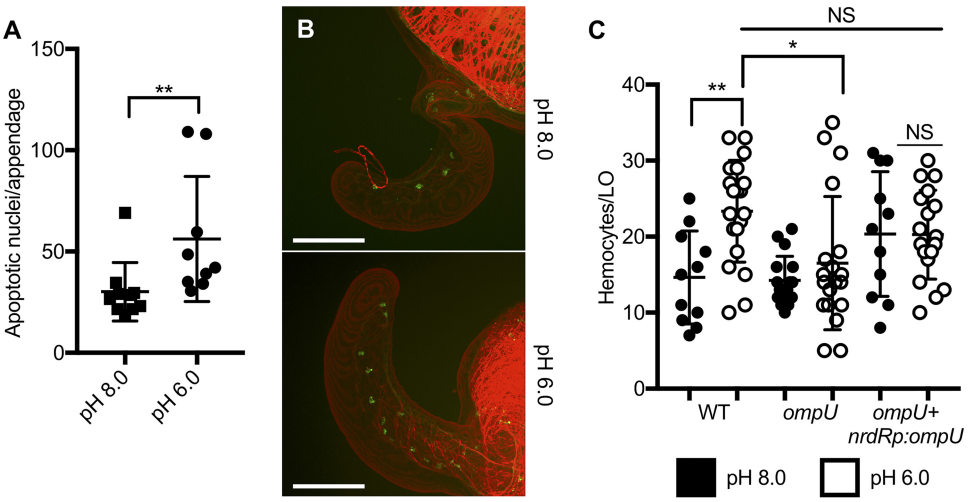

FIG 6 The pH-influenced carriage of OmpU in OMVs alters symbiotic phenotypes. (A) Quantification of developmental apoptotic nuclei in the appendages of OMV-exposed light organs. (B) Representative confocal micrographs of hemocyte trafficking into light organ appendages after exposure to OMVs from *V. fischeri* grown at either pH 6.0 or 8.0. Red, actin; green, hemocytes; scale bars, 100 μm. (C) Quantification of hemocytes in light organ (LO) appendages after exposure to OMVs isolated from wild-type (WT) *V. fischeri* or its *ompU* mutant derivative, as well as the *ompU* strain complemented with a wild-type copy of *ompU* driven by the pH-independent promoter, *nrdRp*. *, $P < 0.05$; **, $P < 0.005$; NS, not statistically significant at a P value of <0.05.

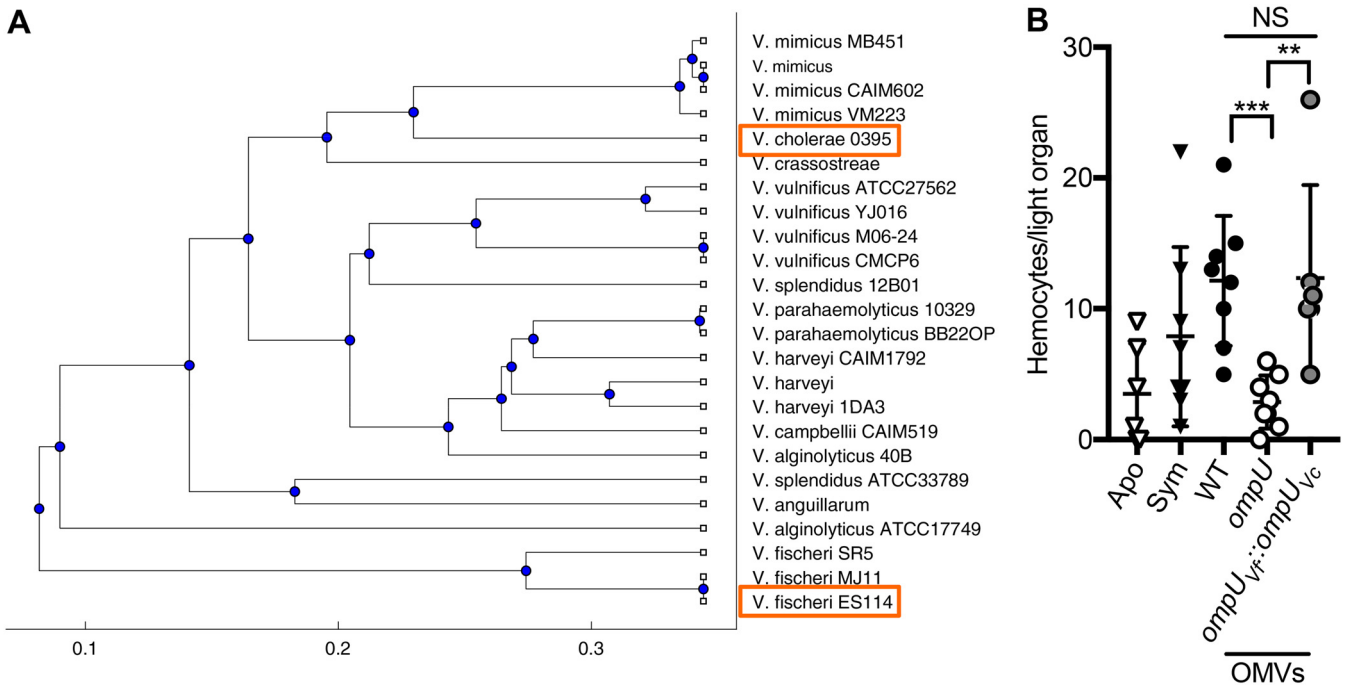

FIG 7 Conserved features of OmpU sufficient for hemocyte trafficking. (A) Phylogenetic tree of OmpU homologs across the genus *Vibrio*. Boxes highlight the positions of OmpU homologs from *V. fischeri* ES114 ($OmpU_{Vf}$) and *V. cholerae* 0395-N1 ($OmpU_{Vc}$). (B) Quantification of hemocytes in light organ appendages of squid exposed to OMVs from wild-type *V. fischeri*, its *ompU* mutant derivative, or the parental strain expressing the *V. cholerae ompU* gene in place of the endogenous *ompU* ($ompU_{Vf}$::$ompU_{Vc}$). All OMVs were from cells grown in LBS medium at pH 7.5. **, $P < 0.005$; ***, $P < 0.0005$; NS, not statistically significant at a P value of <0.05.

pore residues conserved [see Fig. S5 in the supplemental material]) but differ substantially in their extracellular domains (Fig. 7A), we hypothesized that the two proteins would share channel functionality but would differ substantially in their ability to act as surface receptors. To test whether the pore and/or extracellular domain of $OmpU_{Vf}$ is important for signaling in the squid-*Vibrio* symbiosis, we cloned the *V. cholerae ompU* gene into the native *V. fischeri ompU* locus, replacing the endogenous protein-encoding gene sequence but maintaining control by the native expression machinery. We confirmed that this chimeric strain loaded the *V. cholerae* OmpU into *V. fischeri* OMVs (see Fig. S6 in the supplemental material). When measuring the ability of the chimeric OMVs to induce hemocyte trafficking, we found that *V. fischeri* OMVs bearing only the *V. cholerae* version of OmpU were as stimulatory as wild-type *V. fischeri* OMVs, indicating that the specific sequence in the extracellular region of OmpU is not necessary for the symbionts to trigger the normal hemocyte-trafficking response (Fig. 7B).

DISCUSSION

Recently, interest has grown in using OMVs as vehicles for molecular delivery to other bacteria or host tissues (reviewed in references 94 and 95)). This attention has driven a need to understand not only what molecules are typically found in OMVs but also how the occurrence and abundance of this cargo are regulated. Here, we report a proteomic analysis that revealed that *V. fischeri* OMVs share a protein profile broadly similar to those of other bacteria (see examples in references 58 and 96), with envelope-modifying enzymes, flagellar components, and outer membrane proteins (OMPs) being highly enriched. We found that symbiotic strains of *V. fischeri* from divergent hosts produce OMVs with proteomes that are predicted to be functionally similar (Fig. 2). Many of the proteins identified in the OMVs are also found in the proteomes of symbionts in the adult squid light organ, including OmpU, VF_1010, and H-NS (97), indicating that these proteins remain highly expressed throughout the development of the symbiosis. The presence of several components of the flagellar

apparatus was surprising, because these structures were not visible in our sample preparations (Fig. 1D). Further, given that we identified portions of the periplasmic and cytoplasmic motor complex, such as FlgM FlgI, FliK, and FliE, it is unlikely that these peptides are associated with the few contaminating flagellar filaments that may be present (98). Instead, this enrichment could reflect the link between the rotation of the sheathed flagellum of *V. fischeri* and OMV production (39); i.e., as these proteins are introduced into the growing flagellum, they might be captured in OMVs formed near where the flagellar hook stretches the outer membrane into a sheath (76). Regardless of their source, the presence of flagellar components in OMVs may stimulate host responses through sensors analogous to mammalian Toll-like receptors (99).

Elegant work with *Vibrio harveyi* cells has shown the production of distinct populations of OMVs with different functional capacities (44): for instance, certain OMVs carry autoinducer molecules that are involved in quorum-sensing induction. *V. fischeri* cells generate differently sized vesicles dependent on their flagellar activity (39), highlighting a potential concern for many OMV studies, which may selectively deplete certain types of OMVs during processing and purification. Electron micrographs of intact, vesiculating *V. fischeri* cells indicated negligible levels of large (>200-nm diameter) vesicles that have been described in other studies (Fig. 1A); however, experimental enrichment for vesicles in this size range may reveal whether they have unique functional features. Different vesicle populations may (i) include unique cargo molecules or (ii) contain distinct proportions of these molecules due, in part, to the vesicle's different surface area-to-volume ratio (46). As OMVs represent a potent means by which to distribute hydrophobic cargo in aqueous environments, the relative proportion of membrane associated with soluble materials could significantly alter their signaling capacity.

During the initiation of light organ symbiosis, *V. fischeri* cells from the bacterioplankton encounter a change in ambient pH at different locations along their migration path as they colonize host tissue. Specifically, the cells experience a sharp pH decrease from ~8.2 to ~6.0 as they transit from the seawater to the acidic mucus on the surface of the light organ (24); similarly, once established in the light organ crypts, the symbionts of adult animals experience a circadian acidification of their environment to a pH of 5.7 (100). *V. fischeri* is likely to respond physiologically to these spatial and temporal transitions in environmental conditions, optimizing its signaling ability to either initiate symbiotic development of host tissues or adapt to a persistent, cyclic metabolism (101). A principal difference between these two pH transitions is the amount of time *V. fischeri* spends in each; cells only transiently interact with acidic mucus on their way into the light organ, whereas they spend several hours each night in acidic conditions that exist between the accumulation of fermentation products and the morning light organ venting. These distinct temporal scales could affect the bacterium's response and its impact on modes of signaling to the host.

In this study, we discovered that a decrease in ambient pH promoted increased levels of the outer membrane protein OmpU in the OMVs of *V. fischeri*; this increase, in turn, potentiated the symbiont's ability to activate light organ development (Fig. 8). It has not yet been established how OMVs trigger increased hemocyte trafficking into the light organ appendages (Fig. 5B and C); beyond the requirement for OmpU, simply adding peptidoglycan fragments alone will induce this trafficking (1), and their frequent presence in OMVs (102) indicates they are likely to be effectors of this behavior. In the context of natural colonization, few *V. fischeri* cells are needed to initiate symbiotic development (24), and OMVs may be an effective strategy for packaging host-stimulatory cargo that would be otherwise more easily lost to diffusion. Interestingly, while OmpU is required for stimulation of hemocyte trafficking above basal levels (Fig. 7), previous work has found that OMVs from *ompU*-deficient *V. fischeri* induce developmental apoptosis to a level below that with wild-type OMVs but above that in unexposed animals (39). This finding suggests that OmpU might be required for certain host responses while acting as an enhancer alongside other MAMPs such as lipopolysaccharide to potentiate additional phenotypes.

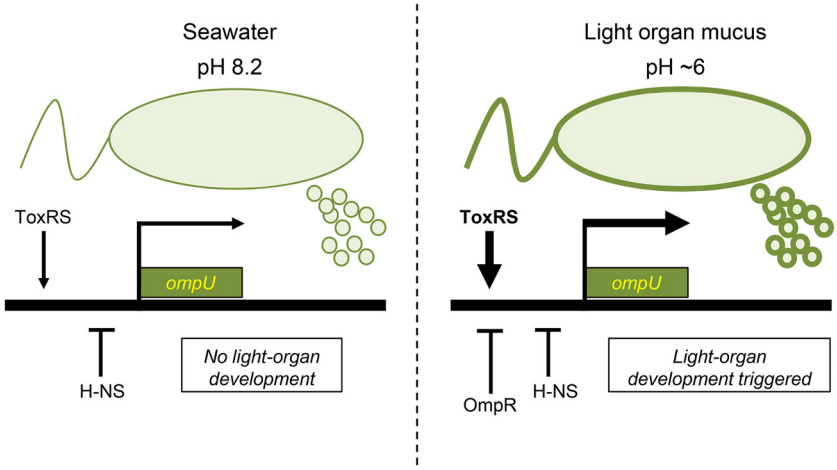

FIG 8 Proposed model of *ompU* regulation in symbiotic *V. fischeri*. At the alkaline pH of seawater (8.2), there is little ToxRS-driven induction of *ompU*, with a mild dampening by H-NS. In contrast, at the acidic pH (~6) characteristic of squid mucus, ToxRS drives more *ompU* expression, but it is tempered by the inhibitory activities of OmpR and H-NS. The net upregulation in *ompU* expression leads to increased presence of OmpU in *V. fischeri* OMVs, which become more potent inducers of symbiotic development in the juvenile squid light organ.

We cannot rule out that some aspects of OmpU-associated activity are caused by OmpU affecting the composition of OMVs by some yet-unknown mechanism, such as shifting the overall protein content of the OMVs. We have not detected obvious differences in the overall OMV proteome between the wild type and *ompU* mutant strains of *V. fischeri* in any of our described media, but these changes could be below our limit of detection. Regardless, our finding that we can complement these phenotypes in *trans* with a nonendogenous promoter (Fig. 6C), as well as our ability to stimulate these responses with a nonendogenous OmpU homolog (Fig. 7B), indicates that OmpU is sufficient to directly or indirectly drive these responses.

Our finding that *V. fischeri* OMVs do not directly affect hemocyte binding of symbionts (see Fig. S4 in the supplemental material) suggests either that the regulation of hemocyte binding is distinct from hemocyte trafficking or that other host cell types play a role as intermediaries in signaling cellular infiltration. We have previously established that hemocytes take up *V. fischeri* OMVs (38), but interactions between OMVs and other cell types remain unknown. For instance, it is possible that epithelial cells in the light organ sense OMVs and emit a cytokine-like signal that attracts hemocytes to the appendages, an activity not unlike that reported in mammalian tissues (103).

Physiologically, OmpU is described as a size-selective porin that dynamically dilates and constricts (61, 63); however, to our knowledge, the specific substrates that pass through the pore under biologically relevant conditions remain unknown. Our work advances an understanding of the role of OMPs in the effector activity of OMVs. An attractive prediction is that the porin function of OMPs allows OMVs to deliver certain cargo molecules to host tissues; however, such a function would require (i) a high initial concentration of those molecules within the lumen of the OMVs or (ii) stringent gating so that the molecule is delivered only under certain conditions. Consistent with this hypothesis is the presence, within the soluble contents of the OMVs, of an activity that induces hemocyte trafficking; this activity acts like, but is not chemically identical to, peptidoglycan monomer (1, 38). If OMVs are able to deliver soluble molecules in such a context-dependent manner, OmpU would be a plausible conduit, as it has two predicted structural gates and adaptively increases the frequency of its "closed" configuration under acidic conditions (63, 104). Defining the soluble molecular contents of OMVs would inform our predictions of the physiological importance of these vesicles to host and bacterium and would likely improve our understanding of the variable

mechanism of their biogenesis. It is notable that some phenotypes conferred by OmpU homologs in *V. cholerae* do not seem to rely on porin activity (105), showing that this family of proteins has functions outside solute transport.

Similarly, the full range of functions of OmpU in vibrio-host associations, both pathogenic and beneficial, remains undefined. While OmpU induces a plethora of host phenotypes, the mechanisms underlying these effects are still largely unresolved. Previous work has shown that trafficking of squid hemocytes can be stimulated by *E. coli* OMVs, which lack an OmpU homolog (38) but bear other OMPs (106). That finding, combined with our OmpU-swapping experiments (Fig. 7), supports the possibility that the exact nature of the OMP is not important but, instead, that certain host cells sense the levels of porins in OMVs as a kind of MAMP, a hypothesis that will require future scrutiny. This notion is especially promising given that some level of specificity in the squid-*Vibrio* symbiosis is determined by access to privileged tissue sites, rather than molecular signatures; i.e., *V. fischeri* induces certain symbiotic phenotypes because it is the only microbe that can reach the deeper tissues of the light organ, not because it releases unique MAMPs. Thus, by exposing squid to OMVs, which we hypothesize diffuse into and throughout the light organ, we can override this spatial specificity.

The expression of *ompU* in *V. fischeri* is determined by the activities of three regulatory proteins, H-NS, OmpR, and ToxR (Fig. 4), which have been reported to control various OMPs in other species. Our finding that the effects of H-NS and ToxR can be muted by changes in medium composition indicates that there is likely some role that nutrient sensing plays in *ompU* expression or OMV composition. Previous work in *V. cholerae* has found that combinations of amino acids—specifically, the mix of asparagine, arginine, glutamic acid, and serine—increase expression of OmpU (107). While both our rich and defined medium formulations include diverse amino acids (from tryptone-yeast extract and Casamino Acids, respectively), it is possible that the concentrations of these nutrients are sufficiently different to generate the conflicting results found here.

While H-NS and ToxR have previously been shown to be key organizers of host-associated phenotypes in pathogenic *Vibrio* species (83, 85, 108, 109), ours is one of the first studies to identify their role in beneficial animal-bacterial symbiosis. Similarly, although *ompU* has been found to be important in regulating the squid-*Vibrio* symbiosis (27, 37, 110), OmpU has been found to be a multifaceted virulence factor in other *Vibrio* species. Our *ompU*-swapping experiments show that the OmpU homolog of a pathogen can complement a beneficial bacterial function, indicating that the effects of "virulence" factors are context specific. In our case, $OmpU_{Vc}$ is able to stimulate the symbiotic response normally induced by $OmpU_{Vf}$, while in its normal pathogenic context, $OmpU_{Vc}$ induces inflammatory immune responses. This result evokes an interesting hypothesis: general host association genes are ancient within the genus *Vibrio* and have more recently been coopted for mutualistic or pathogenic relationships based on the overall lifestyle of the symbiont. Thus, related bacteria can divergently deploy similar regulatory machinery to promote different lifestyles with distinct outcomes. This divergence may make simple homology associations more difficult to interpret across host-associated species.

This study, along with other work from our group (126), positions H-NS as a previously underappreciated regulator of diverse symbiotic phenotypes in *V. fischeri*. Specifically, H-NS suppresses luminescence in both *V. fischeri* (90) and *Photobacterium leiognathi* (111) and regulates *ompU* (Fig. 4 and 5) and other membrane factors, dramatically shaping the symbiotic capacity of *V. fischeri*. The broad regulatory range of H-NS has discouraged its full characterization, and because mutations in the *hns* gene confer a growth defect *in vitro*, it is often excluded from the analysis of mutant screens in *V. fischeri*. Thus, the best way to uncover its regulatory phenotypes may be by using direct biochemical measurements (Table 2), and further analyses of this kind will allow for deeper understanding of its symbiosis-regulating functions.

Here, we have defined an environmentally stimulated regulatory network and a resulting outer membrane reconfiguring that tunes the triggering of host development

via outer membrane vesicles. Given the ubiquity of OMVs across the bacterial domain, our work informs an understanding of a wide range of both beneficial and pathogenic interdomain relationships and guides new questions about the interplay between environmental sensing and interorganismal communications.

MATERIALS AND METHODS

Strains, growth conditions, and molecular genetics. *Vibrio fischeri* strains were all derived from the wild-type *Euprymna scolopes* symbiont isolate ES114 (112) unless noted otherwise. Strains were grown in LBS medium (10 g tryptone liter^{-1}, 5 g yeast extract liter^{-1}, 342 mM NaCl, and 20 mM Tris, pH 7.5) (39) with shaking at 225 rpm in a 28°C incubator. Gene deletions were generated using counterselection allelic exchange with the suicide vector pKV363 as previously described (74) after conjugation from donor *Escherichia coli* β3914. For the *toxRS* mutant, gene deletion was performed using sucrose-based counterselection on LB-sucrose plates (10 g tryptone liter^{-1}, 5 g yeast extract liter^{-1}, 290 mM sucrose, 43 mM NaCl) via *sacB* on the vector pSMV3 conjugated from the donor *E. coli* WM3064 (113, 114). Cells were grown on media solidified with 1.5% agar when appropriate. Antibiotics were used at the following concentrations: chloramphenicol (Cam) at 2.5 μg ml^{-1} or 25 μg ml^{-1} and kanamycin (Kan) at 100 μg ml^{-1} or 50 μg ml^{-1}, for *V. fischeri* or *E. coli*, respectively. Promoter fusions were performed using either traditional restriction digest and ligation or Golden Gate cloning (New England BioLabs).

For experiments defining pH-sensitive responses, cells were grown in a defined artificial seawater-based minimal medium [300 mM NaCl, 100 mM $MgSO_4$, 10 mM $CaCl_2$,10 mM KCl, 287 μM Na_2PO_4, 25 μM NaH_2PO_4, 2% Casamino Acids, 33 μM K_2HPO_4, 20 μM $FeSO_4$, 60 mM glucose, and 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)] (115, 116) buffered to the appropriate final pH using NaOH and/or HCl. "Basic" media were buffered to a final pH of 8.0, and "acidic" media were buffered to either pH 6.5 (for initial *ompU* variation experiments and those involving other *V. fischeri* isolates) or pH 6.0 (for regulatory characterization and OMV experiments). pH 6.5 was used to facilitate growth of more-acid-sensitive strains.

For *ompU* mutant complementation, the ~250-bp region upstream of the *V. fischeri* ES114 *nrdR* gene, which has been previously described as a strong promoter (92), was cloned upstream of the *ompU* open reading frame, and the resulting fragment was inserted into the plasmid pVSV104H (117) via Golden Gate cloning. This construct was transformed into WM3064 cells and conjugated into the appropriate *V. fischeri* strain.

See Table S4 in the supplemental material for full list of strains and genetic constructs used in this study.

Squid husbandry. Adult *E. scolopes* squid were collected around Oahu, HI, and were kept in flowthrough natural oceanic seawater at Kewalo Marine Laboratory according to protocols approved by the University of Hawaii at Manoa. Egg clutches were collected and moved into a 12-h/12-h light/dark schedule within 10 days of being laid. For most experiments, juveniles (<6 h posthatching) were collected, washed, and placed in filter-sterilized ocean water (FSOW) prior to experimental incubation with *V. fischeri* strains or OMVs.

OMV purification for experimentation. OMVs were isolated from saturated *V. fischeri* cultures (24 h of growth) in media as noted. Cells were grown shaking in Erlenmeyer flasks at 28°C and then were pelleted at 5,000 × *g* and 4°C for 10 min. The resultant culture supernatant was filtered through a 0.22-μm-pore-size PES membrane filter (Millipore, Inc.) to remove any contaminating cells. Filtered supernatant was then ultracentrifuged in either an MLA-50 rotor in an Optima-XP centrifuge (Beckman Coulter) or a TLA-45 rotor in a Max-XP centrifuge (Beckman Coulter) at 173,000 × *g* and 4°C for 2 h. Pellets containing OMVs were resuspended in Dulbecco's phosphate-buffered saline (PBS) supplemented with NaCl (422 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 7.7 mM Na_2HPO_4, 900 μM $CaCl_2$, 500 μM $MgCl_2$) (39).

For proteomic analysis and animal response experiments, OMVs were further purified with a discontinuous density gradient (sucrose layers of 25, 35, 40, 45, 50, and 55%, wt/vol), repelleted at 173,000 × *g* and 4°C for 4 h, and resuspended in PBS-NaCl. The total OMV protein concentration was measured using a Qubit 2.0. Samples were diluted to equal concentrations with PBS-NaCl before addition to FSOW for squid exposure experiments. Squid were treated with 25 μg of OMV proteins ml^{-1} for apoptosis measurements and with 50 μg ml^{-1} for hemocyte-trafficking assays. All exposures were for 24 h, on the light schedule mentioned above.

TEM. For transmission electron microscopy (TEM), *V. fischeri* ES114 was grown to mid-log or stationary phase in SWT medium (70% ocean water, 5 g tryptone liter^{-1}, 3 g yeast extract liter^{-1}, 32.5mM glycerol) or LBS, respectively. Because bacteria grown to stationary phase in SWT were found to have extensive cell damage, they were not used in our experiments. Cells were preserved in marine fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 350 mM sucrose, pH 7.6), washed three times in a solution of 100 mM sodium cacodylate and 440 mM sucrose, and postfixed in 1% osmium tetraoxide and 100 mM sodium cacodylate. Cells were then dehydrated in an ethanol series (30%, 50%, 70%, 85%, and 95%), before washing four times in 100% ethanol. Samples were then washed with 100% propylene oxide four times, infiltrated with 1:1 propylene oxide-epoxy resin, immersed in 100% epoxy resin for 2 h, and reimmersed in fresh epoxy resin for 1 h. Samples were then polymerized at 60°C for 2 days and sliced on an ultramicrotome before imaging.

OMV preparations for imaging were spotted onto a plasma-charged Formvar-coated copper grid, fixed and stained with osmium tetraoxide, and washed with distilled water. All imaging was performed on a 120-kV Hitachi HT7700 transmission electron microscope at the University of Hawaii Biological Electron Microscopy Facility.

***V. fischeri* growth at acidic or basic pH.** *V. fischeri* ES114 was grown overnight in LBS medium and then diluted 1:100 in defined minimal medium at the indicated pH. Cells were grown in a 96-well plate at 28°C, and the optical density at 600 nm (OD_{600}) was measured after shaking every 30 min in a Tecan GenIOS Pro plate reader. Reported values are mean ± standard deviation (SD) of four biological replicates.

Processing for proteomic samples. Samples were mixed with NuPAGE LDS sample buffer (Invitrogen) and then boiled at 70°C for 10 min. An aliquot of 50 μl of sample was run on a WedgeWell NuPAGE-SDS gel (Invitrogen) to immobilize proteins for processing, as described previously (118). After Coomassie blue staining, bands were excised, placed into a clean tube, and destained in a mixture of 25 mM NH_4HCO_3 and 50% CH_3CN, followed with a wash in NanoPure water. Samples were dehydrated in 100% CH_3CN and dried, and then disulfide bonds were reduced with 10 mM dithiothreitol in 100 mM NH_4HCO_3 for 1 h at 56°C. Samples were then alkylated for 45 min in the dark at room temperature with 55 mM iodoacetamide in 100 mM NH_4HCO_3 and then washed twice in alternating incubations of 100% CH_3CN and 100 mM NH_4HCO_3. Samples were dried and then incubated in a solution of 5% CH_3CN and 25 mM NH_4HCO_3 containing 12 ng of trypsin μl^{-1} for 18 h. Supernatants were removed to a new tube, and the remaining gel was incubated in 5% formic acid (FA) for 1 h at room temperature. Supernatants were removed again, and the remaining gel was incubated in 50% CH_3CN–5% FA for 1 h at room temperature. All three supernatants were combined, vacuum dried in a Vacufuge Plus (Eppendorf), and kept at 4°C until analysis.

For total OMV proteomic analysis, LDS-suspended OMV samples were minimally run on SDS-polyacrylamide gels, and stained with Coomassie blue, and then the entire protein-containing section of the gel was cut out with a clean razor and processed as described above. For specific band analysis, samples were treated similarly, but samples were run further out on the gel, and individual bands were cut out for proteomic analysis.

LC-MS/MS-based protein identification. All proteomic analysis was performed by the Vermont Genetics Network (VGN) at the University of Vermont. Labeled peptides were resuspended in 2.5% CH_3CN–2.5% FA in water for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Analyses were performed with a Q-Exactive mass spectrometer coupled to an EASY-nLC ULTRA (Thermo Scientific). Peptides were separated using a gradient of 2.5 to 35% CH_3CN–0.1% FA over 150 min, 35 to 100% CH_3CN–0.1% FA for 1 min, and 100% CH_3CN–0.1% FA for 8 min, followed by an immediate return to 2.5% CH_3CN–0.1% FA and a hold at 2.5% CH_3CN–0.1% FA. Peptides were ionized with a nanospray ionization source under voltage of 2.0 kV. Mass spectrometry data were acquired in the "Top 10" acquisition mode with lock mass function activated, in which a survey scan from *m/z* 350 to 1600 at 70,000 resolution was followed by 10 tandem mass spectrometry (MS/MS) scans on the most abundant ions.

Product ion spectra were searched using SEQUEST implemented on the Proteome Discoverer 2.2 (Thermo Scientific) against a curated UniProt (*Alii*)*vibrio fischeri* (strain ES114 or MJ11) protein database (UP000000537 and UP000001857, respectively). Only proteins with >2 identified peptides were considered present in data sets (see Table S2). For protein category (COG) analysis, sequences were categorized according to eggNOG 4.5 using the eggNOG-mapper tool (119).

SDS-PAGE of OMV proteins. OMV samples were diluted to the indicated concentration, boiled at 70°C for 10 min in LDS buffer, loaded onto a 4 to 12% Bis-Tris NuPAGE gel (Invitrogen), and then run for 35 min in morpholineethanesulfonic acid (MES) running buffer at 200 V. Gels were stained with Coomassie brilliant blue 250 (120) and imaged on a ChemiDoc-It² Imager (UVP). The band identified as OmpU was verified through knockout analysis and proteomic analysis (see above). For relative protein quantification, OMVs were isolated from three individual isolates of the indicated strain, and the relative ratio of OmpU to total protein was quantified in ImageJ using densitometry.

RT-qPCR verification. RT-qPCR was used to verify transcriptional variation of *ompU* and *VF_1010* with gene-specific primers designed using Oligoanalyzer (IDT) and a previously established protocol (13). The *polA* transcript was used as a normalizing control.

DNA pulldown experiments. The DNA pulldown protocol was derived from that described by Jutras et al. (121). Briefly, biotinylated primers flanking the region 250 bp upstream of *ompU* were ordered from IDT and used to perform PCR with Phusion DNA polymerase (Invitrogen). PCR mixtures were pooled, and products were precipitated by adding 300 mM sodium acetate, followed by 3 volumes of ethanol, and stored at −20°C overnight. DNA was then pelleted by centrifugation, washed in ice-cold 70% ethanol, repelleted by centrifugation, dried, and resuspended in water. This bait DNA was then diluted to a final concentration of 25 ng μl^{-1} in a 400-μl total volume with water.

Cell lysates were generated by growing *V. fischeri* cells to log phase in LBS medium, pelleting them by centrifugation, and then storing the pellets at −80°C. Pellets were thawed and sonicated eight times for 15 s at 50% amplitude, with 1 min on ice between sonications. Cell debris was then removed by centrifuging lysates at 10,000 × *g* for 10 min at 4°C, after which the supernatants were kept on ice.

Streptavidin-coated Dynabeads (Invitrogen) were washed three times in 2× B/W buffer (2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5) and then twice incubated at room temperature for 20 min with an equal volume of DNA probe. Beads were then washed three times in Tris-EDTA (TE) buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5) and washed three times in BS-THES buffer (BS, 250 mM KCl, 25 mM $CaCl_2$, 60% glycerol, 50 mM HEPES, pH 7.5; THES, 140 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5; BS-THES, 44.3% THES, 20% BS, 35.7% nuclease-free water). Beads were incubated in 400 μl cleared cell lysate twice for 30 min at room temperature and then washed five times in BS-THES buffer. The protein-DNA complexes were eluted with increasing levels of NaCl (25 mM Tris-HCl, pH 7.5, with 100, 200, 300, 500, and 750 mM NaCl). Proteins bound to the DNA probe were processed and identified by proteomic analysis as described above. Negative-control protein identifications were generated by the procedure described

above but using identical nonbiotinylated primers. Proteins were listed as pulled down only if their +biotin score was higher than their −biotin score.

***ompU* reporter assays.** The *ompU* reporter construct was created by cloning the region from bp −400 to bp +200 from the predicted *ompU* start codon in front of a promoterless GFP expression construct in the plasmid pTM267 (122). The resulting construct was conjugated into the indicated *V. fischeri* strains from donor WM3064 *E. coli*, and transconjugants were selected on LBS agar containing 2.5 g Cam ml^{-1}. Cam-resistant isolates were grown overnight in LBS liquid culture containing Cam and then diluted 1:100 in 100 μl of defined minimal medium containing Cam at the indicated pH in a 96-well plate. Cells were grown to mid-log phase at 28°C, and then GFP fluorescence and OD_{600} were measured in a Tecan GenIOS Pro plate reader. Fluorescence measurements for blank media were subtracted, fluorescence levels were divided by the OD_{600} for each well, and GFP/OD_{600} values were normalized to the indicated baseline. Experiments were repeated at least twice on different days with similar results.

Apoptosis analysis using AO staining. Juvenile squid were exposed to 25 μg ml^{-1} OMVs (by protein) in 4 ml of FSOW for 24 h and then were transferred into a solution of 0.0001% acridine orange (AO) in FSOW for 2 min and anesthetized in 2% ethanol. Their mantles were removed, AO staining was visualized by confocal fluorescence microscopy, and the number of condensed nuclei in the light organ's ciliated appendages was counted using the Spots function in the Imaris software (Oxford Instruments). All confocal microscopy was performed using a 40× water objective on a Zeiss 710 upright confocal microscope.

Hemocyte assays. To quantify hemocyte trafficking during normal colonization, newly hatched juvenile squid were inoculated with ~10^4 CFU of *V. fischeri* ml^{-1} FSOW for 16 h. Alternatively, squid were exposed to 50 μg of OMVs (by protein) ml^{-1} FSOW for 24 h. The animals were then fixed in 4% paraformaldehyde and permeabilized in 1% Triton X-100 in marine PBS (mPBS) (450 mM NaCl, 9.5 mM $NaHPO_4$, 4.5 mM $Na2PO_4$, pH 7.4). Hemocytes were stained with 25 μg DNase I-Alexa Fluor 488 ml^{-1}, and actin was stained with 25 μg rhodamine-phalloidin ml^{-1}. Hemocytes that had trafficked into the light organ ciliated appendages were visualized by confocal fluorescence microscopy and counted in Imaris using the Spots function, when possible. Light organs were counted only when both appendages were intact and easily visualized. Experiments were independently performed at least three times over multiple days, and data shown are from either pooled results or representative experiments.

To complement the *ompU* mutation in OMV experiments, *V. fischeri* cultures were grown in LBS medium with Kan to maintain a pVSV104H-derived complementation plasmid. Noncomplemented strains in these experiments received the empty pVSV104H vector and were grown the same way. OMV preparations and exposures were as described above.

Hemocyte-binding assays were performed as previously described (37). Hemocytes isolated from adult wild-caught squid ($n = 3$) were either untreated or exposed to OMVs at a concentration of 50 μg ml^{-1} for 1 h before addition of GFP-expressing ES114 Δ*ompU* (JG01) at a concentration of 25 bacterial cells/hemocyte. Hemocytes were stained with CellTracker deep red dye (Thermo Scientific). Cells were imaged using a Nikon A1R confocal microscope, and bacterial binding was quantified using Fiji software (123).

Phylogenetic relationship and ClustalW alignment. Sequences of *ompU* genes from noted *Vibrio* species were obtained from NCBI and aligned in Matlab using default settings of the seqpdist function (Jukes-Cantor pairwise distance). For *V. fischeri* and *V. cholerae* OmpU alignment, ClustalW alignment was performed on the Protein Information Resource server (https://proteininformationresource.org/pirwww/search/multialn.shtml). Predicted pore residues were identified using two predicted OmpU structures (61, 63).

***ompU* gene swapping.** The *ompU* homolog of nontoxigenic classical Ogawa *V. cholerae* strain 0395-N1 was amplified and swapped into the endogenous *ompU* genomic locus in *V. fischeri* ES114 using allelic exchange. OMVs were purified from the wild-type parent (ES114), the *ompU* mutant derivative, and the *V. fischeri* $ompU_{Vf}$::$ompU_{Vc}$ strain and were analyzed by SDS-PAGE to verify inclusion of the *V. cholerae* $OmpU_{Vc}$ into *V. fischeri* OMVs. Juvenile squid were exposed to 50 μg OMVs (by protein) ml^{-1} of FSOW from each of these three strains, and hemocyte infiltration into the light organ appendages was measured as described above.

Statistical analysis. RT-qPCR measurements were performed on six biological replicates. *ompU* reporter experiments were performed in triplicate, and results shown are from analysis of 3 or 4 biological replicates. All experiments were repeated at least once on a different day with similar results. Gel densitometry was performed on at least 3 biological replicates. Data from different conditions in these experiments were compared with Student's *t* test using Welch's correction.

Hemocyte-trafficking and light organ apoptosis experiments were performed with animals gathered across multiple days ($n \geq 6$ for all hemocyte trafficking and $n \geq 9$ for apoptosis). Hemocyte-binding experiments were performed with hemocytes from three animals. Data were compared with a Mann-Whitney test.

Each data point represents one biological replicate. All graphed data show mean ± SD for that condition.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00319-19.

SUPPLEMENTAL FILE 1, XLSX file, 1.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.04 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.03 MB.
SUPPLEMENTAL FILE 5, PDF file, 12.1 MB.

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